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# INDIRECT FLUORESCENT ANTIBODY TEST (IFAT) TO STUDY THE EFFECT OF THE BIOLOGICAL IMMUNOMODULATOR MANNOPROTEIN OF *CANDIDA ALBICANS* IN THE MICE VACCINATED WITH Rev- 1 BRUCELLA VACCINE

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**ABSTRACT**
A study was carried out to investigate, the immunomodulatory effect of the Mannoproteins of *Candida albicans* Mannoprotein on the Humeral immune response estimated by (IFAT) of mice vaccinated with *BrucellaRev-1* vaccine and considered as biological immunomodulators. This experiment was carried out in animal Farm of National Centre of Drug Control and Research and Central Public Health laboratory in Baghdad from November 2011 to May 2012.The study included eight groups; the first four subgroups were I: treated with distilled water. II: injected subcutaneously with a dose of (300 µg /ml) cell wall mannoprotein only, III: injected subcutaneously with a dose of (200 µg /ml) cell wall mannoprotein only, IV: mice treated with brucella-*Rev-1* vaccine only. The V, VI were treated with combination of Brucella Rev-1 vaccine and mannoprotein while VII and VIII groups were injected with the immunosuppressive drug prednisolone prior to the forthcoming treatment 5 days. All these treatments were carried out on day 1, and then the mice were sacrificed on day 21 and 28 to estimate indirect immunoflourescent test , The anti-Brucella antibodies assessed by indirect immunoflourescent test showed a significant increase titer in immunomodulator-treated and -vaccinated mice in comparison with negative and positive groups . At 21, 28 days, and the best treatment efficiency was recorded in-group VI after 28 days with titer of 512.

**KEYWORDS**: Brucellosis, Mannoproteins, Brucella Rev -1, IFT, Mice.

## INTRODUCTION

Brucellosis is one of the most important zoonetic diseases worldwide, resulting in serious economic losses and public health issues. It is caused by intracellular Gram-negative bacteria of the genus *Brucella*, which are responsible for a debilitating disease in humans and a chronic infection in domestic animals (Vidya *et al*.,2011) . In this regard, biological immunomodulator are materials that mediate the effectors mechanisms of the immune system through immune stimulation to a given antigen or potentiate the effectiveness of a vaccine (Farid *et al*,.2003 ). Recent advances in immunology have led to design vaccines to maximize activation of the humoral or cell-mediated branches of the immune system (Grimble and Grimbl., 1998). *Candida albicans* has been one of the fungal species that share the interest of investigators in the field of immune modulation. Mannoproteins purified from *C. albicans* and administered to mice before or during immunization with viable *C. albicans* developed a significant increased in both humoral and cellular immune response (Ronald and Judith ,1994) Furthermore, (Shigetoshi *et al*.,2000 ) have demonstrated that immunization with mannan (a mannoproteins fraction) and mannoproteins derived from digested cell walls of *C. albicans* induced resistance to a systemic candidiasis. (Farahnejad *et al* ., 2005) Were purified cell wall mannoproteins of intact yeast using a simple treatment of yeast with mercaptoethanol and sodium dodecyl sulfate followed by Concanavalin A chromatography. They are found that Cell wall mannoproteins to be the main cause of adherence of *C.albicans* to epithelial cells in the first step of an infection process.

(Al-jindeel , 2011) was study the immunomodulatory effect of the Mannoproteins of *Candida albicans* cell wall on the immune response of mice vaccinated with *BrucellaRB51* vaccine and considered as biological immunomodulators. The results demonstrated a clear immunomodulatory effect of the mannoproteins of Candida albicans cell wall (improvement of non-specific, and cellular immune response) of the treated mice vaccinated with *Brucella- RB51*.

## MATERIALS & METHODS

### Identification *Candida albicans*

*Candida albicans* was, cultured, and maintained and identified by using *Candida* check (Akpan and Morgan 2002). Identification of Candida albicans was performed according to the method of (. Ha *et al*., 2011) by conducting biochemical test (germ tube) which is considered as specific test for identification the Candida albicans microscopically and crossly (Figure1). Microbiological observations of pseudohyphae, hyphae and chlamydospores were made on cornmeal tween 80 agar incubated at 35°C for 3 days. Culture medium GYEP containing 2% glucose, 0.3% yeast extract and 0.1% peptone (supplemented with penicillin 100 IU/mL and streptomycin 100 µg/mL) were used for *C. albicans* .

