Extraction of RB51 rough lipopolysaccharide antigen for evaluation of locally prepared RB51 vaccine

Sally, M. Abd Elsalam1*, Khaled Al-Amry2, khaled, A, Abd-el-Azeem1, Noha, A. Helmy1, Abd EL Hamid M, I1 and Ahmed Samir2

1Department of Bacterial Sera and Antigens Research, Veterinary Serum and Vaccine Research Institute, Cairo, Egypt
2Department of Microbiology, Faculty of Veterinary Medicine, Cairo University, Cairo, Egypt.

*Correspondence: dr.sally.magdy@gmail.com Accepted: 11 July 2018 Published online: 26 Sep. 2018

Brucella abortus strain RB51 is a cattle vaccine that is approved for use in the U.S. for prevention of brucellosis. At the present time, Egypt is considering the use of SRB51 vaccine in their brucellosis control programs. In the current study, the effect of four stabilizing media, on the viability of lyophilized SRB51 over a 52 week period and the characteristics of the immune responses triggered by vaccination with locally prepared RB51 vaccine are determined. 5 groups of mice immunized with the prepared RB51 vaccines and subsequently challenged with high virulent B. abortus 544 were protected from reinfection. Post vaccination, the early production of gamma interferon seems to have the prominent role in inducing an immunologically based protection.

Keywords: Brucella abortus, RB51, vaccines, Immune response.

INTRODUCTION

Brucellosis is one of the major zoonosis in public and animal health worldwide. Infection by Brucella spp. leads to important economic losses and affects numerous livestock, wildlife and humans (Corbel et al., 2006).

Brucella abortus vaccines play a central role in bovine brucellosis control/eradication programs and have been successfully used worldwide for decades. Smooth Strain 19 and rough strain RB51 are the approved B. abortus vaccines strains most commonly used to protect cattle against infection and abortion (Dorneles et al., 2015).

In fact, the administration of live, attenuated Brucella cells is in contrast with the aim of completely eradicating the infection in animals. In addition, the use of smooth type Brucella strains as vaccines, such as B. abortus S19, induces long lasting antibody responses while interfering with surveillance serological tests. All routine serological tests to detect the reactors, including Rose Bengal, ELISA, Serum Agglutination test and Complement fixation tests, are performed using whole-cell antigens from smooth-type Brucella such as B. abortus S99 that especially identify antibodies directed to the lipopolysaccharide (Alton et al., 1988; MacMillan., 1990).

B. abortus strain RB51 is a stable rough attenuated rifampicin resistant mutant of B. abortus strain 2308 which is used as a licensed drive vaccine for bovine brucellosis (Neha Dabral et al., 2015) used to vaccinate young female cattle against infection with more serious strains of Brucella. Vaccinating cow with the RB51 vaccine helps prevent abortions in cow and reduces the risk of people coming in contact with cows infected with more severe strains of Brucella (CDC, 2017) commonly used to avoid interference with serological tests, because of the lack of S-LPS-side chain. Moreover, this vaccine does not
induce antibodies which can be detected by standard serological tests. (Schurig et al., 1991; Palmer et al., 1997).

The performance of rough lipopolysaccharide antigen used in an indirect enzyme-linked immunoassay test for detecting SRB51 antibodies was evaluated by (Schurig et al., 1991).

In the current study, trials of local preparation of RB51 vaccine and the effect of four stabilizing media: World Health Organization medium (WHO), media- Lactose salt, media- WHO/ lactose salt media, and Sucrose phosphate glutamate albumin (SPGA) were carried out with the aim of investigating the efficacy of the prepared lyophilized SRB51 and evaluation of its protective immune response by measuring the humoral and cellular immunity using mouse model then the results were compared with standard RB51 vaccine.

