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Evaluation of the immunization of camels with *Brucella abortus* vaccine (RB51) in Egypt

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Abstract

Background: Brucellosis is a highly contagious zoonotic disease caused by an intracellular facultative microorganism termed *Brucella* spp. Control of brucellosis depends on test and slaughter policy as well as vaccination programs.

Aim: Estimation of the cell-mediated immunity (CMI) [total leukocytic count (TLC), phagocytic activity, phagocytic index, interleukin 6 (IL-6), and tumor necrosis factor-alpha (TNF-α)] in camels after vaccination with RB51 using real-time polymerase chain reaction (PCR).

Methods: A total of eight camels were grouped into two groups as follows: group (A): vaccinated with RB51 vaccine [1 dose/2 ml S/C (3×10^{10} CFU)] and group (B): control group. IL-6 and TNF-α were used for estimation of the CMI using real-time PCR on serum samples that were collected at 0, 7, 14, 21, 28, and 60 days after vaccination from each group. In addition, TLC, phagocytic activity, and phagocytic index were evaluated on heparinized blood samples at 0 and 60 days post-vaccination.

Results: RB51 vaccine provides a protective immune response which progressively increases from the first week to 60 days after vaccination. Moreover, the levels of TNF-α and IL-6 differed between camels in the vaccinated group.

Conclusion: Vaccination of camels with RB51 vaccine (with dose 3×10^{10} CFU) could induce good protective immune responses and this immunological response will be a good indication for a safe field vaccine that can be used for the control of camel brucellosis.

Keywords: Brucellosis, Camel, IL-6, RB51, TNF-α.

Introduction

Brucella spp. is a Gram-negative, facultative intracellular bacteria that causes severely contagious zoonotic disease and affects domestic and wild mammals. *Brucella melitensis, Brucella abortus,* and *Brucella ovis* are the main causes of camel brucellosis (Gwida *et al.*, 2012; Fekadu and Juhar, 2019). Camel brucellosis causes orchitis, epididymitis, stillbirths, infertility, and abortions (Benkirane, 2006).

The eradication programs for brucellosis are based mainly on the prevalence of the disease in each country. When the prevalence of brucellosis is low (<1%–2% seropositive animals), the test and slaughter policy as well as the application of hygienic and sanitary measures was admitted. While in highly infected countries (the seroprevalence > 10%) control programs depend mainly on vaccination of all livestock animals of different ages (OIE, 2016).

The two most often used vaccines, *B. abortus* S19 and *B. melitensis* Rev. 1, interfere with the serological test used to diagnose brucellosis (Refai, 2002). Due to the intracellular nature of the *Brucella* bacteria, which can survive and proliferate inside the macrophages, cell-mediated immunity (CMI) plays a major part in the protective immune response (Martirosyan *et al.,* 2011). In general, proinflammatory cytokines such as interferon, tumor necrosis factor (TNF), and interleukins (IL-2, IL-4, IL-6, IL-10, IL-12, and IL-1), which promote congenital and acquired immunity and regulate the immune response toward immune-associated cells, play a major role in the infection control and recovery in animals. According to Goenka *et al.* (2011) and Xavier *et al.* (2013), the RB51 vaccine

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was obtained from a serial passage of *B. abortus* strain 2308 on trypticase soy addition with 1.5% agar and variable engrossment of rifampicin or penicillin. The lipopolysaccharide (LPS) extracted from RB51 does not contain an O-chain so it is a rough strain not return to smooth colony morphology *in vitro* or *in vivo*. Thus, this vaccine does not interfere with *Brucella* diagnosis using serological tests so it is valuable in the control and eradication of brucellosis in endemic countries as it produces CMI (Schurig *et al.*, 1991).

Finally, quantitative reverse transcription-polymerase chain reaction (Q-RT-PCR) can calculate various genes even with slight physiologic changes in gene activation and can be done with small amounts of the sample so it can be used to detect cytokine gene expression in ruminants. Q-RT-PCR has high specificity, sensitivity, and accuracy. In addition, the high levels of TNF-α produced later reduced bacterial propagation inside the infected cells (Priyanka *et al.*, 2019).

Therefore, this study was directed mainly to estimate the efficacy of *B. abortus* vaccine strain RB51 in camel using Q-RT-PCR as an indicator for TNF-α and IL-6 gene expression about the antigens of *Brucella*.

