Received: 01 October 2022, Accepted: 21 October 2022

Original Research

Journal of Advanced Veterinary Research
(2023) Volume 13, Issue 1, 1-8

# Comparative Immune Responses and Cytokine Gene Expressions in Sheep Vaccinated with *Brucella abortus* RB51 Vaccine and *Brucella melitensis* Rev. 1 Vaccine

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## Abstract

This work applied three *Brucella* vaccination protocols in adult *Brucella*-free ewes. Serum and blood samples were collected from each group at time points 0, 7, 14, 21, 28, and 60 days post-vaccination. In addition, the humoral immune response was assessed by the Rose Bengal Plate test (RBPT) every week for 16 weeks. Also, cell-mediated immunity was evaluated. Additionally, interleukin 6 (IL-6) and tumor necrosis factor-alpha (TNF- α) were assessed. The results indicated that the cellular immune responses induced by the *B. abortus* RB51 vaccine with a dose of $3.4x10^{10}$ provide a protective immune response near the effect produced by the *B. melitensis* Rev. 1 vaccine. Moreover, the IL-6 was expressed significantly less in the RB51- vaccinated group with a dose of $1.7x10^{10}$ CFU (1 ml subcutaneously), greater in the Rev. 1-vaccinated group with a dose of $1.6\times 10^{9}$ CFU (1 ml subcutaneously), and highest expression in the RB51-vaccinated group with a dose of $3.4x10^{10}$ CFU (2 ml subcutaneously). Also, the expression of TNF- α was lowest in the group vaccinated with RB51 vaccine with a dose of $1.7x10^{10}$ CFU, with greater abundance in the group vaccinated with Rev. 1 vaccine and highest expression in the group vaccinated with RB51 vaccine with a dose of $3.4x10^{10}$ CFU. These findings imply that the RB51 vaccine, given to sheep with a dose of $3.4x10^{10}$ CFU, may trigger protective immune responses and be applied in the field to control brucellosis in sheep.

KEYWORDS
*Brucella melitensis*, Rev. 1, RB51, Immunity, Sheep.

## INTRODUCTION

Brucellosis causes significant financial harm in sheep and goat herds due to abortion and fertility problems. In addition, it causes Malta fever in humans. Brucellosis is endemic in Egypt and continues to be an uncontrollable problem throughout the country (Hegazy *et al*., 2011). Various serological surveys of sheep and goat brucellosis revealed varying levels of infection in various governorates ( Wareth *et al*., 2014; Abdel-Hamid *et al*., 2017; Eltholth *et al*., 2017). Besides that, *B. melitensis* biovar 3 is the most prevalent cause of brucellosis in sheep and goats in Egypt (Abdel-Tawab *et al*., 2018; El-Diasty *et al*., 2021; Hosein *et al*., 2021; El-Diasty *et al*., 2022; Hegazy *et al*., 2022). Ovine brucellosis has been eradicated in many countries thanks to long-term management strategies that include vaccination, serological testing, and culling of positive reactors (Blasco, 1997). Among the vaccines used to protect sheep and goats from *B. melitensis*, is the live *B. melitensis* (Rev. 1 vaccine) (Garin-Bastuji and Blasco, 2004). However, by administering this vaccine subcutaneously, a strong and prolonged immune response can be induced, interfering with serological testing that might be challenging to distinguish from that caused by infection, potentially causing an abortion in female animals given the vaccine while pregnant and possible Rev.1 genital fluids or milk excretion following vaccination (Olsen, 2013; Higgins *et al*., 2017; xie *et al*., 2018). This is problematic because, depending on the challenge, these vaccinations do not always provide complete protection; hence, serological tests may not always reveal whether a specific animal is infected or simply displays post-vaccinal antibodies (Moriyón *et al*., 2004). Besides, Rev-1 is pathogenic to humans (Ebrahimi *et al*., 2012; Goel *et al*., 2013).

Recently, the rough *B. abortus* mutant (RB51) was used to generate a new vaccine for cattle vaccination (Schurig *et al*., 1991). When administered subcutaneously, the vaccine does not result in the development of antibodies (anti-O-side chain) that interfere with serological tests using lipopolysaccharide (LPS) as an antigen, such as the RBPT, complement fixation test, standard tube agglutination test, etc. because the smooth LPS on the bac-


ISSN: 2090-6277/2090-6269/ 

terial cell wall is absent from the *B. abortus* RB51 strain (Stevens *et al*., 1994).

Given RB51's success in immunizing cattle, it may also be beneficial in protecting sheep and goats. A powerful vaccination that has the advantage of not reacting with serological diagnosis would help control and eradicate sheep and goat brucellosis in the countries where it is widespread (Roop *et al*., 1991), and it guarded up to 93% of goats (vaccinated with RB51) against *B. melitensis* infection (WHO, 1998). Conversely, scarce information is available on the use of the RB51 vaccine in sheep.