MATERIALS AND METHODS

Strains:
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:
Genomic DNA was extracted from pure Brucella cultures (B. abortus strain RB51, B. abortus strain 19) by using Genomic DNA Mini Kit. 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 oxyxynucleoside 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 37˚C for 48 hours (OIE., 2016). Slopes were examined visually and all contaminated slopes were discarded. Cultures were harvested with different examined stabilizers (WHO stabilizer consists of casein, sucrose, and glutamate) (Alton et al., 1988), (Lactose salt stabilizer consists of 5-75% lactose, Ascorbic and Thiourea ), (WHO/ Lactose salt 1:1) (Capsel et al., 2000), (SPGA stabilizer consisting of Monopotassium phosphate, Dipotassium phosphate, Monosodium L-glutamate and Sucrose) (Kang et al., 2010). After storage at 4˚C for 72 hrs while viability counts were determined, each pooled bacterial suspension was diluted with a stabilizing medium to a concentration of approximately 1× 10^{11} cfu/ml.

Lyophilization of the vaccine was done through three main stages, first stage was the freezing stage which persisted for about 15 hrs at -46˚C, and second stage was the primary desiccation at -12˚C for 21 hrs. The last stage was the second desiccation at + 25˚C for a period of 8 hrs. The pressure vacuum was 0.5 mbar. The vials were stopped, capped and labeled then stored at -20˚C.

Evaluation of the vaccine:
The vaccine was tested for purity, safety and potency tests according to (OIE, 2016).

Experimental design:
12-14 week-old BALB/c female mice were allocated to experimental groups consisting of 20 animals each. Mice were vaccinated intra peritoneally 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. At 42 days after vaccination, unvaccinated controls and intra peritoneally vaccinated 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, serum was collected by heart puncture for ELISA plate coated with a series of different dilutions of LPS antigen extracted from RB51 strain according to (Galanos et al., 1969). Then, mice were euthanatized and each spleen is excised aseptically. The fat is removed and the
spleen was weighed and two thirds was homogenized aseptically with a glass grinder in nine times its weight in phosphate-buffered saline and an aliquot of the resulting cell suspension was plated in agar plates. Two of the plates were incubated in a 10% CO₂ atmosphere (allows the growth of both vaccine and challenge strains) and the other two plates were incubated in air (inhibits the growth of the B. abortus 544 CO₂ dependent challenge strain) both at 37°C for 5 days. 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).

RESULTS

When PCR was carried out on genomic DNA from B. abortus RB51 and B. abortus strain 19, different size of fragments were amplified, which are: 794 bp, 450 bp with B. abortus (S19), 794bp, 587 bp and 450bp with B. abortus strain RB51. The resulted fragments are shown in photo (1). These results agree with (Lo’pez-Gon’I et al., 2008).


Table (1): Effects of four stabilizers on Brucella abortus strain RB51 during lyophilization at dilution 10⁶ and its physical appearance.

<table>
<thead>
<tr>
<th>Stabilizing Media</th>
<th>Pre-lyophilization Cfu/ml</th>
<th>Post-lyophilization Cfu/ml</th>
<th>Percent Survival</th>
<th>Cfu/ml after 1 month</th>
<th>Frozen pellet</th>
</tr>
</thead>
<tbody>
<tr>
<td>WHO</td>
<td>12</td>
<td>10</td>
<td>92</td>
<td>10</td>
<td>Spherical, of uniform size &amp; separated from the bottle</td>
</tr>
<tr>
<td>SPGA</td>
<td>12</td>
<td>10</td>
<td>92</td>
<td>10</td>
<td></td>
</tr>
<tr>
<td>WHO+ Lactose</td>
<td>14</td>
<td>13</td>
<td>92.8</td>
<td>13</td>
<td>Adhered to the bottle &amp; collapsed</td>
</tr>
<tr>
<td>Lactose salt</td>
<td>10</td>
<td>7</td>
<td>73</td>
<td>7</td>
<td></td>
</tr>
</tbody>
</table>

Viability of strain RB51 stabilized with WHO, WHO /LS and SPGA maintained at 4°C temperatures for 1 month, was greater than strain RB51 stabilized in LS medium. And will maintain greater than 90% of strain RB51 bacteria that were viable after lyophilization.
It was immediately noted during harvest of cell suspensions for vaccine production that the SRB51 suspensions were rapidly autoagglutinated. After final bacterial suspensions were made for distribution into vials, agglutinated SRB51 in the suspensions appeared to disperse readily. All lyophilized vaccines were rehydrated easily and the cells dispersed into a uniform suspension within 30 sec. By examination of sterility, no atypical growth is found in any of the test vessels when compared to a positive control included in the test, so the vaccine is considered satisfactory for purity.

