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The Influence of Stabilizers on Brucella abortus RB51

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***ABSTRACT**--Brucella abortus is one of the most important pathogenic brucella species, RB51 B.abortus vaccine is the best available vaccine used to control and prophylactic of brucellosis, RB51 is live attenuated lyophilized vaccine during freeze drying the bacterial cells exposed to stress due to the lypholization steps which overcomes by using of stabilizers that added to protect the bacterial cells from this stress. **Therefore** this study was conducted to evaluate the efficacy of skimmed milk and WHO as stabilizing compounds for preparation of B.abortus RB51 vaccine**s** and evaluate their humoral and cellular immunity by immunizing the mice with the prepared vaccines .the sera samples were tested by I-ELISA assay and the spleen samples were measured for IFN-ɣ as indicator of cellular immunity then, the results were compared with the licensed RB51 vaccine.*

***Keywords**--Brucella abortus, RB51 vaccine,stabilizer stability ,ELISA, IFN-ɣ*

I. INTRODUCTION

Since century ago ,brucella microorganism had been discovered but brucellosis remains major problem for animals and mankind. (pappas, 2006). It is one of the most important zoonotic disease that affects multiple livestock including wild animal species all over the globe .It also credit as a biological weapon due to its highly infectious nature(Santis, 2011), Brucellosis leads to huge economic losses and major impediment for trade and export of animal and animal by-products (Moriyon et al.,2004) Although the disease has been eliminated and eradicated in some of the dveloped countries but it is stil rooted in many parts of the world causing heavy economic losses which may reflect the significance of the disease worldwide (Dorneles et al., 2017).

Brucella , the etiologic agent of the disease, is gram negative,facultative intracellular bacteria classified upon host preference,biological and biochemical characteristics(WHO 2004) where Brucella melitensis ,Brucella suis and Brucella abortus are the most common agents implicated pathogenic worldwide (Alturi 2011).

Treatment of animal brucellosis is very difficult and after trials it showed that it is not feasible or practical (Radostits et al 2000). The need for a control of animal brucellosis has been a major concern. Vaccination is the only practical means and the cornerstone of controlling the disease specially the countries with high incidence of the disease (Blasco 1990), Although the ideal vaccine is far from those available for the control of animal brucellosis but *Brucella abortus* vaccines have a central role in bovine brucellosis control/eradication programs and have been successfully used worldwide for decades. Strain 19 and RB51 are the approved *B. abortus* vaccines

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strains most commonly used to protect cattle against infection and abortion besides offering long lasting protection. (Dorneles et al., 2015).

Prior to the introduction of vaccine strain RB51 in 1996, *B. abortus* S19 was the official vaccine used in the brucellosis eradication program in the United States. S19 was quite effective in protecting cattle against subsequent infection with virulent strains of *B.abortus*. However, S19 did have several problems that restricted its use within the cattle population. During protection studies, it was discovered that S19, when given to adult cattle (>1yr), often caused persistent titers which could not be distinguished from titers resulting from a natural infection using standard serological tests (Stevens et al., 1994).

B.abortus strain RB51 is a stable rough attenuated rifampicin resistant mutant of *B.abortus* strain 2308 widely used to vaccinate cattle against bovine brucellosis in the U.S.A. (Neha Dabral et al., 2015)**.**RB51 vaccine strain lack o-antigen on the lipid A core of its lipopolysaccharide, sera from RB51 vaccinated animals do not react in the standard serological tests (Shurig et al.,1991**) ,**A rough lipopolysaccharide of *B.abortus* RB51used as a common antigen to detect antibodies against *B.ovis, B.canis* and *B.abortus* RB51 by I-ELISA and fluorescence polarization assay**(** Nielsen et al.,2004) By using I-ELISA coating plate with rough LPS that were extracted from strain RB51. This indicates that there are slight increases in titers that can be detected in third month post vaccination, and decrease until disappeared completely at the day 360 post vaccination.

The stability of the vaccines has a major influence on the success and effectiveness of vaccination programs worldwide and may be responsible for vaccine miscarriage of whole vaccination programs (Kenezvic, 2009). The live attenuated vaccine is mainly obtained in lyophilized form and can be produced with low cost.During lyophilazation and after dilution of Vaccine loss of bacterial numbers was occurred resulting to poor efficacy of vaccine. Stabilizers are therefore mixed to protect from freeze drying stress and heat shock, to enhance the shelf-life of the vaccine, or to improve freeze drying eficiency. Various stabilizers as SPGA, skimmed milk, gelatine, bovine serum albumin, carbohydrates like sorbitol, mannitol, trehalose, starch, sucrose, dextran or glucose and proteins such as albumin or casein, and buffers, such as alkali metal phosphates. These stabilizers are a source of "animal origin" agents/components are used for lyophilization of various microbial vaccines (Warthen et al., 2008).

