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Evaluation of the Immuno- Responses to *Brucella Melitensis*

Abdulaziz. Salih. Abdulaziz[1], Suhair Riyadh Al-Idreesi[1], Jalal Yaseen Mustafa[1]

Professor, Department of Microbiology, College of Veterinary Medicine, Basrah University, Iraq. Email: zoonshadow@gmail.com
Assistant Professor, Department of Microbiology, College of Veterinary Medicine, Basrah University, Iraq. Email: suhair.riyadh@gmail.com
Lecturer, Department of Microbiology, College of Veterinary Medicine, Basrah University, Iraq. Email: dr.jalalyaseen1982@yahoo.com

**Corresponding author's E-mail: zoonshadow@gmail.com*

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Abstract

Brucella melitensis infection remains a serious health threat to humans and livestock in poorer countries in the Middle East. Vaccination with the isolated crude protein protects against B. melitensis infection. In this study, the efficacy of protective and immunogenicity of a crude protein extract of B. melitensis is administered and evaluated in rats. The immunogenicity of crude protein in the animal blood was assessed by SDS-PAGE and Western blot. The results show that certain proteins were found to be more immunogenic; their molecular weights were (21, 23, 24, 25, 28,31, 34.45, 54.66,83 and 157 kDa). ELISA kits were used to measure the concentrations of IL-4 and IFN- in serum. In contrast to IFN-γ, this study found that the group receiving extracted protein had considerably higher serum levels of IL-4 compared to the control healthy and REV1 group.

Keywords: ***Evaluation, ImmunoResponses, Brucella, melitensis***

1. Introduction

Intracellular pathogens such as *Brucella* species are known to preferentially infect macrophages and are classified as facultative intracellular bacteria[30,35]. Human Brucella infection in many underdeveloped nations is endemic[31]. The recent resurgence of the disease increased surveillance in many parts of the world, and the disease's classification as a class B bioterrorist agent have all contributed to the rise in human brucellosis[12]

The three primary pathogens that cause human brucellosis are *Brucella suis*, *Brucella abortus*, and *Brucella melitensis*. *B. melitensis* is the species that is most widespread and virulent in China[38]. Additionally, the prevalence brucellosis in mammals is a major hindrance to the commerce and export of animals due to decreased productivity[20], an increase in abortions, and weak progeny[13,19]. Human brucellosis is a severe, incapacitating illness (undulant fever) that necessitates protracted treatment with a variety of antibiotics and is linked to high medical expenses and lost productivity[9].

The most effective vaccine for the prevention of caprine brucellosis is now *B. melitensis* Rev.1, an attenuated smooth strain used to treat *B. melitensis* infections[2]. It offers heterologous immunity to other Brucella spp[22,32] . Health authorities have, however, prohibited its use for human vaccination because of serious issues, including this strain's capacity to infect humans[11] and the emergence of streptomycin resistance. Due to the need for a subunit vaccine against *B. melitensis*, research on the immunogenicity and protective properties of Brucella outer membrane proteins (OMPs) and cytoplasmic proteins is becoming more and more popular [24].

Scientists still struggle to make a prompt and reliable diagnosis of brucellosis in both humans and animals because of its ambiguous clinical signs and delayed blood culture growth[1]. A core oligosaccharide portion and O-polysaccharide (OPS) section known as O-antigen make up the majority of lipid A, which is a lipophilic component, in practically all Gram-negative bacterial outer membranes[33]. Due to the great immunogenicity and conservation of the O-antigens across many Gram-negative bacteria and species, antibodies produced against the sLPS of E. coli or Yersinia enterocolitica can also bind Brucella LPS. To enhance the current serological tests and better assist disease eradication programs, Brucella-specific immunogenic targets must be identified through investigations of Brucella strains at the protein level [33].