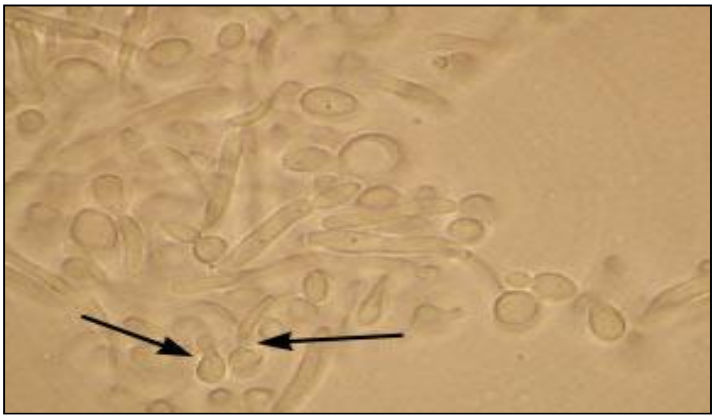

**FIGURE 1:** *Candida albicans* incubated in rabbit serum at 37° (germ tube test). Germ tubes are indicated by arrows and are the beginnings of true hyphae: no constriction is at the origin of the germ tube and the parent cell

**Preparation of cell wall extract of *Candida albicans***

*Candida* colonies were harvested in 2 liters of culture medium, and then they were subjected to a series of laboratory manipulations including ultra-centrifugation to prepare Mannoprotein , which had a final weight of 2.8 grams. Estimation of total protein in the prepared solution of mannoproteins revealed that it was 82 mg/ml, while glucose content was 78 mg/ml estimated by UV spectrophotometer.

**Determination of $LD_{50}$ Mannoprotein *candida albicans***

The evaluation of *C. albicans* Mannoprotein LD50 demonstrated a dose of a wide range safety (100 - 600 µg/kg), also was effective in terms of toxicity and immunomodulatory backgrounds. The *C. albicans* Mannoprotein is essential to nearly every aspect of the microorganism biology and pathogenicity, because it contains materials that are able to mediate interactions with the host immune response (Pietrella *et al* ., 2008 , Al-jindeel ,2011).

**TABLE 1:** Doses of Mannoprotein candida *albicans* that were used in the assessment of LD50

| *C. albicans* | Dose/kg | Dose/mouse | Number of Animals |
|---|---|---|---|
| Mannoprotein | 100 µg | 3.5 mg | 6 |
| | 200 µg | 5 mg | 6 |
| | 300 µg | 7.5 mg | 6 |
| | 400 µg | 10 mg | 6 |
| | 500 µg | 12.5 mg | 6 |
| | 600 µg | 15 g | 6 |

**Experimental Design**

There were eight groups in this experiment, which was designed to evaluate the immunomodulator potential of *C. albicans* Mannoproteins in mice vaccinated with *Brucella* Rev-1 vaccine. The total number of animals in these groups was 200 mice (25 mice in each group).

- Group I: mice were inoculated subcutaneously with a single dose (0.2 ml) of deionized distilled water in day 1.
- Group II: mice were inoculated subcutaneously with a high dose (300 µg /ml) of mannoproteins in a total volume (0.2 ml) day 1.
- Group III: mice were inoculated subcutaneously with a moderate dose (200 µg/ml) of mannoproteins in a total volume (0.2 ml) day 1.

Immunologic parameters in mice vaccinated with combination of Mannoprotein and brucella rev.-1 vaccine

- Group IV: mice were inoculated subcutaneously with a single dose *B*rucella Rev-1 vaccine in day 1.
- Group V and VI: mice were inoculated subcutaneously with a single dose of *combination of B*rucella Rev-1 vaccinated moderate and high dose respectively in day 1.
- Group VII and VIII: mice were inoculated subcutaneously with a single dose of prednisone 5 days prior to the combination of the *Brucella* Rev-1 vaccine and moderate and high dose of mannoprotein in day 1.

