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Protective and immunogenic characters of inactivated oil adjuvant *Brucella melitensis* vaccine versus Rev-1 vaccine

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Owing to the economic and zoonotic importance of *Brucella* melitensis, the present study aimed to evaluate inactivated *Brucella melitensis S-16M* vaccine prepared by using formalin 0.5% then mixed with montanide ISA-206 oil in a ratio of (1:1) as a protective vaccine for control of brucellosis in goats and sheep. Adult *Brucella* free guinea pigs (350-500g) were divided into four groups and the prepared vaccine was evaluated for protection of guinea pigs against challenge infection with virulent *B. melitensis* S-*16M* in comparison to Rev-1 vaccine and to measure the humeral and cellular immune response of the guinea pigs by using different serological tests such as Rose Bengal Plate Test (RBPT), Buffered Acidified plate test (BAPA) and ELISA. Moreover, it was evaluated in susceptible sexually mature goats by using different serological tests such as RBPT, BAPA, Standard tube agglutination and Complement fixation tests to assure that it could be used safely in the field. It was found that the protective activity of the prepared vaccine was 2.944 while that of Rev-1 was 1.66 in guinea pigs. Differences between the protection afforded by the oil adjuvant vaccine and standard Rev1 vaccine were not significant (at $P \geq 0.05$) but the prepared one was potent, safer and stimulated humeral and cellular immunity. In comparison with PBS inoculated (unvaccinated) group, the protection given by the prepared vaccine was significant and also it was safe and immunogenic as antibody titer in sera of vaccinated guinea pigs and goats was monitored with serological tests.

Keywords: Brucellosis, *Brucella melitensis*, vaccine, serological, immunogenic.

INTRODUCTION

Brucella melitensis is the etiological agent of caprine brucellosis, an infectious zoonotic disease with significant economic impact on both the livestock industry and public health. Approximately 90% of goats are located in the developing world, where they are considered one of the important sources of protein for human. Among the different *Brucella* spp. capable of causing disease in humans (*B. abortus, B. melitensis, B. canis and B. suis), B. melitensis* is the most virulent (Corbel, 2006). Nowadays, more than half a million new brucellosis cases per year occur naturally in the populations of developing areas of the world (Pappas et al., 2006). The clinical signs of the disease in animals are characterized by abortions or reproductive failure (infertility, retention of placenta, stillbirth or birth of weak offspring), decreased milk yield and epididymitis in males. While animals typically recover, and will be able to have live offspring following the initial abortion, they may continue to

shed the bacteria. The introduction into the country of foreign cattle breeds in recent years to increase milk production resulted in a raise in the incidence of the disease.

The control and eradication of brucellosis using test and slaughter methods although applied in some countries appeared to be unsuitable for Egypt. Vaccination is an essential step for prevention of further spread of the disease and minimizing the foci of infection. The live *B. melitensis Rev-1* vaccine strain is the most effective vaccinal strain available for prophylaxis and controlling of brucellosis in small ruminants (Elberg, 1981). The standard full dose is 1x 10^9 CFU for young animal (3-6 months) given S/C (Alton, 1985, 1990). However, it displays a number of drawbacks, the interference in the diagnosis with the current serological tests (Blasco et al., 1993). That when it is used in sexually mature animals, it may cause long lasting serological response, interfering with the differentiation between infected and vaccinated animals (Marin et al., 1990). It can also result in abortion if the vaccination is carried out on pregnant females. In addition to the probability of shedding of the vaccinal strain in milk during lactation, consequently human may become infected by consumption of unpasteurized milk and fresh cheese processed from infected milk (Alton, 1985) and when it is given to calf animals, the animal may become *Brucella* carrier for the rest of its life (Blood and Radostits, 1989). So, the aim of this work is to develop a vaccine against *Brucella melitensis* protecting 80% of animals, safe for pregnant animals, effective against *Brucella melitensis* in sheep and goats with potential for a second target species, affordability for small holder farmers, including a sufficiently low cost of manufacturing, provide maximum human and animal safety and therapeutic effect on infected animals.

MATERIALS AND METHODS

Strain*:*
Brucella reference strain 16*M* (*Brucella melitensis biovar1*, virulent strain) was obtained from CITA Institute, Zaragoza, Spain.