Materials and Methods

Animals

Eight camels, aged from 1 to 3 years were used in this study. Before vaccination with RB51, deworming of these animals from ecto and endo parasites was applied for four weeks and also they were examined using mRBPT for antibodies against *Brucella* and proved to be free from brucellosis.

Samples

Whole blood samples

Five ml of heparinized blood samples from the jugular vein were collected after vaccination at 0 and 60 days from all camels for measuring total leukocytic count (TLC), phagocytic activity, and phagocytic index. Specimens were directly transported to the Clinical Pathology Laboratory, Clinical Pathology Unit, AHRI—Zagazig Provincial Laboratory within 1 hour.

Serum samples

Five milliliters of blood samples from the jugular vein of all camels were collected from the two groups after vaccination at 0, 7, 14, 21 28, and 60 days without anticoagulant then centrifuged at 3,000 rpm for 10 minutes, and all collected sera used for TNF-α and IL-6 examination.

Vaccination protocol

A total of eight camels were grouped into two groups as follows.

Group (A): Five camels injected with RB51 vaccine (1 dose/2 ml (3×10^{10} CFU) subcutaneously).

Group (B): Three camels were used as a control group.

Brucella abortus RB51 vaccine

Brucella abortus strains RB51 a vaccinal strain, lyophilized vaccine, the vaccine was used in camels by a dose of 2 ml by S/C in the neck area, the dose of 2 ml containing (3×10^{10} CFU), serial No. 1472 (Vacuna RB51® Becerras, Tornel Laboratorios, Mexico).

Estimation of protection efficacy of vaccination schedules

Cellular immunological analysis

Total and differential leukocytic count

It was manually applied by using an improved Neubauer hemocytometer (Feldman *et al.*, 2000) with a diluting fluid (1:10 dilution) of a blood sample to damage the RBCs and stain the nuclei of leukocytes. One hundred cells were counted and classified.

Phagocytic activity and phagocytic index

Preparation of Candida albicans suspension

Making a suspension of *C. albicans* took only an hour after obtaining the strain from the AHRI Provincial Laboratory's Microbiology Division in Zagazig. The *Candida* was prepared according to Xiong *et al.* (2000). The *Candida* strain was dispersed and grown on Sabouraud's dextrose agar plates for 24 hours at 37°C. To kill the *Candida*, several colonies were transferred to a tube covered with 10 ml of physiological normal saline (0.85%), which was then shaken vigorously and vortexed for 10 minutes (Newman and Holly, 2001). After that, it was centrifuged at 1,500 rpm for 8 minutes after being twice washed with phosphate-buffered saline (pH 7.2). The ultimate concentration of *Candida* (107 cells/ml) was manually adjusted using the hemocytometer.

Candida phagocytic activity test

Since neutrophils must participate in phagocytic activity, *C. albicans* must be quickly killed by heat. Next, with minor modifications, Saikia *et al.* (2003) discussed the staining and counting of intracellular *Candida*. For each sample's 1 ml of heparinized blood, a glass tube was filled with 50 µl of fetal calf serum (Sigma) and 50 µl of the *Candida* solution. After being gently mixed, the samples were allowed to settle at 37°C for 20 minutes. Each tube sample was divided into two blood smears, which were then stained with Geimsa stain, fixed with methanol, and examined under a microscope (100×). On each slide, 100 neutrophils were counted, and the positive cells or the number of neutrophils that had been consumed by *Candida* were determined.

Calculation of the phagocytic activity and phagocytic index

The proportion of phagocytized neutrophils to all neutrophils evaluated was known as phagocytic activity. The average number of particles swallowed by each neutrophil undergoing phagocytosis was used to compute the phagocytic index (Berger and Slapnickova, 2003). Number of positive cells/100 cells is the phagocytic index.

Evaluation of protection efficacy of the vaccination schedules through analysis of cytokine-related genes by real-time PCR

Analysis of cytokine-related genes by real-time PCR

RNA extraction (according to kit instructions)

The experiment was carried out using the QIAamp RNeasy Mini kit (Qiagen, Germany, GmbH). 200 µl of the sample was mixed with 600 µl of RLT buffer,

which included 10 µl of β-mercaptoethanol per 1 ml, and the combination was then left to sit for 10 minutes at room temperature. The purification of total RNA procedures was carried out by the instructions for the QIAamp RNeasy Mini kit (Qiagen, Germany, GmbH) using the cleared lysate and 1 l of 70% ethanol. The column underwent DNase digestion to remove any residual DNA.