Pro-inflammatory cytokines, which are released by cells involved in inflammation, are crucial in controlling immunological reactions (both humoral and cellular immune responses). They induce the release of secondary cytokines through their effects on antigen-presenting cells and T-helper lymphocytes, such as lymphocytes B, and work in conjunction with them to promote the activation and proliferation of lymphocytes B as well the production of various classes of plasma cells and immunoglobulins. Typically membrane-bound and soluble, cytokines interact with their particular receptors (Pietruczuk *et al*., 2001). Therefore, it is generally understood that cytokine expression is necessary for the immune response to function normally and defend itself. The cytokine levels also provide information on the state of the immune system and the progression of the disease. The features of immunological responses to brucellosis have been evaluated using cells and animal models (Goenka *et al*., 2011; Vitry *et al*., 2014). Proinflammatory cytokines that regulate innate and adaptive immunity and mediate the immune response among immune-associated cells, such as TNF- α, interferon-gamma, and interleukins (IL-1, IL-2, IL-4, IL-6, IL-10, and IL-12), are typically expressed during infection control and recovery in animals (Cha *et al*., 2010; Goenka *et al*., 2011; xavier *et al*., 2013). There are various procedures available to evaluate neutrophil functions, many of which, while accurate, need knowledge and money. These techniques involve flow cytometry, chemotaxis assays, spectrophotometric assays, superoxide assays, and immunoblotting. The Nitro blue Tetrazolium reduction test (NBT), however, is less expensive and technically challenging (Jonathan, 2009; Srivani, 2019).

Hence, the goal of the current work was to compare the effectiveness of the local Rev. 1 vaccine, which is primarily used to prevent brucellosis in sheep in Egypt, with the *B. abortus* vaccine strain RB51 in sheep. This was done by measuring the expression of TNF-α and IL-6, via RT-PCR (as a marker for cytokine gene expression) in response to *Brucella* antigens, along with the total leukocytic count (TLC), the mean Nitroblue tetrazolium test (NBT %), phagocytic activity, and the index could offer information for the expansion of new immunodiagnostic tools and immunization approaches for sheep brucellosis.

## MATERIALS AND METHODS

*Ethical approval*

The Faculty of Veterinary Medicine at Mansoura University and the Animal Health Research Institute in Dokki, Egypt's ethics committees' regulations were followed in terms of animal care and study protocols.

*Brucella vaccines and vaccinations*

As recommended by the respective manufacturers, two vaccines were used in this study: *Brucella abortus* strain RB51 vaccinal strain (Vacuna RB51® Becerras, Tornel Laboratorios, Mexico) was used in two doses of 1 and 2 ml, containing $1.7\times10^{10}$ CFU and $3.4\times10^{10}$ CFU, respectively. The vial was reconstituted in 20 ml diluent (0.15M NaCl, pH 6.4) and subcutaneously administered. The second vaccine was *Brucella melitensis* Rev.1 vaccine was obtained from the Veterinary Serum and Vaccine Research Institute (VSVRI), Abbassia, Cairo 11517, Egypt. The Rev. 1 vaccine was subcutaneously administered in the neck in a dose of 1 ml per animal containing $1.6\times10^{9}$ CFU.

*Animals and vaccination protocol*

The Rahmani sheep, which may be recognized by its short ears and red wool, is Egypt's largest breed of animal. It can be found in the Central and Northern Delta (Galal *et al*., 2005). In this study, 20 Rahmani, seminomadic, and brucellosis-free ewes of approximately 12 to 14 months of age were isolated for one month and treated against external and internal parasites. Before vaccination, they were serologically tested for brucellosis by RBPT and then grouped into 4 groups as follows:
Group (A): Animals were subcutaneously vaccinated with the *B. abortus* RB51 vaccine at a dose of 2 ml ($3.4x10^{10}$ CFU).
Group (B): Animals were subcutaneously vaccinated with the *B. abortus* RB51 vaccine at a dose of 1 ml ($1.7x10^{10}$ CFU).
Group (C): Animals were subcutaneously vaccinated with the *B. melitensis* Rev.1 vaccine at a dose of 1 ml ($1.6\times10^{9}$ CFU).
Group (D): Control animals did not receive any vaccines.

*Evaluation of the humoral immune response of vaccinated ewes by using serological tests*

Blood samples were collected from all groups of sheep (Group A, B, C, and D) every week for 4 months. Blood samples were aseptically collected from the jugular vein without anticoagulant, then placed on ice and processed by centrifugation (10 minutes at 3000 rpm). All serum samples were tested for *Brucella* antibodies by *Brucella* antigen (RBPT). The RBPT was done on all sera according to Alton *et al*. (1975). Equal amounts (25 µl each) of the colored Rose Bengal Antigene (Veterinary Serum and Vaccine Research Institute (VSVRI), Abbassia, Cairo, Egypt) and tested sera were gently mixed on a clean glass slide. Then, the slide was observed for 3 minutes for agglutination. The formation of agglutinations indicated a positive result while the absence of clear agglutinations was considered a negative result.

*Evaluation of protective efficacy of vaccination schedules*

Cellular immunological analysis

*Collection of whole blood samples*

Whole blood samples (heparinized) from 5 experimental ewes in every four groups were collected at time points 0, 7, 14, 21, 28, and 60 days post-vaccination via jugular vein puncture to measure some cellular immune responses (TLC, Nitroblue tetrazolium test, neutrophilic activity, and index). Samples were transported immediately to the clinical pathology laboratory, Faculty of Veterinary Medicine, Mansoura University, within one hour.

*Total leukocytic count*

The total leukocytic count was performed manually using an improved Neubauer hemocytometer (Santoro, 2018). The blood sample was diluted (1:20 dilution) with a diluting solution (turkey's solution), which eliminates the red blood cells and stains the leukocyte nuclei. Blood film stained with Giemsa stain was

used to asses the differential leukocytes count.