Experimental animals:
Guinea pigs: Adult *Brucella* free guinea pigs (350-500g) were divided into four groups; 1st group was used to test the potency of the prepared oil adjuvant *B. melitensis 16M* vaccine, 2nd group was inoculated with sterile 10 mM PBS (PH 6.85) kept as negative control, 3rd group was vaccinated with Rev-1 vaccine and 4th group was used for application of safety test among the prepared inactivated oil adjuvant vaccine.
Goats: Local breed *Brucella* free goats of 8 months to one year and half of age were divided into two groups, 1st group was used for evaluation of the immune responses of the prepared inactivated oil adjuvant vaccine and the 2nd group was reared in separated area as a control un-vaccinated group.

Vaccine preparation:
Harvesting and preparation of *B. melitensis* suspension was carried out according to Alton et al. (1988), the bacterial pellet was re-suspended in PBS (PH 7.2). Brucella *melitensis* S-16M culture was inactivated according to Magnani et al., (2009) and Motaharinia et al. (2013) by using formalin 0.5% (Cameron et al., 1972; Brown et al.,1986 and Ebeid et al., 2011) as inactivated agent and incubate the mixture at 37 °C for 48 hours then adjust the density of culture (Alton et al.,1988). An equal volume of antigen solution was incorporated into montanide ISA-206 oil in a ratio 1:1 according to Barnett (1996) and Lyer et al. (2001). Preparation of 1ml of inactivated culture contains 1-2 X 10^{10} CFU/animal (1/4 dose of goat) according to Cameron and Fuls (1973) mixed with montanide ISA -206 oil adjuvant in a ratio 1:1 for 1st group of guinea pigs. Preparation 1ml of Rev-1 vaccine contain 1 X10^8 CFU/animal (1/15 dose of goat) according to British Pharmacopeia (1985) for 3rd group of guinea pigs. Preparation 2ml of inactivated culture contain 1-2 X 10^{11} CFU mixed with montanide ISA-206 oil adjuvant in a ratio 1:1 for goats according to Shafik et al. (2012).

Evaluation of oil adjuvant *Brucella melitensis* strain 16-M vaccine:
It was performed according to Office International des Epizooties (2008) including purity, sterility, safety and potency tests.

Vaccination schedule:

Guinea pig's vaccination:
Adult *Brucella* free guinea pigs (350-500 g) were divided into four groups and managed as follow:
Group1: Each guinea pig vaccinated with 1ml I/D of inactivated *B. melitensis S-16M* culture contains 1-2X10^{10} CFU mixed with montanide ISA 206 in a ratio 1:1, booster dose of the same

vaccine was given 3 weeks after the 1st dose. Guinea pigs were examined for 8 weeks after inoculation to note any post-vaccinal reaction as well as any systemic reactions.

Group2: Each guinea pig inoculated with 1ml I/D of 10 mM PBS (PH 6.85).

Group3: Each guinea pig vaccinated with 1ml S/C of Rev-1 vaccine contains 1 X 10^8 CFU.

Group1,2 and *3* each guinea pig was challenged I/M with 1 X 10^5 CFU of virulent *B. melitensis* S-16M after 8 weeks post vaccination studying the protection level according to (OIE, 2000). Serum samples were collected from guinea pigs pre-vaccinated and weekly post vaccination for 16 weeks.

The guinea pigs were scarified at the end of the experiment and postmortem examination was done.

Group4: Each guinea pig was vaccinated with 10 dose of the prepared vaccine by I/M injection, the animals were placed under observation for any abnormal reaction for 2 weeks.

At the 5th week post vaccination, 3 guinea pigs were taken from 1st group; 2nd group (control negative) and 3rd group were inoculated I/D with 0.1 ml of Brucellin (INRA) for determination of the cell–mediated immune response by Brucellin test.

Goat's vaccination:

Local breed *Brucella* free goats of 8 months to one year and half of age were divided into two groups as follow:

Group1: Each goat was vaccinated with inactivated culture of *B. melitensis (S-16M)* oil adjuvant by inoculation of 2ml contains 1-2 X 10^{11} CFU mixed with montanide ISA-206 in a ratio 1:1 I/M. Booster dose of the same vaccine was given 4 weeks after the first dose.

Group2: Goats were reared in separated areas under hygienic measures and were kept as a control un-vaccinated group. Serum samples were collected weekly till 19 weeks post inoculation.

Evaluation of protection efficiency of the prepared vaccine in guinea pigs:

Determination of the humoral immune response by screening tests such as Rose Bengal Plate test, Buffered Acidified Plate test, micro–agglutination test and the indirect enzyme linked immunosorbent assay (IELISA). This test was conducted by using IELISA coated plates with LPS (prepared from *B. melitensis S-16M*) in carbonate bicarbonate buffer (PH 9.6) for detected *Brucella* antibodies.