The envelope's proteins interact with the host's immune system and trigger the creation of antibodies. To avoid using homologous proteins with the main cross-reactive Gram-negative bacteria, these B. melitensis envelope proteins were examined for their immunogenic potential[29]. The immunological response to these proteinous extracts of B. melitensis was then determined by a study using Western Blotting (WB) analyses to identify particular immune-reactive bands of Brucella, IL-4 is sometimes referred to be a prototypical immune-regulation cytokine due to its significance in the production of antibodies[8]. IFN- is a critical MHC I inducer and macrophage activator. One of the phases in natural immunity, this cytokine is primarily produced by natural killer cells (NK cells) and natural killer T cells (NKT cells). Produced by cytotoxic T lymphocytes and T helper (Th) CD4+ cells, it is crucial for acquired immunity as well. Brucellosis vaccine development is still ongoing, hence this study focused on developing a specific sort of vaccination (protein). Looking into the immunological reactions to this vaccination [14,15], all this elucidates the actual roles of these potent cytokines in the modulation of the initial immune response to Brucella infection[3].

2. Material and Method

2.1. Collection Of Samples and Isolation of Bacteria:

Blood samples were taken from all groups after administering the first dose and the second dose after 14 days after the first dose. After anesthetizing the rats, taking blood from the heart, centrifuging it, and taking the serum to conduct tests on it, the work was done in the laboratories of the University of Basra's College of Veterinary Medicine –Iraq for the period from January 2022 to March 2022.

2.2. Bacterial Strain:

B. melitensis were cultured in tryptose-soy agar (HIMEDIA) containing Brucella selective antibiotics.

2.3. Preparation of Vaccine Antigens:

Preparation of bacterial culture for extraction of vaccine protein antigens using Trichloroacetic acid method as a precipitant for proteins. The extract was collected, dried in an incubator for 24 hours and stored in airtight containers at 4°C until use as a vaccine.

2.4. Experimental Designing:

For this purpose, 24 male Wistar albino rats (8 weeks old and 200-220 grams body weight/animal) were used, which were divided into 4 groups, with 6 animals per group, animal maintenance, handling, and all experiments were performed with strict accordance to institutional ethical guidelines and international protocols. The groups were injected as following table (1):

Table 1: *Types of the experimental groups:*

Groups	Type of Injection
Group 1	Injected subcutaneously with a dose of 1 ml of the prepared Brucella protein by Trichloroacetic acid the injections were repeated after 15 days
Group 2	Injected subcutaneously with *brucella. melitensis* REV1 the injections were repeated after 15 days (C+)
Group 3	1 ml of saline was injected subcutaneously, and the injection was repeated after 15 days (C-)
Group 4	control

Source: authors

2.5. Evaluation of vaccine antigen Protein:
The Biuret method was adopted in the estimation of protein, using a kit supplied by System Bio Company.

2.6. Up-And-Down Method
In this method, after administration of a dose to the experimental animal, it can be reduced if the animal died or elevated if the animal survived and does not respond or exhibit clear clinical signs[23].

2.7. Assessment of the Induced Interleukin-4 (IL-4) and Interferon- (IFN- Γ) in Experimental Groups:
The ELISA assay was used to determine the concentrations of IL4and IFN-γ after each prime and booster doses injection for each prepared vaccine, positive control and negative control including groups vaccinated with attenuated commercial vaccine and PBS, respectively. All ELISA kits were purchased from ELABSCIENCE.

2.8. SDS-PAGE (Polyacrylamide Gel Electrophoresis) and Western Blot Method:
Samples were kept in a container of liquid nitrogen and then removed for homogenization. Fifty microliters of RIPA lysis buffer (E-BC-R327, Elabsciences, China) were added to the bacterial pellet (obtained from a 3 ml bacterial culture). The supernatant of the samples was collected after 10000 RPM for 10 minutes. The samples were mixed with 5X loading sample buffer (E-BC-R288, Elabsciences, China) and the mixture was then heated at 95 °C for 5 minutes. Samples were loaded onto a 10 % SDS-PAGE gel (E-IR-R305, Elabsciences, China). The samples were run on the gel for 45 minutes at 200 volts using the BioRad powerpack electrophoresis power supply (UK) using the electrophoresis buffer (E-BC-R331, Elabsciences, China).