Skimmed milk contains proteins (lactalbumen, lactglobulins, casein, andlactoferrin), lactose and minerals The albumin and globulins of skimmed milk may be recognized as self-antigen in caprine, source of milk casein is not from liver, but is milk acini. In addition to that milk casein is in contrast to other non-hydrolyzed casein which can make anaphylactic shock in animals. (Khokha and Werb 2011).

The present study was planned to evaluate the ability of skimmed milk and WHO as stabilizers to protect *Brucella abortus* RB51 vaccine in a trail to improve the physical property and stability of the vaccine, and evaluate the specific antibody response against extracted rough lipopolysaccharide of *Brucella abortus* RB51 strain.

II. MATERIALS AND METHODS

Strains:

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1- A vaccinal strain *B. abortus* RB51, serial No1472, Professional Biological Company, 4950 York St., Denver, Colorado 8021. USA.

2- *B.* abortus *strain 544* and *B. abortus strain 19* were kindly obtained from Serum and Antigens Department, Veterinary Serum and Vaccine Research Institute, Abbasia, Cairo.

Identification of the strain:

RB51 vaccine was rehydrated with the accompanying sterile diluent according manufactures' instruction.suspension from the strain was grown in tryptose agar(oxoid, England) at 34℃ in 5% Co_2 for 48 hours. Pure colonies were obtained and tested according to classical typing biochemically, morphologically, serologically and genetically.

Genomic DNA was extracted from pure Brucella cultures of (*B. abortus strain RB51, B. abortus strain 19)* by using Genomic DNA Mini Kit (Qiagen). Bruce-ladder identification was based on the numbers and sizes of three products amplified by PCR (Thermo Fischer scientific). Three primer pairs were used in multiplex for molecular typing of *B. abortus* RB51. PCR mixtures were performed in a 25 µl volume containing 1 µl of template DNA, 200 µM of each de oxynucleoside triphosphate, 2.5 units of DreamTaq Green DNA polymerase (Thermo Fischer scientific), 5 µl of its amplification buffer, and 20 pmole of each primer, were added. The PCR amplification was carried out using GeneAmp PCR system 9700 thermal cycler (Perkin Elmer 9700), with 35 cycles of PCR after initial denaturation at 95°C for 10 sec.The PCR profile was as follows: 10 sec. at 95° C for DNA denaturation, 30 sec. at 95°C for DNA annealing, 1 min. and 30 sec. at 72°C for extension, with a final extension at 72°C for 10 min. The PCR products were determined by electrophoresis on 2% agarose gel with ethidium bromide.

Preparation of the vaccine:

Brucella agar slopes containing tryptose soy agar (TSA) with 5% bovine serum were inoculated with *B. abortus* strain RB51 and incubated at 37ºC for 48 hours **(**OIE., 2016). Slopes were examined visually and contaminated slopes were discarded. Cultures were harvested with examined stabilizers (WHO stabilizer consists of casein, sucrose, and glutamate) **(**Alton et al., 1988**),** (skimmed milk consists of 10 gm dry skim milk with 100 ml deionized water and sterilize by autoclaving**.)(OPS diagnostic)** then kept at 4°C for 72 hrs till viability counts were checked, the pooled bacterial suspension was diluted by stabilizing medium to a concentration of approximately 1× 1011 cfu /ml.

Lyophilization of the vaccine has occurred through three main stages, the freezing stage which persisted for about 15 hrs at - 46˚C,followed by the primary desiccation at -12˚C for 21 hrs ended by the second desiccation at + 25˚C for a period of 8 hrs. The pressure vacuum was 0.5 mbar. The vials were stopped, caped and labeled then stored at - 20˚C till evaluation

Evaluation of the vaccine:

The vaccine was tested for purity, safety and potency tests according to **(OIE,**

Mice:

12-14 week-old BALB/c female mice (obtained from veterinary sera and vaccine research institute) were acclimated and distributed into experimental groups. Mice were kept in animal facilities and received water and food .