The cell mediated immune response by delayed hypersensitivity (Brucellin test) was applied at the 5th week post vaccination for three guinea pigs from groups (1), (2) and (3) by using Brucellin (INRA) 0.1 ml inoculated I/D.

Protective test was applied at the 8th week post vaccination for groups (1), (2) and (3) experimentally infected I/M with virulent *B. melitensis (S-16M).*

Spleen count was carried out at 8th week post challenge for groups (1), (2) and (3). The spleens were collected and examined for number of Brucella for spleen following the following formula:

$\underline{Y = \log (x / \log x)}$ **.....** Y= Protective average…. X= number of Brucella for spleen.

Evaluation of the protection efficiency of the prepared vaccine in goats:

Determination of the humoral immune response by screening tests such as Rose Bengal Plate test (RBPT) and Buffered Acidified Plate test (BAPA), Serum agglutination test (SAT) and complement fixation test (CFT).

Statistical analysis:

Statistical analysis using ANOVA test was applied on immunogenic results according to Snedecor and Cochran (1989**)** to compare the efficiency of the prepared vaccine for guinea pigs and goats.

RESULTS and DISCUSSION

The optimal design for a brucellosis vaccine requires a non-living vaccine that confers effective immunity. Here, we demonstrated that inactivation of *Brucella melitensis* by formalin 0.5% and adding adjuvant for efficient triggering of protective responses. Inactivated *Brucella* protects guinea pigs against virulent bacterial challenge. The vaccine presently used for sheep and goats is living attenuated *B. melitensis Rev1* elicit protection but retain unacceptable levels of virulence, with more than 500,000 human infections yearly; however, no human vaccine is currently available. Therefore, the development of new vaccine is one of the principal aims in brucellosis research. Inactivated vaccines against brucellosis were developed by many authors (Montaraz et al., 1986; Khalid et al., 2007; Magnani et al., 2009; Ebeid et al., 2011; Shafik et al., 2012; Shell et al., 2012; Motaharinia et al., 2013; and mahmoud et al., 2016). So, one of the principle objectives of the present investigation is to evaluate the immunity responses of guinea pigs and goats to the prepared vaccine.

The results of quality control of the prepared vaccine indicated that it was free from any contaminants as regards to safety test where the prepared vaccine did not show any abnormalities or adverse reactions during the observation period among the inoculated guinea pigs.

Brucellosis is usually diagnosed based on history, symptoms and serological tests which include RBPT, BAPA, micro-agglutination test and compared with iELISA test and CFT. In the present investigation, the humoral immune responses of the vaccinated guinea pigs against inactivated oil adjuvant *B. melitensis S-16M* (group 1) were measured by using Rose Bengal test and Acidified Plate test as a qualitative method as its positive result was recorded as agglutination (table 1). Meanwhile, the micro-agglutination test and iELISA were quantitative methods as they could detect *Brucella* antibody level (Fig 1&2) respectively.

The data presented by screening tests in table (1) showed that all sera collected from guinea pigs (group1&3) showed clear agglutination within one minute (4+) at five weeks post vaccination then decreased. After challenge, all sera collected from vaccinated animals showed moderate and clear agglutination respectively within one minute (3+ and 4+) at five weeks after challenging then the reaction began to decrease.

The positive serum samples by screening tests were examined by micro-agglutination test, positive samples were taken as those with titers > 1/40 (50%) according to the European technique Alton et al., (1988). The mean of micro-agglutination unit was increased from 0 to 128.6 after three weeks of vaccination, then increased after booster dose to133.3 at five weeks then declined gradually. After challenge, it reached to peak 186.7 at five weeks then began to decline whereas guinea pigs vaccinated with *B. melitensis* Rev1 vaccine (group3) showed increase from 20 to 181 after three weeks of vaccination, then reached to peak 320 at five weeks then declined gradually. After challenge, it reached to peak 213 at five weeks then began to decline. The mean of micro-agglutination unit of the control positive group was significantly increased weekly post infection.