*Phagocytic activity and phagocytic index*

Preparation of Candida albicans suspension

The *C. albicans* strain was kindly provided by the Microbiology Department of Faculty of Veterinary Medicine, Mansoura University, Egypt. The candida was prepared following the guidelines provided by xiong *et al.* (2000). On Sabouraud's dextrose agar plates, the candida strain was strewn, then cultivated for 24 h at 37 °C. Some colonies were well-shacked, transferred to a tube with 10 ml of 0.85% physiological saline, and vortexed for 10 minutes. For 60 minutes, the tube was incubated in a hot water bath at 56 °C to kill the candida (Newman and Holly, 2001). It was then rinsed twice with phosphate-buffered saline (pH 7.2) after being centrifuged for 8 minutes at 1,500 rpm. The ultimate concentration of candida ($10^7$ cells/ml) was manually adjusted using the hemocytometer. The suspension was kept in the refrigerator until it was needed.

Candida phagocytic activity test

Phagocytic activity of the neutrophil leukocytes depends on the engulfing of heat-killed Candida albicans by neutrophils over a brief period (20 minutes). The stained intracellular Candida can be identified within the neutrophils and calculated. It was executed according to Saikia *et al.* (2003) with few modifications. Each sample's one milliliter of heparinized blood was combined with 50 µl of fetal calf serum (Sigma) and 50 µl of the Candida solution in a glass tube. The samples were combined and incubated for 20 minutes at 37 °C. Each tube sample was divided into two blood smears, which were then preserved with methanol, stained with Giemsa stain, and seen under a microscope (100×). One hundred neutrophils/slide were counted. Neutrophils that had been consumed by Candida were counted as positive cells.

Calculation of the phagocytic Index and phagocytic activity

Phagocytic activity was defined as the ratio of phagocytized neutrophils to all neutrophils examined. The phagocytic index was derived from the average number of particles eaten during neutrophil phagocytosis (Berger and Slapničková, 2003). Phagocytic index= number of positive cells/100 cells.

The Nitroblue tetrazolium reduction test (NBT%)

The Nitroblue tetrazolium test (NBT) was made according to Jonathan (2009) with little modification. To create the NBT solution (0.2%), which was freshly made before the test, the NBT dye (Sigma USA) was briefly dissolved in PBS (pH 7.2). Each sample's equal volume of heparinized blood and NBT solution was agitated in a glass tube before being incubated at 37 ºC for 15 minutes. After drying the slides, they were fixed with methanol (97%) for 3 minutes. Then the slides were air-dried and then stained with safranin 0.5% (Sigma USA) for 5 min. The slides were air dried, gently washed with tap water, and examined under an oil immersion lens (VISION 2000, LABOMED, Chennai) to count 100 neutrophil cells and record (using the traditional method) the proportion of cells with clear particles that were blue, black, or coarse in the cytoplasm to cells that did not have any particles. NBT readily enters the cells, and phagosomes reduce the dye intracellularly to produce an insoluble blue crystalline form (formazan crystals). The intracellular reduction is essential for microbicidal activity and depends on the activation of the hexose monophosphate shunt. The results were recorded as NBT%.

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

Collection of serum samples

Blood samples from five experimental ewes in each of the four groups were obtained at time points 0, 7, 14, 21, 28, and 60 days post-vaccination via jugular vein puncture without anticoagulant. After clotting of the blood, all blood samples were centrifuged (10 minutes at 3000 rpm), and sera were collected for analysis of TNF- α and IL-6.

Analysis of cytokine-related genes by real-time PCR

*RNA extraction*

The RNA extraction from serum samples was done using a QIAamp RNeasy Mini kit (Qiagen, Germany, GmbH). A total of 200 µl of the sample were added to 600 µl RLT buffer containing 10 µl β-mercaptoethanol per 1 ml and then incubated at room temperature for 10 min. One volume of 70% ethanol was added to the cleared lysate, and the procedures were completed according to the purification of the total RNA protocol of the QIAamp RNeasy Mini kit (Qiagen, Germany, GmbH).

*SYBR green RT-PCR*

A total of 25- µl mixture containing 10 µl of the 2x HERA SYBR® Green RT-qPCR Master Mix (Willowfort, UK), 1 µl of RT Enzyme Mix (20X), 0.5 µl of each primer of 20 pmol concentration, 5 µl of water, and 3 µl of RNA template were used. The supplied

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

| Target gene | Primers sequences | Reverse transcription | Primary denaturation | Amplification (40 cycles) | | | References |
|---|---|---|---|---|---|---|---|
| | | | | Secondary denaturation | Annealing | Extension | |
| *B- actin* | CGTGGGCCGCCCTAGGCACCA<br>GGGGGCCTCGGTCAGCAGCAC | 50˚C<br>30 min. | 94˚C<br>15 min. | 94˚C<br>15 sec. | 55˚C<br>30 sec. | 72˚C<br>30 sec. | Fitzpatrick *et al*. (2002) |
| IL-6 | CCAGCCACAAACACTGACCT<br>TAGCTCTCAGGCTGAACTGC | | | | | | Wooldridge and Ealy (2019) |
| TNF-α | TCCATCAACAGCCCTCTGGT<br>TGAGGCTTGAGAAGAGGACCTGA | | | | | | Shu *et al*. (2011) |

IL-6; interleukin 6; TNF- α: Tumor necrosis factor-alpha.

primers from Metabion, Germany are mentioned in (Table 1).

*Analysis of the SYBR green RT-PCR results*

The expression of each gene was normalized relative to the expression of β-actin amplification curves, and Ct values were determined by the step one software. The CT of each sample was compared with that of the positive control group according to the "$^{\Delta\Delta}$Ct" method stated by (Yuan *et al.*, 2006), to estimate the variation of gene expression on the RNA of the different samples, using the following ratio ($2^{-DDct}$).

