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

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Abstract

The potential to increase passive transfer of specific *Rhodococcus equi (R.equi)* humoral immunity to newborn by preparturient vaccination of their dams was investigated 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 prior to 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 parturition, the second group was inoculated with adjuvant plus phosphate buffer saline (PBS) twice s.c and kept as control**.** Offspring from the vaccinated dams had revealed high titers of specific *R. equi* antibody as detected by tube agglutination (TA) and passive haemagglutination (PH) test and showed protection against challenge dose. The results revealed that vaccination of pregnant G. pigs with the prepared attenuated vaccine was safe and efficient method to protect their offspring against experimental challenge with virulent *R.equi*. Vaccination was associated with increased humoral immune response in vaccinated group.

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Introduction

Rhodococcusequi formerly named *Corynebacterium equi* is a gram-positive, facultative, intracellular pleomorphic bacterium initially described as a veterinary pathogen that caused a pyogranulomatous lung disease in young foals between 1 and 5months of age (Al-Salihi *et al.*, 2013; Al-Salihi, 2011; Horowitz *et al.*, 2001). It also recognized as a pathogen of human that the organism is being isolated with increasing frequency from sputum and blood cultures of individuals with AIDS and patients

undergoing immune-suppressive therapy (Prescott, 1991). Thus, prevention of infection remains a desirable goal. Affected foals require costly and prolonged antibiotic therapy 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).

Passive transfer of immunity plays a critical role in the foal's resistance to a variety of infectious agents and the failure of passive transfer is associated with increased susceptibility to infections (Kohn *et al.,* 1989). Opsonisation with antibodies against capsular components has been demonstrated in the past, with significant enhancement of *R. equi* killing by alveolar macrophages exposed to immune serum (Hietala and Ardans, 1987). Opsonising IgG antibodies appear to promote *R. equi* phagocytosis and participate in the down-regulation of intracellular growth by enhancing bacterial killing, involving phagosome –lysosome fusion (Cauchard *et al.,* 2004). The clearance of *R. equi* in the horse also coincides with IgG production (Lopez *et al.,* 2002). No vaccines are available, although several immunization strategies have been tested to prevent rhodococcosis (Giguère *et al.,* 2003). Various strategies have been proposed for the development of a safe and effective vaccine against rhodococcosis. For instance, the passive immunization with hyperimmune plasma (Prescott *et al.,* 1997), vaccination procedures with inactivated *R. equi* strains (Varga *et al.,* 1997),VapA DNA vaccine (Haghighi *et al.,* 2005) and, more recently, attenuated *R. equi* vaccine have been reported (van der Geize *et al.,* 2011).Among the different strategies of vaccination for intracellular pathogens, a promising approach may be the use of live vaccines (Lopez *et al.,* 2008).

This study was designed to determine the stimulation and passive transfer of humoral immunity due to immunization of pregnant G. pigs with attenuated *R. equi* that aimed to protect their offspring against challenge infection with virulent *R.equi*.

MATERIALS AND METHODS

1-Bacterial isolates

Rhodococcus equi was isolated from foals suppurative bronchopneumonia in *R.equi* endemic farm .The isolate was identified according to potassium tellurite, gram staining, catalase and oxidase reactions, nitrate test, urease production and fermentation of sugars (glucose, lactose, maltose, rhamnose, sucrose and xylose) as described by Nakazawa, (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; its lethal dose $_{50}$ (LD_{50}) in mice was $2x10^7$bacteria by intravenous (IV) route(Reed and Muench, 1938). A Congo red negative *R.equi* (CR-) kindly provided by Al-Azzawi, W. A., its attenuation was previously described (Al-Azzawi *et al.,* 2000).This strain used to prepare attenuated autogenous vaccine.

2-Vaccine preparation

A CR-*R.equi* strain was used to prepare attenuated autogenous vaccine by inoculation three to five colonies into nutrient broth incubated for 24 hours at 37°C and stored in aliquot at -20°C. A flask containing 250 ml of nutrient broth inoculated with one aliquot

incubated at 37° C for 24 hours. This bacterial suspension was used to inoculate Roux bottles containing nutrient agar, then after 48 hours of growth at 37°C bacteria were harvested by suspending the growth from the agar surface in phosphate buffered saline (PBS)PH.7.2. The bacteria were washed three times by centrifugation using PBS. PH.7.2, then resuspended in PBS. Serial dilutions were prepared for viable bacterial counts according to Cruickshank *et al.,* (1975). Bacterial suspension was adjusted at a concentration of ($1x10^9$/ml) viable bacteria and was determined by standard plate techniques. The potassium alum sulphate (KAL $(SO_4)_2$ 1.1/2 H_2O, Fluka) was used as adjuvant. The vaccine tested for sterility, safety, and potency prior to vaccination.