On the other hand, three weeks after vaccination with the prepared vaccine, the mean ELISA unit reached to 44.2 by using ELISA plate coated with *B.melitensis S-16M* lipopoly-saccharide (LPS) as shown in Fig. (2), when a booster dose was given and evaluated after two weeks, the level reached to 89.9 that was in agreement with Gaidomakova et al. (2012), Tuasikal et al. (2012), Seo (2015) and Mahmoud et al. (2016) who stated that irradiated bacterial vaccines (inactivated vaccines) generates higher humoral immune responses, then the level reached to 130.3 after five weeks of challenge then the level begins to decline at the 6th week post challenge while in guinea pigs vaccinated with *B. melitensis* Rev1 vaccine (group3), the mean ELISA unit was 146 at five weeks post vaccination and reached 122 at five weeks post challenge. Whereas in group (2) the non-vaccinated infected group, the mean ELISA unit was varied between 5.1 and 6 before infection with virulent *B. melitensis S-16M* then the units were significantly increased weekly after infection till reached to 257.8 at the end of experiment that mean there was no clearance for the pathogenic strain and produce an S-LPS antibody responses which has hampered diagnostic efforts to differentiate vaccinated animal from infected one. These results were in agreement with Nielsen et al. (1989) and MacMillan et al. (1990) who concluded that vaccination induces antibody thought to be of lower affinity due to a short exposure time to the antigen because it is eliminated by the immune system. Alternatively, antibody produced in response to natural infection is of higher affinity because the antigen is not removed as quickly by the immune system and, therefore, persists for a much longer period.

In the Delayed Hypersensitivity Test (DHT), Brucellin was used by Bercovich and Muskens (1999) and Saegerman et al. (1999) as an allergen to define the intrinsic parameters of a skin test and to compare its prosperities with serology for diagnosis of brucellosis. The skin test was also evaluated for its capacity to solve problems associated with false positive reaction in serological tests. The optimal reading delay for the skin test was 72 hours. Previously, Jones (1974) demonstrated that the allergic test can differentiate between genera, such as *Brucella* and *Yersinia*, that show serological cross reactivity, indicates that it could be helpful in the diagnosis of animal brucellosis in problem herds with serological reactions and no history of brucellosis.

In the present investigation, intradermal test was performed as described by Alton et al. (1988). The skin diameter was measured prior to infection and 4, 24, 48 and 72 hours thereafter, differences ≥ 7mm were considered to represent positive reaction according to Otitazki (1970).

Table (1): Results of Rose Bengal Plate Test (RBPT) or Buffered acidified plate antigen (BAPA) among guinea pigs vaccinated with inactivated *B. melitensis S-16M* oil adjuvant vaccine in comparison with *B. melitensis* Rev-*1* vaccine.

	Pre-vaccination	Weeks post vaccination								Weeks post challenge							
		1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16
Group(1)	(0)	(0)	(1+)	(3+)	(4+)	(4+)	(3+)	(2+)	(1+)	(0)	(1+)	(2+)	(3+)	(3+)	(2+)	(2+)	(1+)
Group(2)	(0)	(0)	(0)	(0)	(0)	(0)	(0)	(0)	(0)	(0)	(2+)	(3+)	(3+)	(3+)	(4+)	(4+)	(4+)
Group(3)	(0)	(0)	(1+)	(2+)	(3+)	(4+)	(3+)	(2+)	(2+)	(2+)	(1+)	(2+)	(3+)	(4+)	(3+)	(2+)	(2+)

Table (2): Evaluation of humoral immune response of goats vaccinated with inactivated B*. melitensis 16M* vaccine mixed with Montanide-ISA 206 by using BAPA test or RBPT.

	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19
Group(1)	(0)	(4+)	(4+)	(3+)	(4+)	(4+)	(4+)	(4+)	(3+)	(3+)	(3+)	(2+)	(2+)	(1+)	(1+)	(1+)	(0)	(0)	(0)

