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Original article

EVALUATION OF THE HUMORAL IMMUNE RESPONSES OF BRUCELLOSIS VACCINE (REV.1) IN SHEEP: A STUDY FROM IRAN

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Summary

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The live strain of *Brucella melitensis* Rev1 is recognised as the available vaccine for controlling brucellosis in small ruminants. This study aimed to assess the humoral immune responses in sheep when administered the native Rev.1 vaccine. All animals were inoculated with the Rev.1 vaccine, and blood samples were collected on days 0, 14, 28, 44, 55, and 69 post-vaccination. These samples were tested using the modified Rose Bengal plate test (mRBPT), Wright test, 2-mercaptoethanol (2-ME) test, and enzyme-linked immunosorbent assay (ELISA) techniques. After six rounds of sampling, all animals tested negative in the mRBPT evaluation, and the control group exhibited low levels of antibodies to brucellosis when using ELISA. In the test group, most cases of positive mRBPT results were observed on days 14 and 28 of sampling. The Wright, 2-ME, and ELISA tests revealed that the highest antibody levels were detected 14 days after vaccination. In contrast to the Wright and 2-ME findings, the level of antibodies continued to rise until the 44th day after vaccination when using ELISA. The results demonstrated the stimulation of humoral immune responses in the target animals through the native vaccine, which could be employed to immunise livestock in endemic areas like Iran. The ELISA test proved to be more effective than conventional tests in revealing the serological response induced by Rev.1 vaccination, although ELISA may not reliably distinguish between antibodies generated by the vaccine and those resulting from the disease.

Key words: brucellosis, ELISA, Rev.1 vaccine, 2- mercaptoethanol test, Wright test

INTRODUCTION

Brucellosis is one of the most important zoonotic diseases in humans and animals. The disease is also known as contagious abortion in animals. The cause of the disease is a bacterium called *Brucella*, which has different species. This Gram-negative bacterium is immobile and unable to produce spores (Moreno *et al.*, 2023). The main cause of the disease in cattle is *Brucella abortus,* while *Brucella meliten-*

sis is the common species for brucellosis in sheep and goats (Dadar *et al.*, 2023a; Moreno *et al.*, 2023). Brucellosis has economic aspects due to the abortion of animals, reduction of milk production, sterility, loss of economic value of infected animals (Dadar *et al.*, 2021a). In addition to its economic repercussions, it poses a significant public health risk, as it can be transmitted to humans through the consumption of contaminated animal products or direct contact with infected animals.

Sheep, as one of the susceptible hosts for *Brucella* infection, play a crucial role in the epidemiology of brucellosis. Understanding the humoral immune responses in sheep is of paramount significance in disease management and control. Evaluation of humoral immunity allows for the detection of antibodies produced in response to *Brucella* infection, aiding in the early diagnosis and surveillance of infected flocks. Moreover, monitoring the humoral immune responses in sheep is pivotal for implementing effective vaccination strategies, preventing disease transmission to humans, and safeguarding both animal health and the global livestock industry (Higgins *et al.*, 2017).