Oligonucleotide primers

Primers used were obtained from Metabion (Germany) and are listed in Table 1.

SYBR green RT-PCR (according to kit instruction)

The efficiency of the primers was evaluated in a 25 µl experiment that contained 10 µl of the 2× HERA SYBR® Green RT-qPCR Master Mix (Willowfort, UK), 1 µl of RT Enzyme Mix (20×), 0.5 µl of each primer at a concentration of 20 pmol, 5 µl of water, and 3 µl of RNA template. The reaction was run on a real-time PCR machine in step one.

Analysis of the SYBR green RT-PCR results

The normalization of each gene's expression about the expression of β-actin amplification curves and the computation of CT values was done using the step one software. The variance of gene expression on the RNA of the different samples was evaluated using the "Ct" technique, which Yuan *et al.* (2006) described, by comparing the CT of each sample with the CT of the positive control group using the following ratio: ($2^{-}\Delta\Delta^{ct}$).

Statistical analysis

Numbers were given as mean ± SE and count data were expressed as percentages. After the Shapiro-Wilk test, which was successful with a *p*-value more than 0.05, established that the data were normal, one-way analysis of variance was used to analyze the gene expression data. Dennett's test was used to evaluate the significance between each group and the control group. Using a chi-square test for association, the link between brucellosis and each camel's age and sex was evaluated. A one-sample chi-square test was used to examine the significance of different seropositive percentages for different dilutions. At $p < 0.05$, significance was determined. The data were examined using Graph Pad Prism 8.0.2 (Graph Pad Software, Inc.) and SPSS version 25 (Armonk, NY: IBM Corp.).

Ethical approval

The Faculty of Veterinary Medicine's ethical council and the Animal Health Research Institute in Dokki, Egypt (Approval No. ZU-IACUC/2/F/308/2022) guided how to handle and care for the animals.

Results

Post-vaccination observations

Clinical examination of camels revealed that there were no post-vaccinated reactions. There were nonsignificant variations in body temperature and appetite noticed in any of the vaccinated groups throughout the 14 days after vaccination.

Leukogram and phagocytic activity of camels' post-vaccination

The results revealed that there was a significant increase in leukocytes till 60 days post-vaccination. The result of differential leukocytic count found a significant increase in lymphocytes and monocytes, whereas there were no significant changes in neutrophil, basophil, and eosinophil post-vaccination compared with control groups (Table 2). The results of phagocytic % and phagocytic index increased in the vaccinated group than control group (Table 3).

Table 1. Primers sequences, target genes, amplicon sizes, and cycling conditions for SYBR green real-time PCR.

Target gene	Primers sequences	Reverse transcription	Amplification (40 cycles) Primary Denaturation	Secondary denaturation	Annealing (Optics on)	Extension	Reference
B-actin	CGTGGGCCGCCCTAGGCACCA	50°C	94°C	94°C	55°C	72°C	Fitzpatrick *et al.* (2002)
	GGGGGCCTCGGTCAGCAGCAC	30 minutes	15 minutes	15 seconds	30 seconds	30 seconds	
IL-6	CCAGCCACAAACACTGACCT						Wooldridge and Ealy (2019)
	TAGCTCTCAGGCTGAACTGC						
TNF-*α*	TCCATCAACAGCCCTCTGGT						Shu *et al.* (2011)
	TGAGGCTTGAGAAGAGGACCTGA						

Table 2. Leukogram of the experimental camels post-vaccination.

Group	WBCs 103/cm	Lymphocytes	Neutrophils	Monocytes	Eosinophils	Basophils
Vaccinated camels	17.10 ± 3.63*	6.68 ± 1.71*	9.24 ± 1.76^{ns}	0.74 ± 0.14*	0.38 ± 0.08^{ns}	0.05 ± 0.01
Control	12.07 ± 0.45	4.59 ± 0.22	6.47 ± 0.22	0.54 ± 0.03	0.42 ± 0.04	0.04 ± 0.01

*Indicate significance difference with control (*p*-value < 0.001).
ns: nonsignificant difference with control.