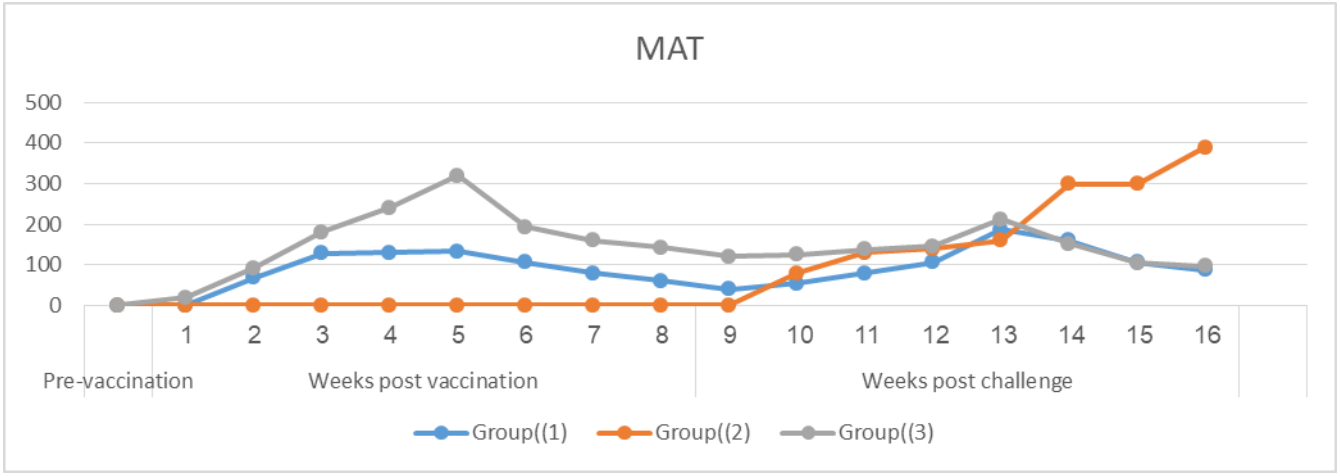


Figure. (1): Mean results of MAT among sera of guinea pigs vaccinated with inactivated *B. melitensis (S-16M)* oil adjuvant vaccine in comparison with *B. melitensis Rev-1* vaccine.

Values in MAT expressed in International Unit (IU).
For vaccinated animals level 50% lower than 40 IU ml^{-1} were considered negative, while with 40 IU ml^{-1} or more were considered positive. (Alton et al., 1988).

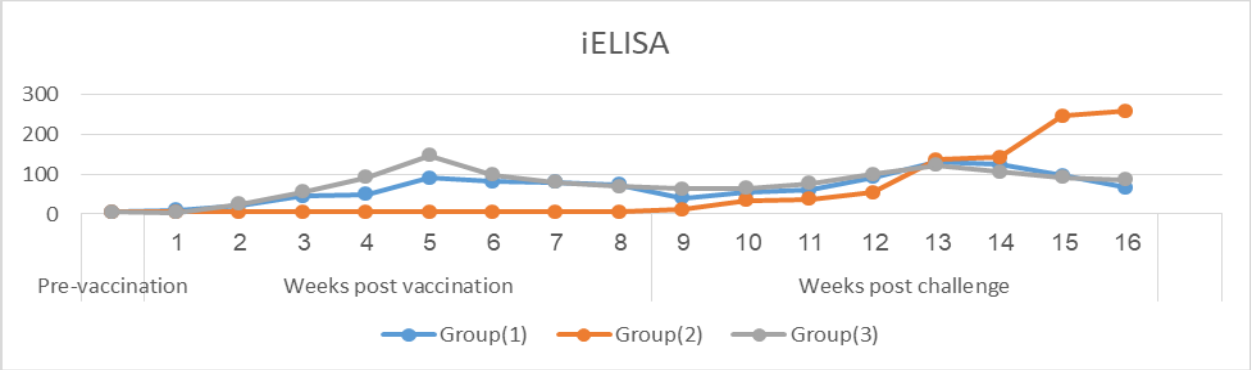
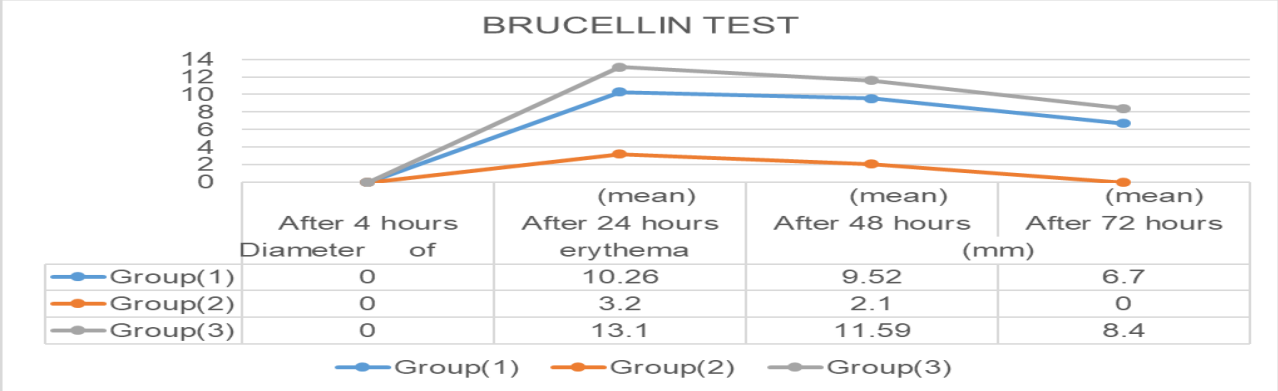