In 1933, for the first time in Iran, Dr. Crandall isolated the bacterium *B. melitensis* from the blood of individuals suspected of having Malta fever at the Pasteur Institute of Iran. Systematic control programmes utilising the Rev.1 vaccine combined with monitoring and control systems and adequate coverage of qualified livestock populations in all sheep epidemiological units could help reduce cases of Malta fever in the human population (Dadar *et al.*, 2021a). The Rev.1 vaccine was first produced in 1957 by Elberg from the acute strain 6056 biovar 1 of *B. millitensis*, which can be distinguished from the acute strain by three characteristics: susceptibility to penicillin, poor growth and formation of smooth colonies smaller than the acute strain on agar medium, and resistance to streptomycin (De Bagüés *et al.*, 1992; Falade, 1983). So far, millions of doses of this vaccine have been used in different countries with very different results without clear explanation of all these differences (Blasco, 1997; Falade, 1983). It is also generally accepted that *B. melitensis* infection in sheep in highly infected countries is not eliminated by testing and culling, and a vaccination programme (Dadar *et al.*, 2021b). Vaccination of animals is the most practical tool for controlling brucellosis in endemic areas. On the other hand, *B. melitensis* Rev.1 is the best available vaccine for this purpose (Ghobrial *et al.*, 2023). The Rev.1 vaccine is an attenuated live vaccine resistant to streptomycin, which is produced in lyophilised form in two types of full doses for young animals (lambs and goats) by different companies. According to the report of the World Organization for Animal Health (WOAH), *B. melitensis* Rev.1 strain vaccine is recognised as the most efficient and effective vaccine for active immunisation and protection against brucellosis in sheep and goats (Blasco, 1997; Lantier & Fensterbank, 1985; Ebrahimi *et al.*, 2012; Hou *et al.*, 2019). According to reports, the *B. melitensis* Rev. 1 vaccine has been found to elicit cellular immunological responses that confer a level of protective immunity comparable to that observed with the *B. abortus* RB51 vaccine, when administered at a dose of 3.4×10^{10} (Ghobrial *et al.*, 2023). Furthermore, the administration of the *B. melitensis* Rev.1 vaccine via conjunctival route demonstrated a robust and safety profile immunological response in sheep and goats (Gurbilek *et al.*, 2023).

Iran is an endemic region for both animal and human brucellosis in the world (Dadar & Godfroid, 2021; Dadar *et al.*, 2021a). The incidence of brucellosis was calculated as 22 cases per 100,000 people in Iran (Zeinali *et al.*, 2022), although the annual global incidence of human brucellosis is estimated to be 2.1 million (Laine *et al.*, 2023). Also, the prevalence of *Brucella* infection in livestock was 10.1% (Dadar *et al.*, 2021a). The disease in livestock is significant due to the infection's transmission to humans (Dadar *et al.*, 2018). Brucellosis has affected a range of livestock species in Iran, such as sheep, goats, cattle, buffalo, and camels, albeit to varying degrees (Dadar *et al.*, 2021b; 2023b). Among the livestock under investigation in Iran, *B. melitensis* and *B. abortus* are the most frequently reported species (Dadar *et al.*, 2021a). Different laboratory methods are used to detect brucellosis in animals in Iran. In serology-based methods, the modified Rose Bengal Plate test (mRBPT), Wright standard tube agglutination test (SAT), 2-mercaptoethanol (2-ME), and enzyme-linked immunosorbent assay (ELISA) are the most common (Adabi *et al.*, 2022). Nowadays, ELISA with 100% sensitivity and 99.2% specificity is been used as a suitable and reliable technique. Additionally, SAT accounts for aggregated quantities of IgM and IgG, while IgG to *Brucella* infection is calculated using the treatment of sera samples. IgG tracing is important for determining the active stage of brucellosis (Baltierra-Uribe *et al.*, 2019). Furthermore, it has been reported that the mRBPT or iELISA tests have the potential to be more favourable and sensitive alternatives to the existing RBPT process employed as screening test of brucellosis in sheep (Ferreira *et al.*, 2003).

In this study, the effectiveness of the brucellosis vaccine in the lamb population of Hamedan province was evaluated with the aim of brucellosis control in sheep of rural and nomadic areas and the executive instructions for technical supervision of its implementation. The purpose of this study was to determine the immunogenicity of the Rev. 1 vaccine. The results of this study can be used in annual and long-term planning to control brucellosis in small ruminants.

MATERIALS AND METHODS

Study area and target population

In Hamedan province (an endemic region for brucellosis in the western part of Iran), livestock breeding is considered one of the most important and main animal husbandry activities in rural areas and plays an important role in providing animal protein and employment for villagers. Most of the small ruminate livestock are raised in pasture and rural systems. Considering that the spring season has the most eligible livestock (lambs and goats) for immunity coverage in Hamedan province with the full dose Rev.1 vaccine, the eligible livestock were vaccinated against brucellosis in this season. In this study, 80 sheep were investigated to evaluate the effectiveness of vaccination. All of the animals were Mehraban (a native breed), females under 1-year-old (3 to 8 months), negative for brucellosis by using modified RBPT as well as without a history of vaccination against brucellosis. At the same time as vaccination, ear clipping was done in vaccinated animals.