Immunization and Protection:

Mice were anesthetized and immunized by the intra peritoneal route with 0.2 ml of PBS containing 2×10^8 CFU of the prepared vaccines and commercial *B. abortus* RB51 vaccine. Unvaccinated control animals remained untreated throughout the experiment. 42 days after vaccination, mice were challenged intra peritoneally with 0.2 ml of PBS containing 2×10^4 CFU of *B. abortus* 544. At 14, 28, and 42 days after vaccination and 3, 6, and 10 days after challenge mice were euthanatized and each spleen is excised aseptically, weighed and two thirds was grinded and homogenized aseptically in nine times its weight in phosphate-buffered saline.

Cytokine responses:

The remaining one third of the spleens was used for Cytokine expression in culture supernatants of splenocytes (according to Quantitect SYBR green PCR kit) and Extraction of RNA (according to RNeasy Mini Kit**) (Pasquali., 2001).**

Elisa Test:

At 30, 60, and 90 days after vaccination, serum was collected and assayed at 1:100 dilution by ELISA in plate coated with a series of different dilutions of LPS antigen extracted from RB51 strain according to **(Galanos et al., 1969)**.

III. RESULTS

This strain showed typical characteristics of B.abortus rough strain species when it exhibited to biotyping assays , analyzed by agglutination test with brucella sera (A,M,R) , growth on dyes and roughness by acriflavin .their unique characteristics was identical to B.abortus rough chain.

In the PCR , *B.* abortus *RB51 and B. abortus strain 19* showed species- specific amplification fragments which are: 794 bp, 450 bp for *B. abortus* (S19)**,** 794bp, 587 bp and 450bp for *B. abortus* strain RB51. The resulted fragments are shown in photo (1). These results agree with **(Lo´pez-Gon˜I et al., 2008).**

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Figure 1: PCR product amplified from 2 *B. abortus* strains using Bruce ladder multiplex PCR: Lane (1): PCR marker, Lane (2): *B. abortus* strain 19, Lane (3): *B. abortus* strain RB51

All lyophilized vaccines were rehydrated easily and the cells dispersed into a uniform suspension within 30 sec. No atypical growth or other bacterial or fungal contamination were observed in any of the test vessels compared to a positive control test, so the vaccine proved to be pure and sterile.

Examination of two stabilizers used in RB51 vaccine preparation and their effect on bacterial count and physical appearance of lyophilized cake were shown in table (1).

Table 1: Effects of two stabilizers on *Brucella abortus* strain RB51 during lyophilization at dilution 10^8 and its physical appearance.

Stabilizing Media	Pre-lyophilization Cfu/ml	Post-lyophilization Cfu/ml	Percent Survival	Cfu/ml after 1 month	Frozen pellet
Skimmed milk	14	13	92.8	13	Spherical, of uniform size & separated from the bottle
WHO	12	10	83	10	

Rough lipopolysaccharide (LPS) antigen extracted from *B. abortus* strain RB51 using phenol, chloroform, petroleum ether method according to **(Bhattacharjee *et al.*, 2002) used** as antigen for coating ELISA plate for evaluation of prepared vaccines as shown in chart (1).

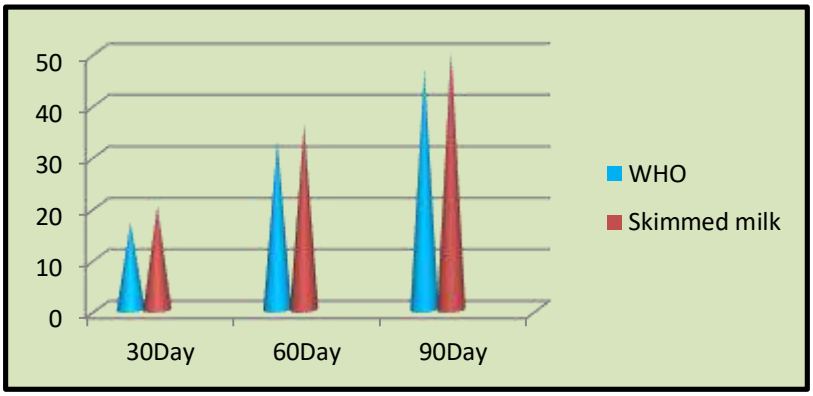

Figure 1: Result of I- ELISA test using LPS coated plates

Estimation of potency and safety of all prepared vaccines Inoculation the prepared vaccines in mice as shown in table (2) spleen weight did not show any increase after challenge or any undesirable symptoms from non-challenged mice till42 days. On the other hand. spleen weights of unvaccinated and infected mice showed a significant enlargement as early as 6 days after infection. Vaccinated mice were almost refractory to a subsequent challenge infection with ***B. abortus* 544** cells, exhibiting significantly lower levels of infection compared to unvaccinated infected animals 3, 6 and 10 days after infection (Data are mean values of five animals of each group)