After electrophoresis, the gels of the proteins of *B.melitensis* were separated and transported to the nitrocellulose membrane (diameter of 0.4μm pores) Nitrocellulose membrane was shifted using the semi-liquid western blot system for 15 minutes, the application of a temperature of 4 ° and a 250 mA electric current for 2.5 hours. using nitrocellulose membrane was washed with distilled water twice, each time for five minutes, to remove the gel residue and the residue of the transfer buffer. The membrane was immersed with a blocking solution, consisting of 3% of the BBS albumin (BSA) dissolved in the TBS-Buffer, for 1 hour at room temperature or overnight at +4 ° C on a circular vibrator to satisfy non-specific sites on the membrane. primary antibodies to B. melitensis were incubated for one hour, then by secondary antibodies that were labeled. horse radish peroxidase (Invitrogen Company / California / USA) directed against *B.melitensis* antibodies, TBS-T solution for one hour at room temperature and on a circular vibrator, all previous stages are separated by membrane wash 3 times with TBS-T (pH = 7.5) to dispose of unrelated substances.

The membrane was incubated with the OPD substrate in 10 mL of the substrate buffer with stirring gently using a circular vibrator until the appearance of the bands indicating the antigen for (15-20 seconds) that stimulated antibodies the reaction was stopped, wash the membrane with distilled water gently twice for 2 minutes. The Gel Analyzer program was used to estimate the molecular weight.
All experiments were performed in strict accordance with institutional ethical guidelines and international protocols.

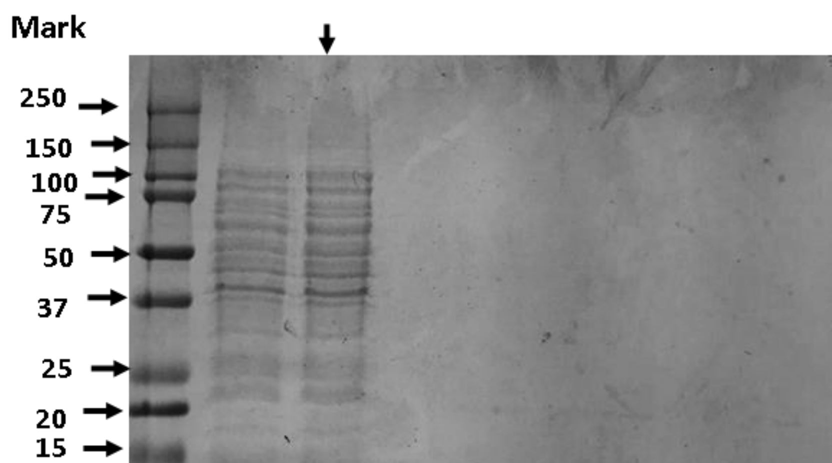
3. Result And Discussion

3.1. Protein Concentration

Using the biuret method, it was found that the protein concentration in the vaccine antigens prepared by the method of trichloroacetic acid was 62 µg \ml.

3.2. Antigens Analysis

To separate, SDS-PAGE was used. B.melitensis proteins produced the polypeptides needed for the assay. Eleven polypeptides were stained with Coomassie blue in particular. (15-125 kDa) Molecular weights were (Fig 1) In antigens prepared by the tri-chloroacetic acid technique, electrophoresis in a polyacrylamide gel with major proteins with molecular masses of 25 to 27 kDa and 36 to 38 kDa (porin) and minor proteins with molecular masses of 10, 16.5, 19, 31 to 34, and 89 kDa recognizing the Brucella27. Two major outer-membrane proteins (OMP) of 25–27 kDa and 31–34 kDa, peptidoglycan (PG), and a negligible amount (1.5%) of LPS were present in the B. melitensis SDS–cell wall fraction7. This result supports the finding 17 that the outer membrane of the Brucella family contains three major protein aggregates: the first group has a molecular weight between 88 and 94 (kDa), the second group has a molecular weight between 35 and 40 (kDa) and the third group has a molecular weight of 25 to 40 (kDa)21



Source: authors

Fig- 1: *SDS-Page analysis of crud extract of B.melitensis with coomassie blue staining*

Western blot with molecular weight 21, 23,24, 25, 28,31, 34.45, 54.66,83 and 157 kDa (Fig 2) (Table1) was discovered with immune serum (30 days) after infection by the defense im-mune.