Study and sampling plan

A cross-sectional study between May and July of 2020 was conducted using the full

dose Rev.1 vaccine of Razi Vaccine and Serum Research Institute, Iran. The study was performed in a farm in Bahar district, Hamedan, West of Iran. The group of examined animals (n=80) was divided into two groups of 40 each: one serving as control group and the other: as test group. A dose of Rev.1 vaccine (1 mL, subcutaneous in scapula area) and placebo (physiological saline) was inocculated in all animals of test and control groups, respectively. A dose of full dose Rev.1 vaccine contained $(1–4)\times10^9$ live *B. melitensis* bacteria with bactocasion preservative. Antibodies to *Brucella* were evaluated at different times using serology techniques.

Sampling and conducting tests

In 6 series of examinations (80 blood samples in each series), a 5 mL blood sample was collected from lambs at different times after vaccination (1, 14, 28, 44, 55, and 69 days). The first stage of sampling (stage 1) was taken before the vaccination of animals. After separating the serum (centrifuging blood samples at 1,400×g for 12 minutes), the samples were transferred to the central laboratory of the Veterinary Service of Hamedan and were kept at a temperature of minus 20°C until the test was performed. At first, all animals were tested using modified-RBPT (mRBPT) and ELISA in different stages of sampling. In the next step, mRBPT and ELISA-positive samples were re-tested by SAT and 2-ME.

Serology

mRBPT. In this assay, 90 µL of sera and 30 µL of RBPT antigen (Vaccine and Serum Research Institute of Razi Co., Iran) were mixed on a rapid test white plate and shaken for 4 minutes. The appearance of any pink agglutination was detected as a positive reaction (Blasco *et al.*, 1994).

SAT. In summary, the first agglutination tube received 0.8 mL of buffer phosphate saline + phenol 0.5% (Pourquier Co., France). The amount for tubes 2–7 was 0.5 mL. The first tube also received 0.2 mL of the sera sample. Pipetting 0.5 mL of the first tube's contents into the succeeding tubes was used to perform serial dilution. Then, 0.5 mL of the solution was discarded from the end tube. The final stage involved adding 0.5 mL of 10% Wright antigen from the Razi Co., Iran, Vaccine and Serum Research Institute, and incubating all tubes for 24 hours at 37 °C. (Alton *et al.*, 1988).

2-ME test. The protocol was adjusted similarly to SAT. Briefly, 0.3 mL and 0.5 mL of buffer phosphate saline plus phenol 0.5% (Pourquier Co., France) were poured into the first and other tubes, respectively. The first tube also received 0.2 mL of the sera sample. The second step involved adding 0.5 mL of 2-ME solution (Merck, Germany) to the first tube, shaking it, and incubating it at 37 °C for an hour. Pipetting 0.5 mL of the contents of the first tube into the succeeding tubes was done to perform serial dilution. Then, 0.5 mL of the solution was then removed from the end tube and discarded. Finally, 0.5 mL of 10% Wright antigen (Razi Co., Iran, Vaccine and Serum Research Institute), was added to each tube, and all tubes were incubated at 37 °C for 24 h (Alton *et al.*, 1988).

ELISA. A commercial ELISA kit belonging to ID-Vet company, France (ID Screen® Brucellosis serum indirect multispecies, Lot No: 144) was used for detecting the antibodies to *Brucella* in animals. The procedures were conducted according

to the instructions recommended by the ID-Vet company. For all samples, the optical density (OD) of the sample and controls [S/P% = 100×(OD sample−OD negative control)/(OD positive control−OD negative control)] was used for calculation of sample's positive percentage (S/P%). A S/P% ≥120 was accepted as positive for brucellosis.

Data analysis

A *t*-test was conducted using SPSS version 18 (SPSS Inc., Chicago, IL, USA) to compare the results of serological tests between different groups. The results were reported statistically significant, with a P-value of less than 0.05.