*Statistical analysis*

The data were investigated using SPSS software. The mean and standard deviation are used to express the results of the numerical data. An unpaired t-test and a one-way ANOVA were done to analyze the statistical significance. $P < 0.05$ was taken as the level of significance. The Chi-square test was used to assess the relationship between categorical variables. Proportions are used to express categorical data.

## RESULTS

*Post-vaccination findings*

There were no clinical signs post-vaccination. Neither loss of appetite nor febrile response were detected in any of the treated groups during the 14 days following up vaccination. About 80% of vaccinated ewes with Rev. 1 showed a discrete nodule at the

Table 2. Results of humoral immune response of vaccinated ewes using RBPT

| Groups | Pre-vaccination | Weeks post-vaccination | | | | | | | | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 10 | 11 | 12 | 13 | 14 | 15 | 16 | 17 | 18 | 19 | 20 |
| Group (A) | 0 | 0/5 | 0/5 | 0/5 | 0/5 | 0/5 | 0/5 | 0/5 | 0/5 | 0/5 | 0/5 | 0/5 | 0/5 | 0/5 | 0/5 | 0/5 | 0/5 | 0/5 | 0/5 | 0/5 | 0/5 |
| Group (B) | 0 | 0/5 | 0/5 | 0/5 | 0/5 | 0/5 | 0/5 | 0/5 | 0/5 | 0/5 | 0/5 | 0/5 | 0/5 | 0/5 | 0/5 | 0/5 | 0/5 | 0/5 | 0/5 | 0/5 | 0/5 |
| Group (C) | 0 | 1/5 | 3/5 | 5/5 | 5/5 | 5/5 | 5/5 | 4/5 | 4/5 | 4/5 | 3/5 | 3/5 | 2/5 | 3/5 | 2/5 | 1/5 | 1/5 | 0/5 | 0/5 | 0/5 | 0/5 |
| Group (D) | 0 | 0/5 | 0/5 | 0/5 | 0/5 | 0/5 | 0/5 | 0/5 | 0/5 | 0/5 | 0/5 | 0/5 | 0/5 | 0/5 | 0/5 | 0/5 | 0/5 | 0/5 | 0/5 | 0/5 | 0/5 |

Table 3. The leukogram in post-vaccination for the different groups of the experimental ewes.

| DPV | Group | TLC $10^3/\mu L$ | Lymph. $10^3/\mu L$ | Neut. $10^3/\mu L$ | Mono. $10^3/\mu L$ | Eosin. $10^3/\mu L$ | Baso. $10^3/\mu L$ |
|---|---|---|---|---|---|---|---|
| Zero day | A | 8.86±0.79$^a$ | 5.23±1.16$^a$ | 3.55±0.88$^a$ | 0.05±0.07$^a$ | 0.02±0.02$^a$ | 0.02±0.04$^a$ |
| | B | 9.21±2.04$^a$ | 5.65±1.37$^a$ | 3.36±0.83$^a$ | 0.12±0.14$^a$ | 0.05±0.08$_a$ | 0.02±0.04$^a$ |
| | C | 8.25±1.36$^a$ | 4.79±1.33$^a$ | 3.38±0.66$^a$ | 0.03±0.04$^a$ | 0.03±0.04$^a$ | 0.02±0.04$^a$ |
| | Control | 8.75±1.29$^a$ | 5.61±0.89$^a$ | 2.97±0.56$^a$ | 0.09±0.06$^a$ | 0.04±0.09$^a$ | 0.04±0.09$^a$ |
| | *P* value | 0.768 | 0.65 | 0.659 | 0.361 | 0.867 | 0.917 |
| 7$^{th}$ day | A | 15.63±3.29$^a$ | 11.54±1.28$^a$ | 3.94±2.52$^a$ | 0.13±0.13$^a$ | 0.03±0.06$^a$ | 0.00±0.00$^a$ |
| | B | 13.37±1.38$^a$ | 10.14±2.20$^a$ | 2.99±1.73$^a$ | 0.19±0.19$^a$ | 0.04±0.07$^a$ | 0.00±0.00$^a$ |
| | C | 13.61±1.38$^a$ | 9.75±2.04$^a$ | 3.66±1.57$^a$ | 0.15±0.16$^a$ | 0.05±0.07$^a$ | 0.00±0.00$^a$ |
| | Control | 9.08±1.77$^b$ | 5.77±1.59$^b$ | 3.17±0.43$^a$ | 0.08±0.09$^a$ | 0.04±0.05$^a$ | 0.02±0.05$^a$ |
| | *P* value | 0.001 | 0.001 | 0.816 | 0.682 | 0.914 | 0.418 |
| 14$^{th}$ day | A | 9.75±2.57$^a$ | 6.56±1.86$^a$ | 3.04±1.10$^a$ | 0.12±0.13$^a$ | 0.02±0.05$^a$ | 0.00±0.00$^a$ |
| | B | 8.33±4.19$^a$ | 5.54±3.03$^a$ | 3.68±1.24$^a$ | 0.068±0.07$^a$ | 0.04±0.06$^a$ | 0.00±0.00$^a$ |
| | C | 10.37±3.66$^a$ | 7.54±3.09$^a$ | 2.74±1.47$^a$ | 0.09±0.09$^a$ | 0.00±0.00$^a$ | 0.00±0.00$^a$ |
| | Control | 8.26±3.03$^a$ | 4.88±2.10$^a$ | 3.26±1.18$^a$ | 0.08±0.10$^a$ | 0.02±0.05$^a$ | 0.02±0.44$^a$ |
| | *P* value | 0.738 | 0.386 | 0.749 | 0.837 | 0.647 | 0.418 |
| 21$^{st}$ day | A | 10.21±1.82$^a$ | 7.10±0.74$^a$ | 2.95±1.30$^a$ | 0.12±0.11$^a$ | 0.04±0.05$^a$ | 0.00±0.00$^a$ |
| | B | 8.00±3.53$^a$ | 5.85±2.59$^a$ | 2.01±1.41$^a$ | 0.096±0.07$^a$ | 0.04±0.08$^a$ | 0.00±0.00$^a$ |
| | C | 9.77±2.79$^a$ | 7.09±1.28$^a$ | 2.55±1.59$^a$ | 0.09±0.09$^a$ | 0.02±0.052$^a$ | 0.02±0.05$^a$ |
| | Control | 8.02±1.73$^a$ | 4.40±1.21$^a$ | 3.43±1.33$^a$ | 0.09±0.073$^a$ | 0.07±0.08$^a$ | 0.0±0.05$^a$ |
| | *P* value | 0.569 | 0.047 | 0.421 | 0.937 | 0.801 | 0.338 |
| 28$^{th}$ day | A | 10.21±3.18$^a$ | 5.74±2.32$^a$ | 4.42±1.18$^a$ | 0.02±0.04$^a$ | 0.03±0.04$^a$ | 0.00±0.00$^a$ |
| | B | 7.90±2.20$^a$ | 4.44±0.13$^a$ | 3.28±1.32$^a$ | 0.05±0.08$^a$ | 0.11±0.08$^a$ | 0.02±0.05$^a$ |
| | C | 8.88±2.37$^a$ | 5.53±1.48$^a$ | 3.12±0.89$^a$ | 0.10±0.11$^a$ | 0.10±0.11$^a$ | 0.02±0.04$^a$ |
| | Control | 9.46±3.06$^a$ | 5.09±2.22$^a$ | 4.11±1.24$^a$ | 0.10±0.09$^a$ | 0.12±0.10$^a$ | 0.04±0.05$^a$ |
| | *P* value | 0.604 | 0.709 | 0.261 | 0.377 | 0.345 | 0.554 |
| 60$^{th}$ day | A | 7.68±3.13$^a$ | 4.65±1.84$^a$ | 2.98±1.37$^a$ | 0.02±0.03$^a$ | 0.02±0.03$^a$ | 0.01±0.02$^a$ |
| | B | 8.54±2.18$^a$ | 4.94±1.46$^a$ | 3.48±1.09$^a$ | 0.08±0.08$^a$ | 0.03±005$^a$ | 0.00±0.00$^a$ |
| | C | 8.55±1.18$^a$ | 5.09±0.78$^a$ | 3.31±0.53$^a$ | 0.11±0.13$^a$ | 0.04±0.08$^a$ | 0.00±0.00$^a$ |
| | Control | 9.18±1.32$^a$ | 4.89±1.14$^a$ | 4.05±0.37$^a$ | 0.10±0.13$^a$ | 0.11±0.08$^a$ | 0.02±0.05$^a$ |
| | *P* value | 0.736 | 0.964 | 0.352 | 0.518 | 0.134 | 0.54 |