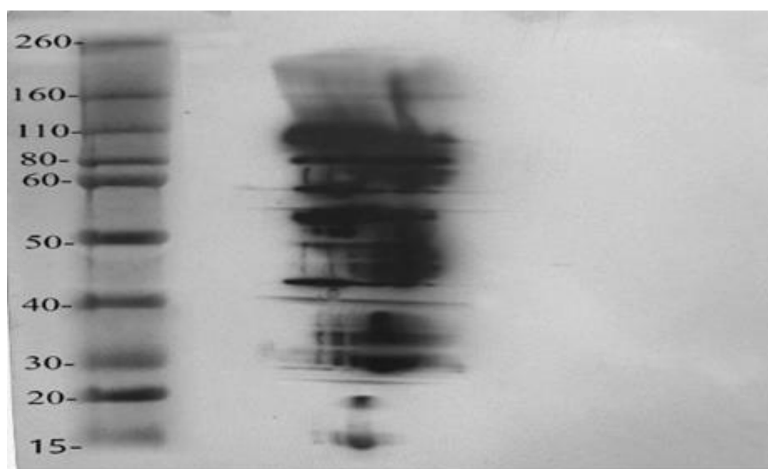


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***Fig -2**: Western blot of Brucella melitensis antigens against a serum from a B. melitensis infected rats untreated*

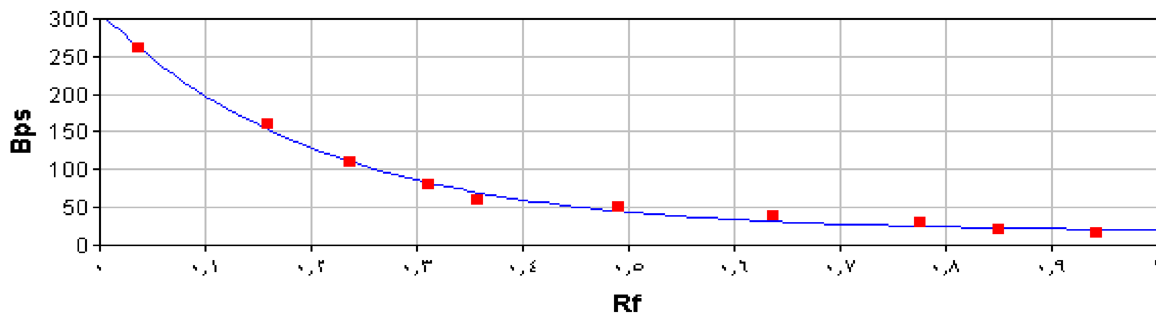


***Fig-3:** Standard curve for protein bands*

Y=291,599285*exp(-4,845033*X)+18,317416

R2=0,994

***Table-1**: the molecular weight of B.melitensis protein bands*

Lane #	Band #	Rf	Raw volume	Cal. volume	MW		
2	1	0.153	4165	-	157		
2	2	0.311	1296	-	83		
2	3	0.372	1180	-	66		
2	4	0.431	1114	-	54		
2	5	0.497	892	-	45		
2	6	0.587	1710	-	35		
2	7	0.641	3691	-	31		
2	8	0.705	2389	-	28		
2	9	0.766	1818	-	25		
2	10	0.817	3122	-	24		
2	11	0.853	4832	-	23		
2	12	0.944	145	-	21		