Result of cytokine analysis (CMI response) of vaccinated camels

Q-RT-PCR was used for the detection of the mRNA expressions of TNF-α and IL-6. The fold differences were used to determine the expressing variance in the vaccinated and the control group and there was significant variation in the levels of TNF-α and IL-6 among the vaccinated group that appeared 21 days post-vaccination (Table 4).

Discussion

Controlling camel brucellosis is a huge problem and needs extensive struggles in developing countries. It is established that vaccination increases the population's resistance to the disease. Effective vaccine almost eradicates the clinical symptoms of the disease and reduce the contamination of the environment and population at risk to the infectious agent as well.

This study aimed to estimate the efficacy of *B. abortus* vaccine strain RB51 in camels. The efficacy was determined by the cellular immune response. *Brucella* microorganisms can survive and multiply inside the macrophages because of their facultative intracellular nature so CMI is regarded as essential for immune system protection (Nicoletti and Winter, 1990; Martirosyan *et al.*, 2011). CMI is characterized by the activation of macrophages by antigen-specific T cells leading to the destruction of the microorganism (Oliveira *et al.*, 1998).

The ideal vaccine against brucellosis should be easily produced and stored, stable, long immunogenic duration, have minimum interference with serological tests, least undesirable effects in vaccinated animals, and not cause brucellosis in men in case of accidental exposure (Nicoletti and Winter, 1990), eliminate the clinical symptoms of the disease and decrease the contamination of the environment (Olsen, 2013).

The TLC was observed to be the highest in camel (1) in comparison to the others and this might be attributed to the microbial pressure (dose 3×10^{10} CFU) (Table 2). Whereas the phagocytic activity and phagocytic index in camel (4) was higher than other camels (Table 3). These results are similar to Higgins *et al.* (2018) who found an increase in pro-inflammatory response characterized by an increased number of lymphocytes after Rev.1 vaccination in goats. Moreover, CMI is characterized by the stimulation of macrophages through antigen-specific T-cells. The cellular immune response indicated by TLC was shown to be significantly greater in vaccinated camels than in control. In addition, phagocytic activity and phagocytic index in vaccinated camels were significantly greater than control. This may be attributed to the virulence of the RB51 live vaccine. This result agreed with Jonathon (2009).

Recently, Q-RT-PCR was used to estimate the cytokine gene expression because it can quantify messenger RNA (mRNA) of any gene and can use a minor amount of sample with high sensitivity, specificity, and accuracy (Giulietti *et al.*, 2001; Priyanka *et al.*, 2019). Cytokine evaluation has a main role in determining the status of the immune response and detecting the severity of the infection. Both TNF-α and IL-6 elevated in acute or chronic brucellosis and had a significant role in the elimination of *Brucella* microorganisms from the macrophages (David *et al.*, 2018; Lin *et al.*, 2020).

Our results revealed that the RB51 vaccine stimulated the production of a highly immune response represented by high levels of IL6 and TNF-α inside the body. There was a significant fold change of IL-6 and TNF-α at 21st, 28th, and 60th day. There was a progressive expression of the IL-6 was found to be increased from the 1st week till the 60th day post-vaccination (Table 4 and Fig. 1).

Table 3. The phagocytic activity and phagocytic index post vaccination.

Group	Phagocytic %	Phagocytic index
Vaccinated camels	76.67 ± 2.27	4.72 ± 0.13*
Control	63.67 ± 1.08	2.33 ± 0.06

*Indicate significance difference with control (p-value < 0.001).
ns: nonsignificant difference with control.

Table 4. Cytokine gene expressions of IL-6 and TNF-α as a comparison between the vaccinating group with the control group, results in the table represent mean ± SE.