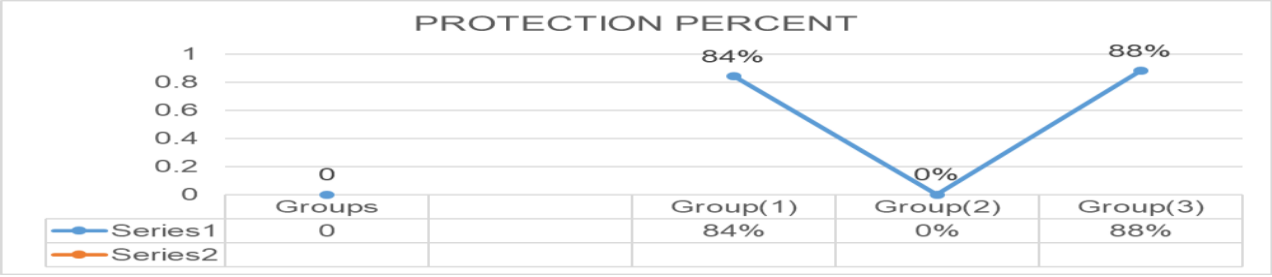
Figure. (2): Mean results of ELISA units among sera of guinea pigs vaccinated with inactivated B. *melitensis (S- 16M)* oil adjuvanated vaccine in comparison with *B. melitensis Rev-1* vaccine by using LPS coated plates.

ELISA unit= tested serum sample - control negative serum x100= ≥ 20
Control positive serum- control negative serum
NB: Positive ELISA unit must be ≥ 20 unit (Alton et al., 1988).



Diameter of	After 4 hours	(mean) After 24 hours erythema	(mean) After 48 hours	(mean) After 72 hours
		(mm)		
Group(1)	0	10.26	9.52	6.7
Group(2)	0	3.2	2.1	0
Group(3)	0	13.1	11.59	8.4

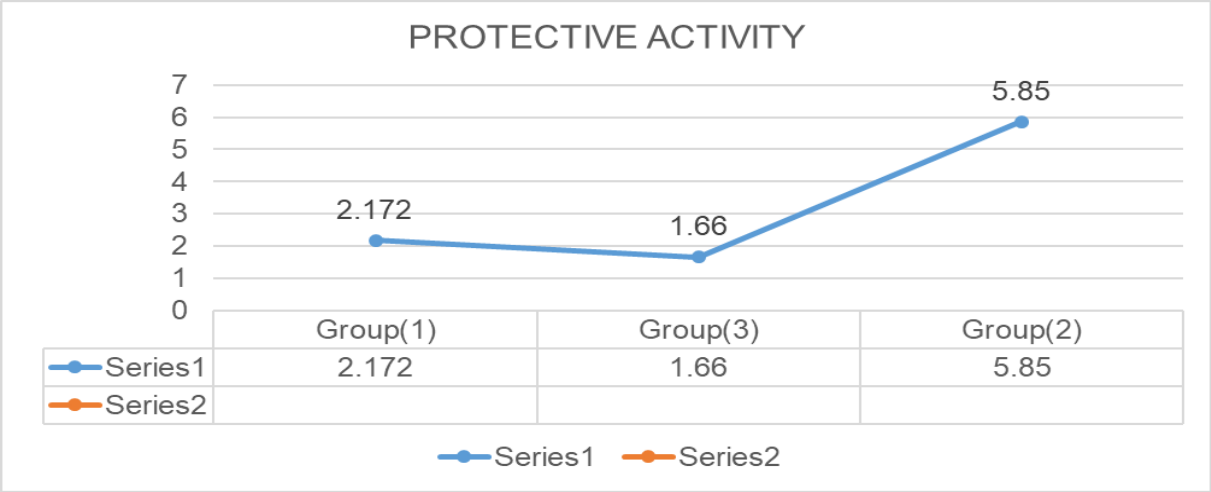
Figure. (3): Evaluation of the cell mediated immune response for inactivated *Brucella melitensis 16M* oil adjuvanated in comparison with *B. melitensis Rev-1* vaccine in guinea pigs by the delayed type of hypersensitivity (Brucellin test).



Groups		Group(1)	Group(2)	Group(3)
Series1	0	84%	0%	88%
Series2				

Figure. (4): Mean of protection% of the prepared *B. melitensis S- (16M)* oil adjuvanated vaccine in comparison to *B. melitensis Rev-1*vaccine in guinea pigs after challenged with virulent *B. melitensis S-16M.*

Protective% was calculated = number of animals had negative *Brucella* spleen count X 100
Total number of the examined animals



Figue. (5): Mean of protective activity level of inactivated *B. melitensis S-16M* oil adjuvant vaccine and *B. melitensis Rev-1* vaccine in guinea pigs in comparison to un-vaccinated infected group.
Mean (SD) Y= Log (x / Log x) of unvaccinated guinea pigs is at least of 4.5 (OIE, 2008).