Source: Authors

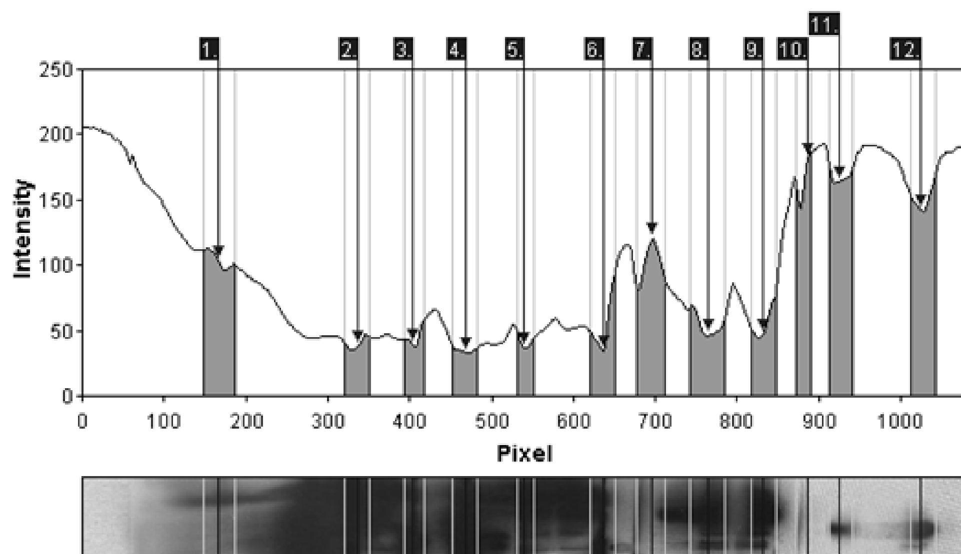


***Fig-4:** Sample western blot by using a gel analyzer*

Source: Authors

The total serum proteins from our experiment reacted with proteins with a molecular weight between 160 and 15 kDa with distinct bands at (21, 23, 24, 25, 28, 31, 34.45, 54.66, 83 and 157 kDa which is in agreement with [29]who reported proteins with a molecular weight range between 15 - 160 kDa in the Western blot for *Brucella. melitensis* total serum antigens from infected sheep[16]. Were recognized by western blot as hypothetical *B. melitensis* cell envelope proteins, referring to a total of ten proteins, the total or crud protein data will be helpful in the future development of innovative vaccines and accurate diagnostic tests. Interestingly, immunoreactive proteins include eight recognized virulence-related proteins, including OMP25 and other proteins[33]. Mentioned the discovery of 12 immunogenic proteins from *B. melitensis,* may which can be used to differentiate between animals that have Brucella infection and those with *Yersinia. enterocolitica* and *E. coli* infections. Seven of these should be outer membrane proteins (OMPs).

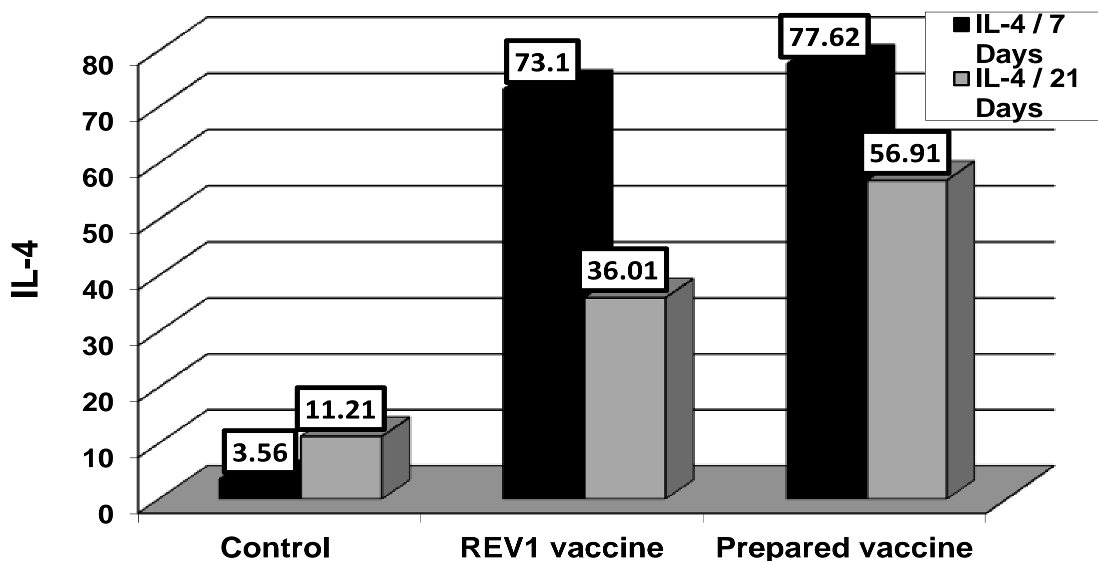
OMP25 and OMP31, both considered highly immunogenic substances in *B. mlitensis*, appeared in our Western blot results. OMP25 is a member of the OMP A protein family, which accounts for approximately 30–40% of the outer membrane in the geum of *B. melitensis* OMP25 regulates immunological responses and may act as a mediator that prevents host cells from producing TNF-alpha[6]. In Brucella species, the bcsp31 gene is highly conserved, according to numerous studies. Given this high antigenicity and conservation, the diagnostic potential of the omp31 product could be explored[37]. The protein product of the (BCSP31) gene of omp31 is extremely immunogenic, selective, and conserved. BCSP31 and anti-BCSP31 MAbs have the potential to be effective brucellosis diagnostic tools. Due to variable clinical features and slow growth in blood cultures, scientists still have difficulty diagnosing human brucellosis rapidly and accurately[36].