DPV: Days post vaccination; TLC: Total leukocyte count; Lymph: Lymphocytes; Neut: Neutrophils; Mono: Monocytes; Eosin: Eosinophil; Baso: Basophils. Means ± SD with different superscript letters ($^{a, b, c}$) in the same column are significantly different at $P < 0.05$.

injection site, that disappeared within two weeks.

*The humoral immune response of vaccinated ewes by using serological tests*

After vaccination, determination of the humoral immune response by RBPT, as a screening test, revealed that all the Rev. 1 vaccinated ewes became serologically positive to RBPT at 3 weeks, ranging between 3 and 8 weeks. After that, the percentage of seropositive ewes decreased and was zero at 16th weeks post-vaccination (Table 2). While ewes were vaccinated with RB51 (Group A and B), and non-vaccinated control (Group D) ewes did not produce seroconversion as measured by RBPT.

*The cell-mediated immune response of vaccinated groups*

Before treatment, the total leukocytic count and blood lymphocyte proliferative responses were nearly similar in all groups. However, after immunization, lymphocyte proliferative responses varied between immunized groups. On the 7th day post-vaccination, lymphocyte responses were significantly higher in Group (A) than in other Groups (B and C). While the mean NBT %, phagocytic activity, and phagocytic Index in Group C (vaccinated with Rev. 1) were higher than in other Groups (A and B) (Table 3).

At the 14th day and follow-ups days post-vaccination, the total leukocytic count, the mean NBT, phagocytic activity, and phagocytic index showed that the RB51 vaccine with a dose of $3.4x10^{10}$ in Group (A) provides a protective immune response near to that effect produced by Rev. 1 vaccine in Group (C) (Tables 4 and 5).

*Cytokine gene analysis*

The mRNA expressions of TNF- α and IL-6 were detected by qRT–PCR. Variation between the vaccinated groups and control is exposed by expressing the corrected values as fold differences (Table 5). The relative levels of TNF- α and IL-6 differed across the vaccinated groups. The IL-6 was expressed less in Group (B), greater in Group (C), and the highest expression in Group (A). Also, the mRNA expression of TNF-α was lowest in Group (B), with greater abundance in Group (C), and the highest expression in Group (A). Generally, the fold differences of TNF- α were higher than those of IL-6.