RESULTS

In the control group, all animals were negative in mRBPT evaluation in six consecutive samplings. Additionally, low levels of antibodies to brucellosis, as measured by ELISA, were observed. The maximum antibody level detected was 43, and all S/P% values were below 120. The average antibody levels, as determined by ELISA, varied at different stages of sampling: 11.1±6.9, 10±6.9, 17.5±9.2, 13.3±7.2, 15.5±9.2, and 18.5±9.2 for the first through sixth sampling stages, respectively. In the test group, all animals initially tested negative using mRBPT before vaccination in the first sampling. The highest number of animals with positive mRBPT results (100%) was observed on days 14 and 28 after vaccination which was significantly greater compared to that days 44, 55, and 69 (P<0.05). Afterward, the number of positive cases decreased significantly (P<0.05) (Fig. 1).

Wright and 2-ME results showed that the average level of antibodies related to the vaccine increased to a titre of 1:160 after 14 days of vaccination (Fig. 2 and 3). However, this value decreased significantly to 1:10 on the 69th day of sampling (P<0.05). In contrast to the Wright and 2-ME findings, the level of antibodies measured by ELISA, increased significantly (P<0.05) until the 44th day after vaccination (Fig. 4). After vaccination, the average antibody levels on days 28, 44, and 55 were determined to be 197, 358, and 230 respectively using the ELISA method. In summary, the results suggested that the test group of animals exhibited an antibody response to brucellosis following vaccination, with peak antibody levels observed around day 14 and day 28, as indicated by positive mRBPT results and increased titres. However, the antibody levels subsequently decreased over time, with variations depending on the testing method used (Wright and 2-ME vs. ELISA). The control group remained negative throughout the study, indicating the effectiveness of the vaccination in inducing an immune response in the test group.

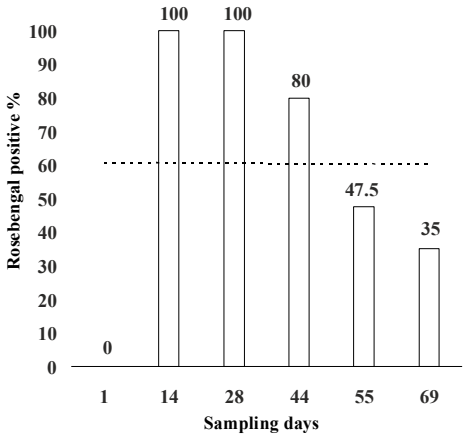

Fig. 1. The frequency of the number of positive mRBPT according to the date of blood sampling in the test group.

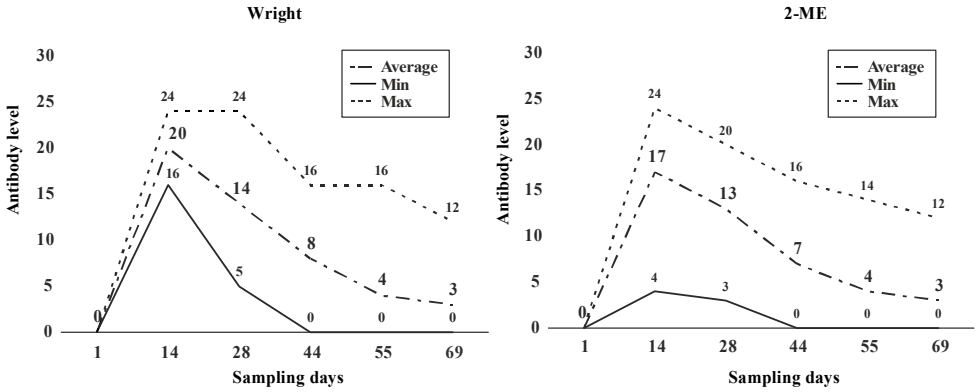

Fig. 2. Calculation of average, minimum, and maximum titre values after vaccine inoculation using Wright and 2-ME techniques based on blood sampling date.
[**24**=++++/320, **20**=++++/160, **17**=+/160, **16**=++++/80, **14**=++/80, **13**=+/80, **12**=++++/40, **8**=++++/20, **7**=+++/20, **5**=+/20, **4**=++++/10, **3**=+++/10].