OMP31, an outer membrane protein of *B.melitensis*, was also cloned, prepared, and purified. This antigen has been shown to react with some, but not all, blood samples from humans, dogs, sheep, and rams infected with *Brucella* spp. In addition, has been clearly shown to react with positive pooled human serum[10]. In Addition, it has been reported that immunization of animals with rOmp31 encoded for *B. ovis*, alone or in combination with *B. ovis* -type R-LPS, resulted in the development of tolerable defenses against *B. ovis* infection in the immunized mice[25]. Analysis of the interactions of rOmp31 with vaccinated rabbit serum in the current study revealed that pre-vaccinated rabbit serum was not the strongest ELISA reactant of rOmp31. These results may indicate that Omp31 is a promising option for a *B. melitensis* subunit vaccine. CD4+ Tcells are one of the most important sources of IL-4 because they respond directly to foreign antigens and activate them for a rapid immunologic response to these antigens. In agreement with[26], which showed that IL-4, IFN-γ, and TNF-α levels were higher in brucellosis patients than in control subjects in terms of disease progression, showed that groups vaccinated with prepared vaccines had a significant increase in the level of IL-4, which may indicate stimulation of Th2 cells and promotion of humoral immune response. In addition, the results support previous research showing that Th1 cytokines help the body initially fight infection. And the result of [3]suggest that these CW fractions most likely stimulated Th2-dependent rather than Thl-dependent antibody responses in BALB/c mice. Immunization with the pepsin-digested SDS-I cell wall fraction of *B. melitensis* strain 16M (S) resulted in the highest protection rates. We agree with previous studies that found protection against Brucella. spp may be obtained through immunization with proteins extract (recombinant Omp31-enriched preparations) that elicit strong Ab responses but weak cellular responses. Significant total serum IgG levels and astonishing IgG1 and IgG2a responses to omp16 protein were observed[5].

3.3. ELISA:

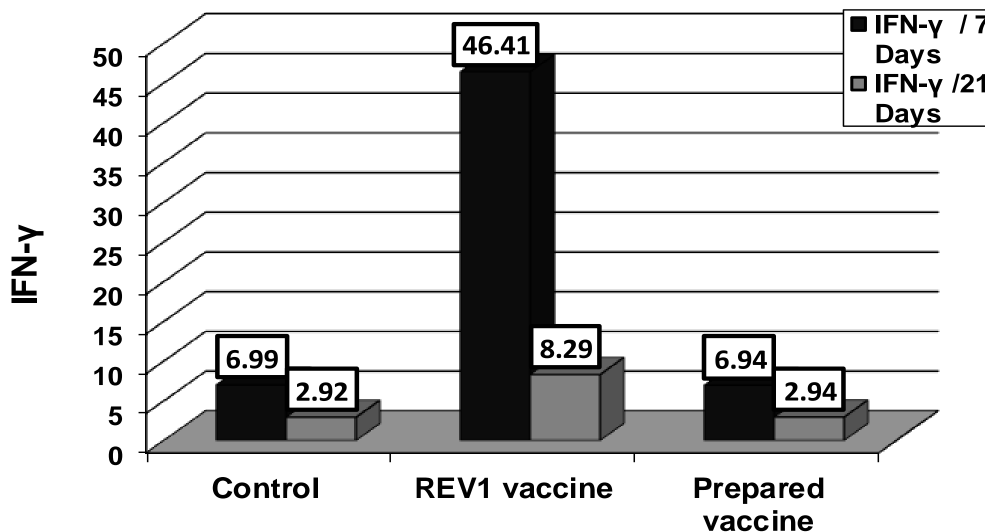
The results revealed that the experimental conditions employed increased significantly the antigenic properties of the protein extract, leading to the benefit of the industrial scale-up. Data were recorded, and these differences between study groups displayed significant differences. experimental groups and control, and Our results demonstrated that groups vaccinated with prepared vaccines produced a significant increase in IL-4 concentrations Harvested data show the study group to have significant differences at $p \leq 0.01$ (prepared vaccine) compared with the controls and group vaccinated with REV1 vaccine Fig (5).