## DISCUSSION

Ovine brucellosis in underdeveloped countries will require a lot of effort to be successfully eradicated. The main goal of vaccination, on the other hand, is to strengthen a community's

Table 4. The mean NBT%, phagocytic activity, and phagocytic indexpost-vaccination of different groups.

| DPV | Group | Mean NBT % | phagocytic activity ratio | phagocytic index |
|---|---|---|---|---|
| Zero-day | A | 13.16±6.99 [a] | 11.92±9.45[a] | 2.31±1.62[a] |
| | B | 10.24±5.55[a] | 15.99±7.88[a] | 2.16±1.75[a] |
| | C | 9.98±5.19 [a] | 16.76±10.56 [a] | 2.29±1.49[a] |
| | Control | 9.98±4.74[a] | 17.50±6.07[a] | 2.19±1.46[a] |
| | *P* value | 0.774 | 0.745 | 0.998 |
| 7th day | A | 37.20±9.15[ab] | 38.22±12.99[ab] | 3.14±2.16[a] |
| | B | 29.73±13.18[a] | 19.28±6.56[b] | 1.77±0.60[a] |
| | C | 61.18±19.88[a] | 49.72±27.01[a] | 3.51±1.77[a] |
| | Control | 20.78±13.36[b] | 16.57±5.44[b] | 1.72±0.93[a] |
| | *P* value | 0.002 | 0.016 | 0.17 |
| 14th day | A | 48.62±14.49[ab] | 31.50±25.33[a] | 2.56±1.18[a] |
| | B | 38.10±29.53[ab] | 27.90±17.23[a] | 1.72±0.89[a] |
| | C | 49.34±27.12[a] | 32.79±23.72[a] | 3.15±1.69[a] |
| | Control | 9.44±6.20[b] | 17.16±9.13[a] | 2.75±1.58[a] |
| | *P* value | 0.033 | 0.6 | 0.432 |
| 21st day | A | 24.54±8.14[b] | 21.17±12.49 [a] | 1.57±0.32[a] |
| | B | 11.74±10.81[ab] | 13.02±24.99 [a] | 1.25±0.41[a] |
| | C | 35.38±12.03[a] | 26.60±9.42 [a] | 1.87±0.40 [a] |
| | Control | 11.48±8.83[b] | 21.69±14.06 [a] | 1.38±0.51[a] |
| | *P* value | 0.004 | 0.689 | 0.143 |
| 28th day | A | 16.72±16.14[a] | 8.27±2.42 [a] | 1.60±0.27[a] |
| | B | 14.68±9.22[a] | 7.47±5.77[a] | 1.44±0.55[a] |
| | C | 15.64±10.62[a] | 9.69±7.30 [a] | 1.79±0.76[a] |
| | Control | 11.56±5.52[a] | 16.61±7.99 [a] | 1.67±0.98 [a] |
| | *P* value | 0.892 | 0.125 | 0.838 |
| 60th day | A | 11.96±4.29[a] | 13.28±7.44[a] | 1.31±0.26[a] |
| | B | 8.94±8.05[a] | 11.39±10.27[a] | 1.13±0.42[a] |
| | C | 12.56±4.88[a] | 14.63±6.73[a] | 1.49±0.40[a] |
| | Control | 9.02±6.30[a] | 12.79±4.97[a] | 1.29±0.27[a] |
| | *P* value | 0.693 | 0.951 | 0.472 |

DPV: days post-vaccination; NBT: Nitroblue tetrazolium test; Means ± SD with different superscript letters ([a, b, c]) in the same column are significantly different at $P < 0.05$.

resilience to disease. An efficient vaccine not only eliminates the disease's clinical symptoms but also lessens environmental pollution and the number of people that are susceptible to getting the infectious agent (Nicoletti, 2010; Olsen, 2013). The Rev. 1 (*B. melitensis* strain) vaccination was used in endemic nations to lower the incidence of brucellosis among sheep herds (Garin-Bastuji and Blasco, 2004). The drawback of this approach is that pregnant animals cannot be vaccinated because the vaccine is not safe enough for them (WHO, 1998). Additionally, the subcutaneous injection of Rev. 1 demonstrates a post-vaccinal antibody response that obstructs serological assays, making it challenging to distinguish from infections. Therefore, a different strategy is needed to prevent brucellosis in small ruminants. To be able to mass-vaccinate a flock or area (young and adults) when the disease frequency is high, to quickly build up an immune stock, and to lower the rate of abortions, hence reducing environmental contamination and disease transmission (Ward *et al*., 2012). The present work examined the effectiveness of the Rev. 1 vaccine and RB51 (*B. abortus* strain) vaccine in sheep under controlled circumstances. The effectiveness was measured by both cellular and humoral immune reactions. Although *Brucella* spp. is a facultative intracellular microorganism and could survive and replicate within macrophages, cell-mediated immunity (CMI) is considered essential for protective immune response (Martirosyan *et al*., 2011; Goel *et al*., 2013). Furthermore, cell-mediated immunity is characterized by antigen-specific T-cell-mediated activation of macrophages and is the major effector of the cell-mediated killing of this organism (Oliveira *et al*., 1998). Clinical observation of vaccinated ewes showed some tissue reactions at the injection site with the Rev. 1 vaccine (Group C). This is likely due to bacteremia and a generalized infection with the live vaccine (Higgins *et al*., 2018). Also, goats showed some tissue reactions after subcutaneous vaccination with Rev.1 vaccine, which disappeared within 14 weeks post-vaccination (Elberg and Meyer, 1959).