**Indirect Fluorescent Antibody Test (IFAT):**

The IFAT was used to assess anti-*Brucella* antibody titer in the sera of mice that were immunized with *Brucella* Rev-1 vaccine in different treatment regimens. The procedure of (WHO 1997) was adopted to determine such titer.

1. One milliliter of blood was obtained by scarified mouse and transferred to Eppendorf tube. The blood was left at room temperature to clot for 15 minutes, and then it was centrifuged at 2000 rpm for 15 minutes. After that, the serum was collected, divided into aliquots (50 µl) and frozen at -20°C until use in this assay and other assays.
2. Five microliters of formularized *Brucella* Rev-1 strain 89 * $10^6$/ml (the bacteria were treated with 10% formalin) were applied to the wells of a slide containing 12 circle, and after air-drying; the bacterial cells were fixed with acetone for 5 minutes.

3. One aliquot of the frozen serum was thawed at room temperature for around 15 minutes, and then serial dilutions (1:16, 1:32, 1:64, 1:128 and 1:256) were made using the PBS (pH 7.2) as a diluting solution. Normally, the test started with the dilution 1:16, and if the serum was positive, the next dilution was assessed and so on.
4. Ten microliters of diluted serum was added to the circle that contained the fixed antigens, and the slide was incubated for 30 minutes in a humid chamber at 37ºC.
5. The circles were washed with PBS for 10 minutes, and then the slide was dried using Whattman filter paper No. 1.
6. Ten microliters of Rabbit Anti-Mouse antibody conjugated with fluorescin were added to each well, and the slide was incubated for 30 minutes in a humid chamber at 37ºC.
7. Step no. 5 was repeated, but this time the PBS was mixed with Evan's blue.
8. The slide circles were examined with fluorescent microscope (Ceti, Germany), and the well that emitted green rays was considered positive, while those that emitted red rays were considered negative.

**RESULTS**

The sera of mice in groups II, III, and I showed no anti-*Brucella* antibodies at the start titer 16 after 21 days, while the other groups showed some variations. All mice of group V and VI showed a higher positive immunoflourescent reaction at the titer 128, while the other group VIII showed a positive reaction which was observed at the titer 64. These results are given in (Table 2). After 28 days the sera of mice in groups II, III, and I showed no anti-*Brucella* antibodies, while the highest anti brucella antibodies titer was recorded in mice of group VI at the titer 512 and at the titer 256 in mice of group V. After that positive Immunoflourescent reaction at the titer 64 was observed in mice of groups IV and VIII After. These results were given in (Table 3).

**TABLE 2:** Anti-*Brucella* antibody titers of mice vaccinated with *Brucella* Rev-1 vaccine and inoculated with *C. albicans* Mannprotein after 21 days

| Groups | Anti Brucella antibodies Titers after 21 days | | | | |
|---|---|---|---|---|---|
| | 16 | 32 | 64 | 128 | 256 |
| I | 0 | 0 | 0 | 0 | 0 |
| II | 0 | 0 | 0 | 0 | 0 |
| III | 0 | 0 | 0 | 0 | 0 |
| IV | 16 | 32 | 0 | 0 | 0 |
| V | 16 | 32 | 64 | 128 | 0 |
| VI | 16 | 32 | 64 | 128 | 0 |
| VII | 16 | 32 | 0 | 0 | 0 |
| VIII | 16 | 32 | 64 | 0 | 0 |

**TABLE 3**: Anti-*Brucella* antibody titers of mice vaccinated with *Brucella* Rev-1 vaccine and inoculated with *C. albicans* Mannoprotein after 28 days.

| Groups | Antibrucella antibodies Titers after 28 | | | | | |
|---|---|---|---|---|---|---|
| | 16 | 32 | 64 | 128 | 256 | 512 |
| I | 0 | 0 | 0 | 0 | 0 | 0 |
| II | 0 | 0 | 0 | 0 | 0 | 0 |
| III | 0 | 0 | 0 | 0 | 0 | 0 |
| IV | 16 | 32 | 64 | 0 | 0 | 0 |
| V | 16 | 32 | 64 | 128 | 256 | 0 |
| VI | 16 | 32 | 64 | 128 | 256 | 512 |
| VII | 16 | 32 | 0 | 0 | 0 | 0 |
| VIII | 16 | 32 | 64 | 0 | 0 | 0 |