Table 2 : Effect of vaccination with *B. abortus* RB51 and challenge with *B. abortus* 544 on the spleen weight

Type of stabilizer	**Spleen weight (mg) Post vaccination**			**Spleen weight (mg) After challenge**		
	14 day	**28 day**	**42 day**	**3 day**	**6 day**	**10 day**
WHO	13	21	15	17	25	18
Skimmed milk	23	30	15	16	24	16
Commercial RB51	10	35	10	15	18	13
Control	10	25	25	3	96	95

Specific IFN- Ɣ response from Spleen cells of injected mice with the prepared vaccines and killed at 14 and 28 days after vaccination produced similar amounts of IFN-Ɣ, and the level tented to increase in mice killed 42 days after vaccination ($P < 0.05$). Peak induction of IFN-Ɣ in spleen cells from mice killed 42 days after vaccination corresponded to an observed reduction in spleen weight and bacterial counts in mice. While, IFN-Ɣ production was detectable as early as 3 days after challenge in spleen cells from both vaccinated and unvaccinated mice, although levels were significantly higher in vaccinated mice. Levels of IFN-Ɣ in spleen cells from challenged and unvaccinated group are the same throughout the experiment, In contrast to spleen cells from

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unvaccinated, challenged mice showed increased in IFN-Ɣ production that reached levels were seen in spleen cells from vaccinated, challenged mice 6 days after challenge as shown in table

Table 3: Production of IFN- γ in stimulated spleen cells fromvaccinated or unvaccinated mice challenged with *B. abortus*544 and killed 3, 6, and 10 days after challenge

Treatment	Post vaccination			After challenge		
	14 day	28 day	42 day	3 day	6 day	10 day
None + challenge	0.800	0.1670	0.1918	0.804	0.1676	0.1518
Skimmed milk	0.456	0.850	0.2176	0.1649	0.1885	0.1480
WHO	0.429	0.822	0.2035	0.1353	0.1900	0.1440
Commercial RB51	0.570	0.920	0.2568	0.1616	0.1919	0.1404

Ethical considerations:

This study agrees with the U.S. Government rules for the employment and nursing of animals intended for experimental, training, and research objectives. The study design was accepted by
The Sera and Antigens Department, Veterinary Serum and Vaccine Research Institute

IV. DISCUSSION

It is widely accepted that vaccination has been one of the most successful medicine tools. Vaccine stability is a key factor to keep vaccine efficiency and potency therefore stabilizers are added to protect microorganisms from stresses they exposed like freeze drying, heat shock during storage and transportation The aim of Freeze drying, or lyophilization process is to remove the solvent from the material to a level where the product shows significantly increased in stability and remain viable after rehydration without any change in structure. It is applied for the preservation of many different types of materials even small molecules like microorganisms.

In this study we prepared local *Brucella abortus* RB51 vaccine by using two different stabilizers and evaluate its cellular and humoral immune response.

The RB51 vaccinal strain in this study was identified microscopically, macroscopically, serologically and biochemically. It was resembled the mother seed strain (**Alton et al., 1988)**

Molecular characterization (PCR-based assays) has been employed for molecular typing of *Brucella* species. Although this PCR assay cannot differentiate among biovars from the same species but Bruce-ladder was species specific for most strains and biovars from *Brucella* species. (**Yoldi,et al.,2006)**.When Bruce ladder multiplex PCR assay was carried out on genomic DNA from *B.abortus* RB51 and *B. abortus* strain 19, the results showed 794 bp, 450 bp for *B. abortus* strain 19and 794bp, 587 bp and 450bp for *B.abortus* strain RB51, thus, they were confirmed the strains genetically and this result agree with (**Lo´pez et al.,2008).**

The prepared vaccines match to OIE for sterility as shown to be free from bacterial and fungal contamination