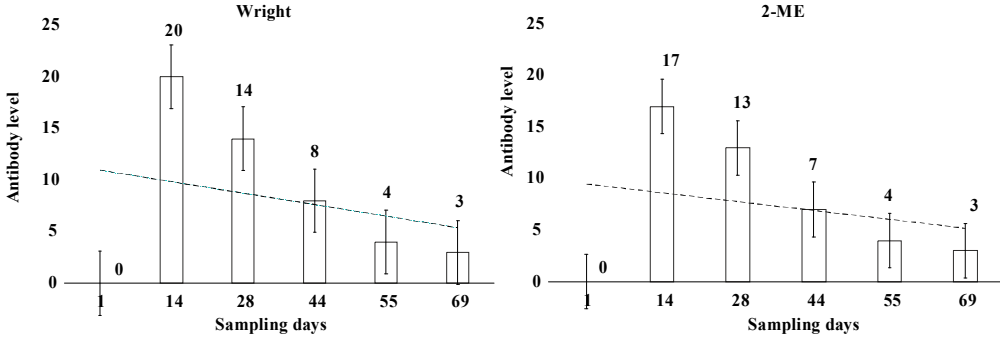

Fig. 3. Average titres with standard error after vaccination using Wright and 2-ME techniques based on the date of blood sampling [**20**=++++/160, **17**=+/160, **14**=++/80, **13**=+/80, **8**=++++/20, **7**=+++/20, **4**=++++/10, **3**=+++/10].

DISCUSSION

Vaccination is one of the most effective tools to prevent and control brucellosis. Vaccination against brucellosis has been able to significantly reduce the incidence of the disease and associated abortions in livestock. Knowledge about the mechanism and function of a vaccine is so important for selecting the right type of vaccine (Dadar *et al.*, 2021b).

The *B. melitensis* attenuated strain of Rev.1 vaccine is stable, live and has been found to stimulate good protection in small ruminants against *B. melitensis*-induced abortion. However, the major risks of vaccination are possible abortion in females vaccinated during pregnancy and possible genital or lactational shedding of the Rev.1 strain after vaccination (Higgins *et al.*, 2017; Xie *et al.*, 2018). Little is known about the immune system's response to the most-used Rev.1

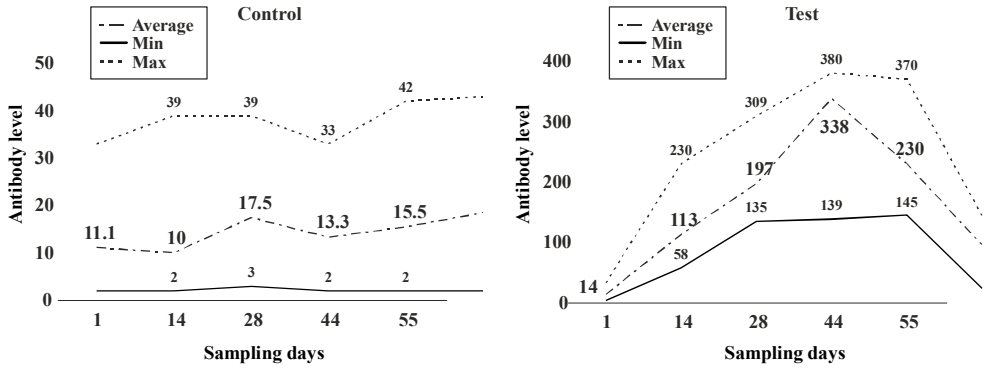

Fig. 4. Calculation of average, minimum, and maximum levels of antibodies to brucellosis after vaccine inoculation using ELISA based on blood sampling date.