After vaccination, the humoral immune response was assessed by RBPT as a qualitative method every week for 16 weeks, and its positive results were recorded as agglutination. The obtained results showed that sheep vaccinated with Rev. 1 vaccine (Group C) had 100% positive seroconversion, and this agreed with previous findings in cattle, sheep, and goats (El Idrissi *et al*., 2001; Abdel-Tawab *et al*., 2018). However, unlike responses in Rev. 1-vaccinated ewes, sheep vaccinated with RB51 (Group A and B) did not develop an antibody reaction, and this lack of antibody production has previously been demonstrated in a variety of animal species, including cattle, goats, sheep, rabbits, and mice (Schurig *et al*., 1991). The negative seroconversion of RB51-vaccinated sheep is similar to that of the non-vaccinated sheep (Group D) and appears long-lasting. The ideal brucellosis vaccine candidate should have the following characteristics: (1) lengthy immunogenic duration; (2) little interference with diagnostic testing; (3) ease of manufacture and storage; (4) minimal side effects in treated animals; and (5) no risk to humans in case of contact (Goel *et al*., 2013).

On the 7th day post-vaccination, when the RB51 vaccine was subcutaneously administered to ewes with a dose of $3.4x10^{10}$ CFU (Group A) similar to the dose of cattle, the cellular immune response represented by total leukocytic count was noticed to be higher than other groups (B and C), and this could be due to the microbial load (dose $3.4x10^{10}$ CFU). While the mean NBT %, phagocytic activity, and phagocytic Index in Group C (vaccinated with Rev. 1) were higher than in other Groups (A and B), this point may be due to the high virulence of Rev. 1 live vaccine. These results agreed with (Higgins *et al*., 2018) who reported developing a pro-inflammatory response characterized by increased lymphocyte population post-vaccination of goats with Rev. 1. At the 14-day and follow-ups days post-vaccination, the total leukocytic

Table 5. Cytokine gene expressions of IL6 and TNF- α in different vaccinated groups.

| Days post-vaccination | Grouping | *B- actin* | IL6 | | TNF- α | |
|---|---|---|---|---|---|---|
| | | Ct | Ct | Fold change | Ct | Fold change |
| Day 7 | Control | 29.8± 0.5$^{ab}$ | 31.2±1.1$^{a}$ | 0.0±0.0$^{d}$ | 29.8± 0.5$^{a}$ | 0.0±0.0$^{b}$ |
| | Group A | 30.6±0.3$^{a}$ | 30. 2±0.1$^{ac}$ | 4.8± 0.5$^{a}$ | 26.7± 0.5$^{b}$ | 13.6± 1$^{a}$ |
| | Group B | 28.9± 0.9$^{b}$ | 29.3±0.3$^{bc}$ | 2.1± 0.2$^{b}$ | 26.2± 1.5$^{b}$ | 4.9± 0.6$^{bc}$ |
| | Group C | 30.1± 0.7$^{ab}$ | 29.9±0.4$^{bc}$ | 3.5± 0.6$^{c}$ | 27.7±1.4$^{ab}$ | 5.8± 1.6$^{c}$ |
| Day 14 | Control | 29.8±0.5$^{a}$ | 31.2±1.1$^{a}$ | 0.0±0.0$^{b}$ | 29. ±0.5$^{a}$ | 0.0±0.0$^{b}$ |
| | Group A | 30.4±0.4$^{a}$ | 29.2±0.4$^{b}$ | 6.9±2.2$^{a}$ | 24.4±0.4$^{b}$ | 59.8±18.9$^{a}$ |
| | Group B | 30.5±0.3$^{a}$ | 30.0±0.2$^{b}$ | 5.0±3.2$^{b}$ | 26.0 ±0.6$^{c}$ | 17.2 ±5.8$^{b}$ |
| | Group C | 30.1±0.5$^{a}$ | 29.3±0.5$^{b}$ | 5.2±1.9$^{a}$ | 25.8 ±0.2$^{c}$ | 22.6 ±9.9$^{a}$ |
| Day 21 | Control | 29.8 ±0.5$^{a}$ | 31.2 ±1.1$^{a}$ | 0.0±0.0$^{b}$ | 29.8 ±0.5$^{a}$ | 0.0±0.0$^{c}$ |
| | Group A | 30.5 ±0.4$^{a}$ | 28.2 ±0.7$^{b}$ | 11.2 ±3.8$^{a}$ | 24.4 ±0.5$^{b}$ | 68.5 ± 13.24$^{a}$ |
| | Group B | 30.0 ±0.5$^{a}$ | 28.8 ±0.6$^{b}$ | 7.1 ±2.1$^{a}$ | 24.9 ±0.8$^{b}$ | 21.0 ±2.9$^{b}$ |
| | Group C | 30.1 ±0.5$^{a}$ | 28.0 ±0.8$^{b}$ | 9.4 ±1.9$^{a}$ | 25.3 ±0.9$^{b}$ | 30.4 ±8.0$^{b}$ |
| Day 28 | Control | 29.8 ±0.5$^{ab}$ | 31.2±1.1$^{a}$ | 0.0±0.0$^{b}$ | 29.8±0.5$^{a}$ | 0.0±0.0$^{b}$ |
| | Group A | 30.6±0.3$^{b}$ | 28.8±0.5$^{b}$ | 11.5±5.6$^{a}$ | 22.6±0.9$^{b}$ | 253.5±143.5$^{a}$ |
| | Group B | 30.1±0.7$^{ab}$ | 29.5±1.0$^{a}$ | 4.8 ±1.9$^{b}$ | 24.6±0.7$^{b}$ | 47.3±25.8$^{b}$ |
| | Group C | 29.0±1.0$^{a}$ | 27.9±0.7$^{b}$ | 6.3 ±1.7$^{ab}$ | 22.7±1.6$^{b}$ | 69.4±16.6$^{b}$ |
| Day 60 | Control | 29.8±0.6$^{b}$ | 31.2±1.1$^{a}$ | 0.0±0.0$^{b}$ | 29.8±0.5a | 0.0±0.0$^{b}$ |
| | Group A | 31.1±0.6$^{a}$ | 27.5±0.8$^{b}$ | 36.0±3.4$^{a}$ | 21.7±1.5$^{b}$ | 784.7±143.2$^{a}$ |
| | Group B | 30. 1±0.5$^{b}$ | 27.2±1.0$^{b}$ | 22.7 ±10.7$^{a}$ | 22.6 ±1.3$^{b}$ | 140.3 ±8.6$^{b}$ |
| | Group C | 30. 5±0.3$^{ab}$ | 27.3±0.2$^{b}$ | 30.4 ±11.7$^{a}$ | 22.7 ±0.6$^{b}$ | 150.6 ±63.8$^{b}$ |