Bacteria need a lyoprotectant which helps them survive during freeze drying process which could be obtained either by simple media as 10% skim milk, or complicated one as animal sera. Good media for lyophilization should be lyoprotectant to stabilize the cells when water is removed and to have matrix agent that allows the entire cell to retain its shape during and after processing. The ideal solution will have a component that helps to form a solid "cake" which gives body to the bacterial suspension once freeze dried. (**OPS Diagnostics 2016)**Comparing the experimental vaccines with the licensed one for their physical properties, both are looking same and similar to each other and to the licensed RB51 vaccine as shown in table 1. Vaccines are combination of biological and non biological components i.e.they are sensitive to environmental and many other factors .So changes could be occurred in live vaccines like viability which consequently affects on their potency.(Capsel etal ., 2000) mentioned that stabilizing media and storage temperature can have significant effects on the viability of lyophilized SRB51 during long-term storage. The WHO stabilizer is less qualified in maintaining the viability /stability of the brucella RB51 strain.Skimmed milk in the suspension shows superior level resulting in minimal percent reduction in brucella cell viability (92.8%) with cfu(14 :13) at 4°C in all stages, during lyophilization and storage for 1 month when compared to other medium and proved to be an effective preservative because it contains millk casein is in contrast to other non hydrolyzed casein which could make anaphylactic shock in animals , lactose and minerals which resulted in minimal percent reduction in salmonella cell viability(18.9%) after exposure to heat stress and gives best thermal stabilization of salmonella enteritidis attenuated vaccine, as mentioned by barbur et al(2001)

To determine the role of the humoral response elicited by these two prepared vaccines.Anti rough LPS titers were measured by I- ELISA in sera from immunized mice .As shown in chart the sera of both vaccines scored positive and induce production of antibodies against rough LPS antigen extracted from *B. abortus* strain RB51 which were recorded at day 30,60 and 90 respectively.Interestingly, no preferential bias appeared to exist by both groups with slight increase in titers at day 90 of the skimmed milk stabilizes vaccine .

Mice are currently used as a model for studying some aspects of bovine brucellosis **(**Teane et al., 2011).

In this study we investigated the protection elicited by the prepared vaccines against B. abortus infection, the results shown in table (2) clarify that vaccinated and infected mice spleen weight did not increase after challenge and were not significantly different from non-challenged, vaccinated animals killed 42 day after vaccination. In contrast, spleen weights of unvaccinated and infected mice showed a significant enlargement as early as 6 days after infection. Vaccinated mice were almost refractory to a subsequent challenge infection with ***B. abortus* 544** cells, exhibiting significantly lower levels of infection compared to unvaccinated infected animals 3, 6 and 10 days after infection (Data are mean values of five animals).

To confirm the effect of WHO and skimmed mil k stabilizers on cellular immunity, cytokine response was measured by pcr as described above and spleen from individual mice were harvested to evaluate IFN- . INF-γ is very important in the control of *Brucella* infections. INF-γ is responsible for macrophage activation, increased expression of the major histocompatibility complex (MHC) molecules and other antigen processing components as well as facilitating immunoglobulin (Ig) class switching. INF-γ is also responsible for the up regulation of the production of oxidative metabolites and other molecules toxic to bacteria **(Janeway et al., 1999).** To evaluate the effect of stabilizers on the cellular immune response,IFN- production was analyzed since it is mainly secreted by Th1,CD8T and CD4 T

Our results indicate that the levels of IFN- produced in response to the vaccines prepared from mice slaughtered at day 14 and 28 days after vaccination are similar.while at day 42 after vaccination the level of IFN- produced slight significant increase (P0.05)with peak accompanied by observed reduction in spleen weight as well as decrease in bacterial counts. On the opposite, IFN- production was observable as early as 3 days post challenge in spleen cells from all groups (vaccinated and un vaccinated mice,in spite of the significant higher levels in vaccinated mice.No difference in IFN- levels were detected in spleen cells of vaccinated challenged groups while the spleen cells of unvaccinated challenged one showed increase of IFN- production to level could be seen in spleen cells of the vaccinated challenged group at day 6 after challenge as shown in table (3) these result agree with **Pasquali et al 2001, Dorneles et al 2015.**

Now days, the production of cheap, efficient and stable brucella vaccine comprises strategic, technical and economical challenges to health agencies worldwide. Current development in vaccine formulation focuses on the importance of structural characterization of the vaccine and the need of a systematic formulation analysis to avoid failure resulting in sub optimal formulation (more field2011).

V. CONCLUSION

Current study approach for stabilizers evaluation. It proved that skimmed milk 10% is like or more efficient than WHO stabilizer in reducing the loss of cfu colony count during lyophilization and during thermo stability in addition to better physical appearance of final product and costless in compare to WHO stabilizer. Both vaccines showed protective level of antibodies with slight increase in titer with that of skimmed milk stabilizer one. Protection test and cytokine response indicate that skimmed milk stabilizer RB51 vaccine is nearly better or similar to the commercial one and perceed the WHO RB51one in IFN production so it is found that skimmed milk stabilizer is suitable and effective for B.abortusRB51 vaccine as stabilizer due to its impaction on lyophilization and after reconstitution

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