	Fold change		CT		
	IL-6	TNF-α	β-actin	IL-6	TNF-α
Control	1 ± 0	1 ± 0	27.83 ± 0.31	28.93 ± 0.36	27.89 ± 0.27
7th day	4.96 ± 0.67 ns	10.19 ± 0.98 ns	27.83 ± 0.46 ns	26.69 ± 0.49**	24.37 ± 0.74**
14th day	14.09 ± 1.63 ns	30.11 ± 4.17 ns	28.63 ± 0.155 ns	25.69 ± 0.22***	23.64 ± 0.24***
21th day	40.63 ± 7.75***	82.96 ± 13.45*	26.07 ± 0.24**	21.96 ± 0.48***	19.64 ± 0.35***
28th day	55.08 ± 8.46***	193.6 ± 28.34***	27.9 ± 0.31 ns	23.04 ± 0.29***	20.23 ± 0.42***
60th day	72.44 ± 6.72***	221.4 ± 18.07***	27.58 ± 0.20 ns	22.61 ± 0.22***	19.87 ± 0.47***

*, **, ***Indicate significance difference with control.
ns: nonsignificant difference with control; TNF-α: tumor necrosis factor-alpha; IL-6: interleukin 6; CT: cycle threshold.

These results revealed a constant release of Th1 cytokine response in RB51 vaccinated camel with a dose of 3 × 10^{10} CFU, which may be useful for developing long-term immunity against *Brucella* infections. Our findings agreed with that reported by Hop *et al.* (2019) who found that great levels of IL-6 stimulate the bactericidal activity of macrophages and differentiation of $CD8^+$ T cells, leading to the Th1 immune response against *Brucella* infection. In addition, Ramy *et al.* (2022) found that the level of IL-6 begins to increase from first-week post-vaccination of ewes with RB51. Moreover, Odbileg *et al.* (2008) found a significant increase in the level of IL-6 from first-week post-vaccination of camel with *B. abortus* S19.

The progressive expression of TNF-α reflected the stimulation of Th1-type immune cells by LPS or endotoxin (Table 4 and Fig. 2). The high production of TNF-α leads to successive reduction of proliferation of the bacteria inside the infected cells (Ottones *et al.*, 2000; Dornand *et al.*, 2002). In addition, Ghalib *et al.* (1995) and Ko *et al.* (2002) reported that stimulation of TNF-α cytokine mRNA played a main role in cellular immune response regulation against brucellosis and other intracellular infections.

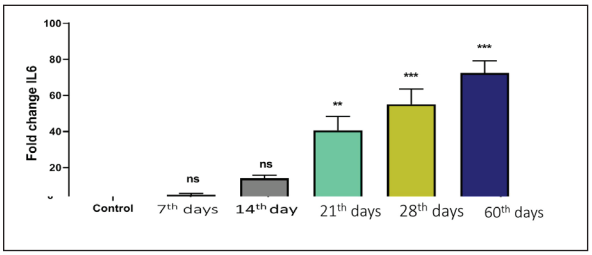

Fig. 1. Comparing the effect of gene expression on IL-6 in vaccinated and control groups. Bars represent mean ± SE. bars carrying ** and *** represent significant differences with the control group, $p < 0.001$, and bars with ns represent nonsignificant differences with the control.

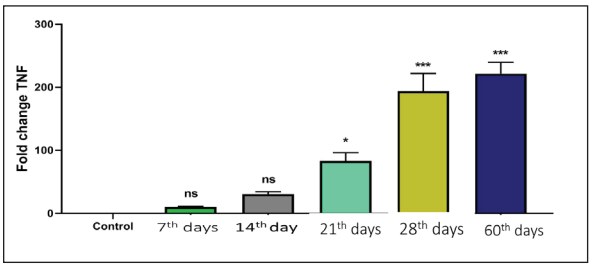

Fig. 2. Comparing the effect of gene expression on TNF-α in vaccinated and control groups. Bars represent mean ± SE. bars carrying ** and *** represent a highly significant difference with the control group, $p < 0.001$. These results revealed a constant release of Th1 cytokine (especially TNF-α) response in RB51 vaccinated camel with a dose of 3 × 1010 CFU, which may be useful for developing long-term immunity against *Brucella* infections.

Conclusion

To the best of our knowledge, this is the first report that has evaluated the cell-mediated response post RB51 vaccination in camels in Arabian countries. We found that RB51 (3 × 10^{10} CFU) could activate a cellular Th1 immune response against camel brucellosis and prove that the control strategy against *B. melitensis* in Egypt needs rethinking to improve vaccine efficacy.

Conflict of interest

The authors declare that they have no conflict of interest.

Data availability

All data are provided in the manuscript. Any extra data needed can be provided by the corresponding author upon reasonable request.

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