vaccine for brucellosis prevention in sheep and goats. Moreover, the need to evaluate the immune responses to the various variants of this vaccine further complicates the situation. On the other hands, utilising multiple serological tests

increases the detection of antibodies at different stages of infection. No single serological test is perfect, and each has its limitations, including the potential for false negatives or false positives. By using a combination of serological tests, the overall accuracy of diagnosis is improved, as the strengths of one test can compensate for the weaknesses of another. Some serological tests such as RBPR may cross-react with antibodies from other infections, leading to false-positive results. By employing multiple tests, it becomes possible to verify the presence of *Brucella* antibodies through confirmation and cross-checking, reducing the likelihood of misdiagnosis. Therefore, the use of multiple tests in the serodiagnosis of brucellosis is considered indispensable in endemic areas (El-Diasty*et al.*, 2021). The RBPT is a quick, accurate, and reliable screening test used to detect *Brucella* infection. This seroagglutination assay is preferred due to its specificity and involves staining an acidic solution of *B. abortus* as an antigen source. Furthermore, the modified test of RBPT (mRBPT) is more sensitive, detecting *Brucella* antibodies at an earlier stage of infection, thereby reducing the likelihood of false negatives. In the context of brucellosis testing in sheep and goats in Asia, RBPT, buffered acidified plate antigen test (BAPAT), and complement fixation test (CFT) are important tools for monitoring and controlling the disease in vaccinated and non-vaccinated animals (Al-Sherida *et al.*, 2020). It has been revealed that the implementation of vaccination using the *B. melitensis* Rev.1 vaccine, along with test-and-slaughter measures, resulted in a reduction of seroprevalence in herds located in Kuwait (Al-Sherida *et al.*, 2020). Following inoculation of the Rev. 1 vaccine, another research has shown that all serum samples tested positive for the infection at both post-inoculation days 30 and 60 (Doostdari *et al.*, 2019). These results are in line with our findings that showed that after 69 days of being vaccinated, 35% of the samples tested positive on the mRBPT. According to Blasco *et al.* (1984), 98% of the cases had positive results in the RBPT in the first week after inoculation with the Rev.1 vaccine, this rate decreased to 80% in the eighth week and even further to 28% after

22 weeks (154 days). According to the findings of Daz-Aparicio *et al.* (1994), 80% of the vaccinated sheep tested positive in the RBPT 6 months after receiving the Rev.1 vaccination. Another study found that the RBPT in sheep aged 3-6 months and adults, was positive 21 days after being vaccinated with the Rev.1 vaccine supporting the current study's findings. They also noted that 4 months after receiving the Rev.1 vaccine, the 3- to 6-month-old sheep had a negative RBPT result (Stournara *et al.*, 2007). However, another study has shown that 95% of the sheep samples tested using the RBPT assay displayed a positive reaction for six months after being inoculated with the Rev.1 vaccine (Shome *et al.*, 2014). Benkirane *et al.* (2014) revealed that the administration of the Rev.1 vaccine resulted in positive RBPT results for a period of two months, consistent with the findings of our study. However, one of the limitations of this study was the relatively short duration of the antibody response survey, which spanned only 69 days.

Brucella species are a type of bacteria that, during infection, trigger both cellular (T cell) and humoral (antibody) responses. The S-LPS *Brucella* major antigen, in particular, elicits a T cell-dependent immune response, with IgG1 playing a prominent role. The transition from IgM to IgG can occur within a week. This means that the primary humoral response can typically be detected through IgG (Ducrotoy *et al.* 2016). The seroagglutination test of Wright is one of the most frequently used tests for the diagnosis of brucellosis. It has been a fundamental method in many early brucellosis prevention programmes in different countries (Dadar *et al.*, 2021b). There have been concerns regarding the seroagglutination test reliability due to the possibility of nonspecific agglutinins against *Brucella* and the limited ability of most IgG antibodies to agglutinate antigens. Consequently, this test may yield false negative results (Allan *et al.*, 1976). Our findings reveal that the serum sample titres in the Wright analysis increased from zero on the first day to 160 after 14 days following the vaccination of sheep with the Rev. 1 vaccine. After one month, these titres began to decline, reaching 80%. Another study demonstrated that Wright's analysis in sheep vaccinated with Rev.1 yielded a titre of 160 after one month (Doostdari *et al.*, 2019). Furthermore, the Wright titres reported in our investigation after 1 month of inoculation with Rev.1 vaccination were 120, which was lower than the titres observed in other studies (Mambini *et al.*, 2014; Doostdari *et al.*, 2019). The difference between the documented titres could be due to the discrepancy in the bacterial cell counts found in the injected Rev. 1 vaccines. Another study showed the titres of Wright as 80 for sheep after 24 weeks of Rev.1 vaccination (Aldomy *et al.*, 2009), however, the Wright titres for the vaccines in the present study were 80 and 10 after the 28 and 69 days, respectively. Moreover, it has been reported that after 7 months of Rev.1 inoculation, Wright results were negative (Benkirane *et al.*, 2014). Furthermore, antibodies titres were still measurable after 6 months of vaccination (Doostdari *et al.*, 2019). However, our research revealed that antibodies remained detectable for up to 69 days following vaccination. Shome *et al.* (2014) reported that after administering the Rev.1 vaccine, 65% of the sheep exhibited Wright titres exceeding 40 for a duration of six months. Notably, this outcome differed from the results obtained in our study.