3-Antigens preparation

A-Whole cell preparation

The antigen used for tube agglutination test (TA) was prepared according to Nakazawa, (1980),

B-Soluble antigen

Antigen used for passive haemagglutination (PH) was prepared according to Prescott et al., (1979). Protein content was determined using the method of Lowry *et al.,* (1951).

4-Experimental animals

Two groups of apparently healthy pregnant guinea pigs of similar age (6-9 months) weighing about 400 grams were brought to the animal experimental house of the College of Veterinary Medicine and kept there for three weeks for acclimatization before starting the experiment. The animals were examined serologically for the detection of any antibody titer to *R.equi* prior to vaccination. Several fecal samples were taken from each guinea pig for bacterial isolation. These tests proved that these guinea pigs were *R.equi* free and were not exposed previously to this organism .They were reared in the separated cages and fed commercial assorted pellets and alfalfa.

A-First group (vaccinated group)

Twelve pregnant guinea pigs vaccinated twice five and three weeks prior the expected parturition date s.c 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 LD_{50} of CR+ *R.equi* three weeks after birth.

5-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. Blood samples were collected from the vaccinated and control groups at zero time and weekly thereafter till birth. Also, afterbirth, samples were obtained from the dams and their offspring till two weeks after challenge. Serum samples were separated and frozen at -20 for further investigation.

6-Evaluation of vaccine efficiency

In addition to the resistance of newborn G. pigs from the vaccinated dams to challenge, vaccine was evaluated for humoral immune response in the dams and their offspring by TA according to method described previously by (Nakazawa, 1980) and PH according to method described previously by (Prescott *et al.*, 1979).

Ethical approval: This study was approved by the Ethical and Research Committee of the College of Veterinary Medicine – University of Baghdad.

RESULTS

1- Guinea pigs

A-Clinical observations post vaccination

The pregnant guinea pigs were slightly depressed and listless 72 hours after vaccination. Transient elevation of temperature (39.2 ± 0.2 C), pulse rate (160 ± 5 beat/min) and respiration rate (88 ± 4 breath/min) were recorded for three to five days and returned to normal range in the 6^{th} day post vaccination. Localized swelling was detected during palpation of injected sites after 48 hours, some of them developed to small abscesses and disappeared within two weeks. *R. equi* CR- was isolated from these abscesses. There were no adverse effects in vaccinated pregnant guinea pigs, birth of normal and healthy offspring was given.

The means of body temperature, pulse and respiration rates remained within the normal ranges in the control group, except localized swelling was detected during palpation of the injected sites with adjuvant and PBS which developed to small nodule and disappeared within 10 days. No bacteria were isolated from these nodules.

B- Humoral immune response of dams

All vaccinated and control pregnant guinea pigs had no antibody titer to *R. equi* prior to vaccination. *R. equi* specific antibodies were detected during the third week of the first immunizing, reached their peak three weeks after boosting then declined gradually by the seventh week. Antibody titers to *R. equi* were not detected in control groups (Figure.1).