Recorded data showed no significant differences in IFN-γ concentration between prepared vaccine group and other research groups based on the data collected. The result referred to the concentrations of IFN-γ is highly in animal group vaccinated with REV1 vaccine with significant differences ($p>0.01$) compared with our prepared vaccine group while the IL-4 concentration was no significant values Fig (6).



Source: Authors

Fig- 5: *The IL-4 values in different groups of experiment after 7 and 21 days from prime dose*



Source: Authors

Fig-6: *The IFN-γ values in different groups of experiment after 7 and 21 days from prime dose*

In the current study, serum levels of IL-4 were significantly higher in our study group than in REV1 and controls. However, there was no discernible difference in the serum levels of IFN-γ between our research group and the healthy limit. It is suggested that the decrease in IFN-γ during *B. melitensis* infection limits macrophage production of nitric oxide during primary infection as well as cytokine induction in the late stages of infection.[16] In general, after activation, CD4+ T-cellscan differentiate into either Th1 or Th2cells, which are responsible for the humoral immune response. Therefore, the differentiated Th2 cells may produce IL-4 through a feedback mechanism known as the positional feedback loop, producing additional amounts of IL-4, which may explain the low IFN-γ production in patients infected with *B. melitensis*[18].

When live *Brucella* are present, the host immune system must rely on cell-mediated immunity, which primarily includes activated antigen-presenting cells (macrophages and dendritic cells), CD4+ T helper (Th) cells, and CD8+ cytotoxic T cells. The recorded data showed significant differences at p0.01 between the REV1 group in our study and the control group[28]. Previous studies have shown that IFN-plays a vital role in cell-mediated immunity to brucellosis and can predict future susceptibility to symptomatic brucellosis. Our studies revealed that the protein extract vaccination group had significantly higher serum levels of IL-4 than the REV1 group. Different effects on Brucella pathogenesis are caused by a Th-2 cell phenotype that stimulates Brucella-triggered inflammation and the release of IL-4. When these cytokines are released, the bactericidal function of macrophages can be downregulated, leading to tissue damage by triggering the synthesis of necrotizing enzyme[19]. In addition, B. melitensis has been shown to prevent antigen presentation and respiratory bursts of macrophages. Th-2 cytokines cause mucosal inflammation, which helps to control Brucella infection early and prevent broad damage. Because live attenuated Brucella vaccines have several limitations, many scientists are interested in developing a safe and effective subunit vaccine against brucellosis. Identification of potential vaccine candidates is critical in this process. In this regard, several Brucella protein antigens have been evaluated for resistance to experimental infection; only a small number of protein antigens have demonstrated a significant protective immune response[34].

4. Conclusion

Crude vaccine from *B.melitensis* by tri-chloro-acetic acid method showed a potent immune response where serum levels of IL-4 were significantly higher in our study group than in REV1 and controls. A polyacrylamide gel revealed many protein bundles with molecular weights of 15 to 75 (kDa). In western blot, the total serum proteins from our experiment reacted with proteins with a molecular weight between 160 and 15 kDa with distinct bands at (21, 23, 24, 25, 28, 31, 34.45,54.66, 83 and 157 kDa. Further research might study vaccines against brucellosis, focusing on the outer membrane protein groups (OMP), especially OMP31.

Conflict of Interest

The authors declare no conflict of interest.

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None.

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