The examination of the *Brucella* immune response mechanism and advancements in diagnostic technologies contribute to a deeper comprehension of efficacious control tactics (Elrashedy *et al.*, 2022). In our study, a 2-ME test was performed to investigate the presence of IgG in serum samples exposed to brucellosis. The 2-ME can break down the disulfide bonds in IgM molecules, resulting in the degradation of IgM in the serum, leaving only IgG molecules as the predominant component. Our findings showed lower titres of the serum samples tested in the 2-ME test compared to the Wright test. After one month, the serum samples had 2-ME titres of 80, which were comparable to the Wright titres, The 2-ME assay results indicated the presence of IgG antibodies in the examined samples for up to 69 days after the Rev.1 inoculation. The highest IgG titres in the tested sera were observed one month after the Rev.1 vaccination, gradually declining in the subsequent months. The the 2-ME test results from the study of Mambini *et al.* (2014) showed that one month after receiving the Razi Rev.1 vaccine, the serum titres were 40, which were lower than the titres observed in the current study. The ELISA assay results in our study revealed that all groups exhibited a significant antibody titre response 44 days after vaccination. During this period, there was a slight increase in protective antibody titres between days 28 and 44 after vaccination. However, the Wright and 2-ME findings indicated lower antibody titres at the 44-day mark post-vaccination. As a result, the ELISA test has demonstrated superior effectiveness compared to the traditional RBPT and CF tests in assessing the serological response following Rev.1 vaccination. It has shown equal efficacy to standard tests when evaluating sera from both negative and positive controls. The quantity of antibodies generated as a result of the Rev.1 vaccination depends on both the amount of inoculum administered and the vaccination method used (De Bagüés *et al.*, 1992). Furthermore, the detection of post-vaccinal antibodies can be accomplished through the utilisation of in-house fluorescence polarisation assay (FPA) and competitive enzyme-linked immunosorbent assay (cELISA) (Elrashedy *et al.*, 2022). However, the reported titres after vaccination may vary depending on numerous factors including the viable counts of bacteria in vaccine administered, the breed and age of the sheep studied, their living conditions, as well as individual differences.

CONCLUSION

In conclusion, the outcomes obtained from Rev.1 vaccine for inducing humoral immune responses in the target animals indicate that the native vaccine can effectively be used to immunise livestock in endemic regions. ELISA proved to be more effective than the conventional tests for revealing the serological reactions produced following vaccination with Rev.1, but ELISA is not a reliable method to diagnose the differences between antibodies to the vaccine and the disease. Therefore, the specific challenges and drawbacks associated with ELISA for distinguishing antibodies produced in response to vaccination versus those resulting from natural infection should be considered. This may include issues related to cross-reactivity, sensitivity, and specificity of the assay. Additionally, alternative diagnostic procedures such as culture or sequencing techniques may provide greater discriminatory accuracy. These methods can be employed to differentiate

between vaccine-induced antibodies and disease-induced antibodies, potentially influencing the interpretation of results, public health policies, and the monitoring of vaccine efficacy. In conclusion, while this study provides valuable insights into the effectiveness of the Rev.1 vaccine against brucellosis in a specific region and sheep population, its limitations, including limited sample size, low duration of study, and limited diversity in the study population, should be acknowledged when interpreting the results and considering their generalisability to broader contexts.

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