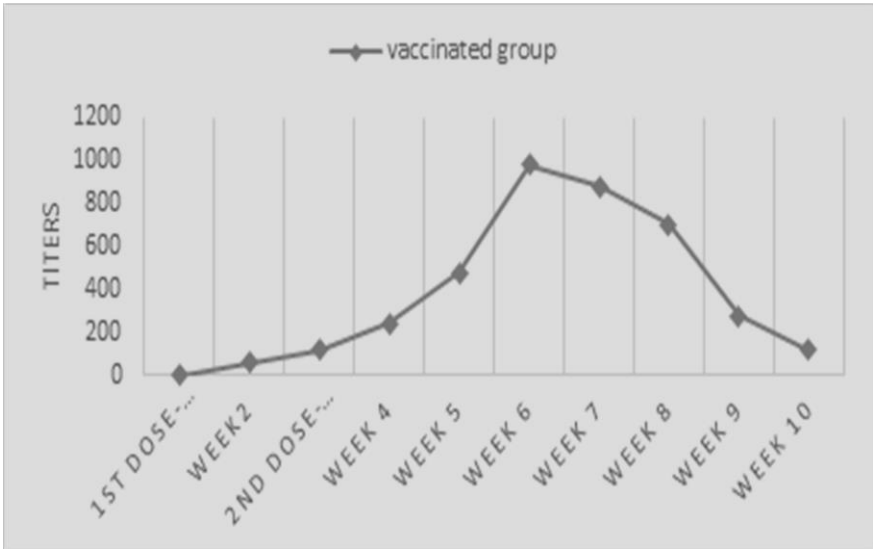

Figure 1. Antibody titer of vaccinated pregnant G. pigs.

C- 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.

D- Humoral immune response of offspring

The *R. equi* antibody titers in sera of offspring from vaccinated dams which reflected maternal immunity are shown in (Figure.2). Low titers of antibodies were detected in sera of offspring from control dams two weeks post challenge only.

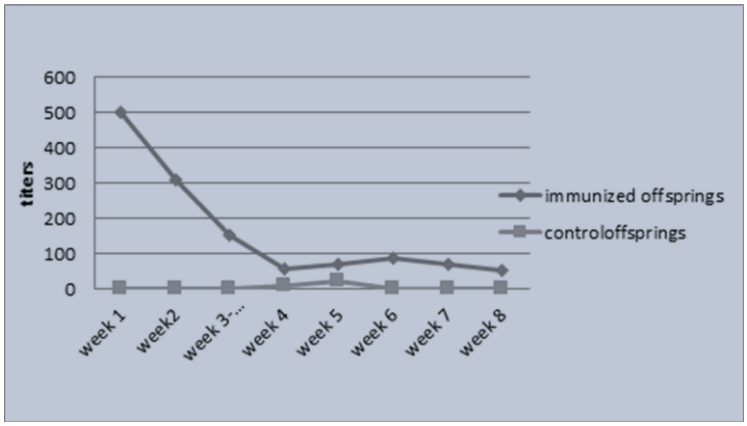

Figure 2. Antibody titers in newborn G. pigs of vaccinated and control dams by PHA.

DISCUSSION

Rhodococcus equi is a facultative, intracellular, gram-positive pathogen that survives and replicates within macrophages causing granulomatous inflammation (Hondalus and Mosser, 1994). Effective immunization against intracellular pathogens such as mycobacteria requires the use of live organism rather than killed one to promote 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).
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. 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.

Subcutaneous vaccination of pregnant guinea pigs with prepared vaccine did not manifest adverse systemic reactions except slight depression and listless for 72 hours and transient elevation in body temperature, increased pulse and respiratory rates for three to five days, which might be the expression of immunological and inflammatory reactions and was consistent with those observed by others (Prescott et al. 1979, 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 (Prescott, 1991). The vaccination procedure appeared safe for the pregnant guinea pigs and without any adverse effects. The vaccinated pregnant guinea pigs with the prepared vaccine revealed elevated levels of *R. equi* antibody titer, which might provide passive protection to their offspring against experimental challenge with the virulent organism. In the present study, antibodies were higher than those determined by Nakazawa, (1980). The discrepancy might be due to the difference between bacterial isolates, dose of *R. equi* exposure, the age and immunological status of animals and the time of exposure relative

to serological test. The results of this study were compatible with those of previous studies (Chirino-Trejo *et al.,* 1987; and Al-Azzawi *et al.,* 2000). They reported that oral and parenteral administration of foals and guinea pigs with attenuated *R. equi* isolates resulted in high titer of *R. equi* antibody which protected foals and guinea pigs against *R. equi* challenge. 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 offspring 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 *et al.,* (2000). While the systemic response was more severe and of longer duration in offspring of 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, 1993).

The offspring of the vaccinated pregnant dams have high titers of *R. equi* antibody which reflected maternal immunity. The passive transfer of humoral immunity is relied upon to protect offspring from many neonatal diseases (Martens *et al.,* 1989). Antigen specific IgG plays a significant role in opsonisation, promoting phagocytosis of virulent *R. equi* by alveolar macrophages and down-regulation of intracellular growth through enhanced bacterial killing capacity (Cauchard *et al.,* 2004).

It appeared that vaccination of pregnant guinea pigs with attenuated *R. equi* vaccine resulted in the production of humoral immunity which was transferred to their offspring and protected them 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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