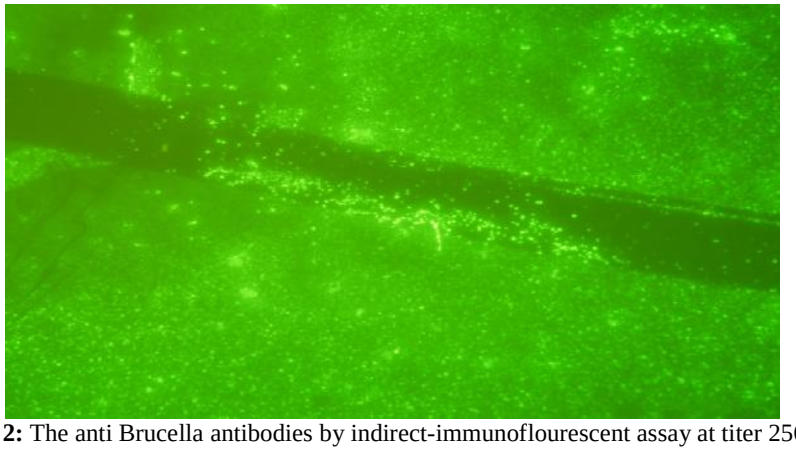

**FIGURE 2:** The anti Brucella antibodies by indirect-immunoflourescent assay at titer 256.

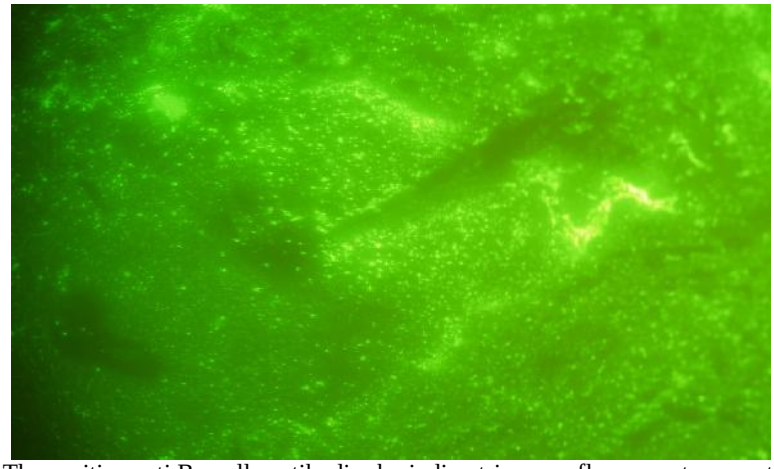

FIGURE 3: The positive anti Brucella antibodies by indirect-immunoflourescent assay at titer 128.

**FIGURE 4:** The negative anti Brucella antibodies by indirect- immunoflourescent assay.

## DISCUSSION

Immunofluorescence is the visualization of antigens within cells using antibodies as fluorescent probes; The method has achieved the status of combining high sensitivity with high resolution in the visualization of antigens and will be a major tool for many years that any pathologist studying cells or molecules cannot afford to ignore; For a methodology article on immunofluorescence labeling of formalin-fixed, paraffin-embedded tissue (Robertson *et al*., 2008). Anti-Brucellin antibodies showed an increased titer in all immunized groups treated with the immunomodulators used in the study, especially groups V1 and IV as compared to the control group that received vaccine only. Such observation suggests that the immunomodulation also involved the humoral immune response, although the pathway may be through the modulation of macrophages and T lymphocytes as both types of cells are required to enhance the B-lymphocytes to produce immunoglobulin (Takahashi, 2003). These are in agreement with this conclusion; several researchers suggested the potentional use of *C. albicans* cell wall mannoproteins in this line of experimental immunology by using different laboratory approaches and animals (Donatella *et al* , .2008 **(**Farid *et al*., 2003 **,** Grimble and Grimble., 1998 **;** Ronald and Judith 1994). Figure (2, 3,4) shows the positive and negative results for *anti brucella antibodies* by immunoflourescent assay.

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