As shown in figure (3), the diameter of erythema in the group (1) was 10.26, 9.52 and 6.7 mm after 24, 48 and 72 hours of infection of Brucellin (INRA) respectively, its highest degree was at 24 hours after injection, this result was in agreement with Alton et al. (1988) and Bercovich et al., (1999) but the diameter reached to 13.1, 11.59 and 8.4mm in the group vaccinated with *B. melitensis* Rev1 vaccine in the same times respectively**.** There was significant difference between the vaccinated groups and control negative group confirming that the prepared vaccine is capable to induce cellular immune response which plays a role in protection against the organism as facultative mononuclear phagocytes intracellular pathogen as reported by Yingst et al. (2003).

Protective activity was measured by average *Brucella* spleen count among the vaccinated guinea pigs. The current results indicated that the prepared vaccine showed acceptable degree of potency, it presented reduced pathogen colonization for virulent *Brucella melitensis S-16M* and absence of splenomegaly throughout vaccinated guinea pigs. Figure (4) showed that protection percent conferred by the prepared vaccine inoculated group was (84%) in comparison with standard *B. melitensis* Rev1 vaccine (88%). The differences between the protection afforded by the adjuvant vaccine or Rev1 vaccine were not significant.

OIE considers a vaccine to be protective when it has a protective activity 2.5 and not more than 4.5 according to the results of infected control group and according to the dose of challenge and virulence of strain used in challenge as shown in Figure (5). These results agreed with Magnani et al. (2009) who reported that inactivated *Brucella melitensis* vaccine is protective against virulent *B. melitensis* challenge in mice.

The purpose of this study was to evaluate the humoral immune responses of the vaccinated goats against inactivated oil adjuvant *B. melitensis S-16M* (group 1) by using Rose Bengal Plate test (RBPT) and Buffered Acidified Plate test (BAPA)), Serum agglutination test (SAT) and compared with complement fixation test (CFT) according to Alton et al., (1988).

Mean serum antibody responses of vaccinated goats were measured with Rose Bengal Plate test and Acidified Plate test and recorded as a degree of agglutination (2). The positive serum samples by screening tests were examined by Serum Agglutination Test. The mean of agglutination unit was increased from 0 to 146.7 after three weeks of vaccination, then increased after booster dose to 266.7 at five weeks then declined gradually till the end of study (Figure 6). These results agreed with Plommet et al. (1970) who confirmed that inactivated vaccine induced agglutinin antibody titers that persist for long time in vaccinated cattle, sheep and goats. Complement fixation at a dilution of (1:8), as recommended by Australian Bureau of Animal Health (1979), was regarded as a positive reaction. Serum samples were titrated 1:4 to 1:1024 in CFT.

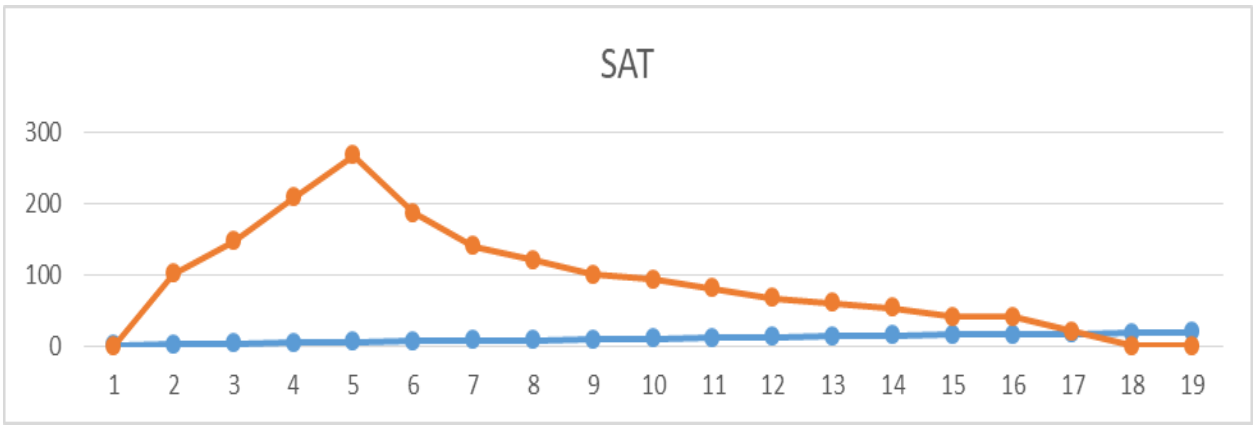


Figure. (6): Evaluation of humoral immune response of goats vaccinated with inactivated *B. melitensis 16M* vaccine mixed with Montanide-ISA 206 by using serum agglutination test

Mean serum antibody responses of goats group (1) using serum agglutination test (SAT).
Values in SAT expressed in International Unit (IU).
For vaccinated animals level 50% lower than 40 IU ml^{-1} were considered negative, while with 40 IU ml^{-1} or more were considered positive. (Alton et al., 1988).