TNF-α: tumor necrosis factor-alpha, IL-6: interleukin 6, Ct: cycle threshold, A: group vaccinated subcutaneously with the *B. abortus* RB51 vaccine at a dose of 2 ml ($3.4 x10^{10}$ CFU), B: group vaccinated subcutaneously with the *B. abortus* RB51 vaccine at a dose of 1 ml ($1.7 x10^{10}$ CFU), C: group vaccinated subcutaneously with the *B. melitensis* Rev.1 vaccine at a dose of 2 ml ($1.6 \times 10^{9}$ CFU), and D: control ewes did not receive any vaccines. Means ± SD with different superscript letters ($^{a, b, c, d}$) in the same column are significantly different at $P < 0.05$.

count, the mean NBT%, phagocytic activity, and phagocytic index showed that the RB51 vaccine with a dose of $3.4x10^{10}$ in the group (A) provides a protective immune response near to that effect produced by Rev. 1 vaccine ($1.6\times10^{9}$ CFU/dose) in Group (C).

As of right now, quantitative reverse transcription-polymerase chain reaction (Q-RT-PCR) is the standard technique used to study cytokine gene expression in ruminants because it can quantify various genes even with slight physiological variations in gene activation by detecting messenger RNA (mRNA) for cytokine-specific proteins and it also allows for the use of small amounts of the sample with high specificity, sensitivity, and accuracy (Priyanka *et al*., 2021). Therefore, cytokine assessment was a very useful way of investigating the immune response in ruminants or predicting the severity of the infection; the IL-6 and TNF- α also increased in acute or chronic brucellosis (Lin *et al*., 2020). Besides that, they were vital in *Brucella*'s elimination from the macrophages (Pascual *et al*., 2018).

The present results showed higher transcriptional expressions of the TNF- α and IL-6 levels in harmony with the studied cellular parameters (TLC, phagocytic activity, index, and the mean NBT %) along the studied experimental periods, and this means a good response of the studied vaccines. Furthermore, our results showed that the RB51 vaccine with a dose of $3.4x10^{10}$ showed a higher immune response and can induce the body to produce significant levels of IL-6 and TNF-α than Rev. 1 vaccine along the studied experimental periods meanwhile the RB51 vaccine with a half dose showed lower immune response than Rev. 1. T1-type immune cell activation by lipopolysaccharide or endotoxin is thought to be characterized by increased expression of TNF- α in Group (A) (Coussens *et al*., 2004). Additionally, it was demonstrated that in mice infected with *Brucella*, functioning TNF- α links the proinflammatory and adaptive immune responses (Zhan and Cheers, 1998). Furthermore, the increased TNF- α production reduced subsequent bacterial proliferation within the infected cells (Dornand *et al*., 2002; Priyanka *et al*., 2021). Several studies provide a strong index that induction of TNF- α cytokine mRNA significantly regulated cellular immune responses against brucellosis and other intracellular infections (Ko *et al*., 2002). Transcriptional expressions of the IL-6 in Group (A) were higher than in other vaccinated groups. High levels of IL-6 aid in the Th1 immune response against *Brucella* infection by regulating the bactericidal activity of CD8 + T cell development and macrophage cells (Hop *et al*., 2019).

Along the studied experimental periods, IL-6 and TNF- α cytokine expression in the RB51 vaccinated ewes (group A) were still greater than those in other groups. These results demonstrated a persistent release of Th1 cytokine response in sheep receiving a dosage of $3.4x10^{10}$ CFU of the RB51 vaccine, suggesting potential therapeutic targets for enhancing long-term protection against *Brucella* infections. According to TLC, phagocytic activity and index, and the mean NBT percent, subcutaneous vaccination with Rev. 1 can trigger potent humoral and cellular-oriented immune responses. Also, subcutaneous vaccination of RB51 with a dose of $3.4x10^{10}$ CFU could induce a cellular immune response almost close to the Rev. 1 vaccine providing significant levels of protection. However, since the humoral reaction is lacking, it is possible to distinguish between the serological responses brought on by infection and those brought on by vaccination using diagnostic assays with a reasonable level of specificity. However, our findings support the idea that the RB51 vaccine, at a dose of $3.4x10^{10}$ CFU, can provide a safe and effective defense against ovine brucellosis and highlight the need to reevaluate Egypt's *B. melitensis* control strategy to maximize vaccine effectiveness.

## CONCLUSION

*B. melitensis* infection should be avoided and managed to reduce financial losses in the small ruminant industry and stop the global spread of zoonoses. The RB51 vaccine would be an additional method of ovine brucellosis prevention.

## CONFLICT OF INTEREST

The authors declare that they have no conflict of interest.

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