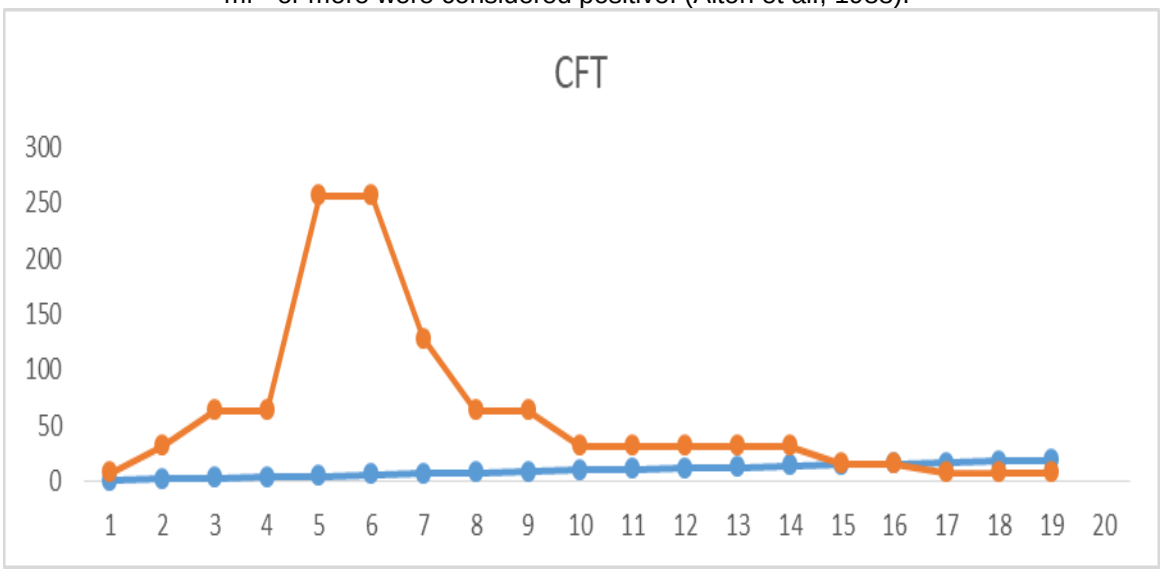


Figure. (7): Evaluation of humoral immune response of goats vaccinated with inactivated *B. melitensis 16M* vaccine mixed with Montanide-ISA 206 by using CFT.

Reading is interpreted according to presence or absence of hemolysis (the positive reaction is shown with the last dilution give no hemolysis).
The antibodies titer began high from 2nd week post vaccination and reached to peak at 5th & 6th week then decrease gradually till reach to negative titer at last 3 weeks.

Titers determined by the CFT were expressed as the reciprocal of the last dilution at which a positive reaction occurred. When CFT was done on the serum samples of goats vaccinated with *B. melitensis (S-16M)* oil adjuvant vaccine, antibodies responses were absent in 1st week post-vaccination and began to appear and became satisfactory from 2nd week post-vaccination and reached the peak at 5th and 6th weeks post-vaccination then began to decline gradually till the 16 weeks of the study but the titers remained satisfactory (Figure 7). So, this approach provides a promising strategy of safe vaccination against *Brucella* infection with satisfactory protective responses.

CONCLUSION

From these results, we can recommend the use of inactivated oil adjuvant *B. melitensis* vaccine as a potent, safe vaccine and immune-stimulant when inoculated in combination with oily adjuvant which will increase the duration of immune responses against infection with virulent *B. melitensis*. Furthermore, it is suitable for our country to prevent the further spread of the disease and minimizing the foci of infection. Future work needs further investigations in cattle host to provide maximum human and animal safety.

CONFLICT OF INTEREST

The authors declared that present study was performed in absence of any conflict of interest.

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AUTHOR CONTRIBUTIONS

Prof. Ashraf Abd El-Tawab, Prof. Fatma El Hofy and Prof. Medhat Sadk designed the experiments; Dr. Rania Abo-Sakaya and Dr. Amany Baghdadi performed the experiments, analyzed the data and wrote the paper.

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