A vaccine is mixed with stabilizers to protect degradation-prone components from being degraded, to enhance the shelf-life of the vaccine, or to improve freeze drying efficiency. Useful stabilizers are: SPGA, skimmed milk, gelatine, bovine serum albumin, carbohydrates; e.g. sorbitol, mannitol, trehalose, starch, sucrose, dextran or glucose, proteins such as albumin or casein or degradation products thereof, and buffers, such as alkali metal phosphates. These stabilizers are a source of “animal origin” agents/components (Warthen et al., 2008). In our study, examination of four stabilizers used in RB51 vaccine preparation and their effect on bacterial count and physical appearance of lyophilized cake were shown in table (1).

Viability of strain RB51 stabilized with WHO, WHO /LS and SPGA maintained at 4°C temperatures for 1 month, was greater than strain RB51 stabilized in LS medium. And will maintain greater than 90% of strain RB51 bacteria that were viable after lyophilization.

Since LPS is the main expressed antigen on the surface, the serological responses following Brucella infection are directed against LPS thus the serological diagnosis in human and animal is mainly based on the detection of specific anti-LPS antibodies (Salmani et al., 2008). Rough LPS of \textit{B. abortus} strain RB51 could be used as antigen for the detection of antibodies against \textit{B. abortus} strain RB51 by ELISA and fluorescence polarization assay (Nielsen et al., 2005). In our study, after using rough lipopolysaccharide (LPS) antigen extracted from \textit{B. abortus} strain RB51 by using phenol, chloroform, petroleum ether method according to (Bhattacharjee et al., 2002) as antigen for coating ELISA plate for evaluation of prepared vaccines - it was noticed that the four stabilizers used are similar in its immune response as there is a slight increase in titers which can be detected until day 90 after which they decrease until day 365 post vaccination.

Mice are currently used as a model for studying some aspects of bovine brucellosis (Shurig et al., 1991). After injection of the prepared vaccines in mice, the results shown

<table>
<thead>
<tr>
<th>Type of stabilizer</th>
<th>Spleen weight (mg) Post vaccination</th>
<th>Spleen weight (mg) After challenge</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>14 day</td>
<td>28 day</td>
</tr>
<tr>
<td>SPGA</td>
<td>13</td>
<td>26</td>
</tr>
<tr>
<td>WHO</td>
<td>13</td>
<td>21</td>
</tr>
<tr>
<td>Lactose salt</td>
<td>12</td>
<td>17</td>
</tr>
<tr>
<td>WHO + Lactose</td>
<td>23</td>
<td>30</td>
</tr>
<tr>
<td>Commercial RB51</td>
<td>10</td>
<td>35</td>
</tr>
<tr>
<td>Control</td>
<td>10</td>
<td>25</td>
</tr>
</tbody>
</table>
Mice are currently used as a model for studying some aspects of bovine brucellosis (Shurig et al., 1991). After injection of the prepared vaccines in mice, 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).

in table (3) Vaccination of mice with live *B. abortus* RB51 cells resulted in a pattern of bacterial growth in which numbers $4 \cdot 7 \times 10^5$ CFU/spleen were seen after 6 days, and peak numbers ($8 \cdot 10^6$ CFU/spleen) were seen 18 days after vaccination, followed by a progressive decline. Bacteria were absent in the spleen at 42 days post-vaccination. The results agree with (Schurig et al., 1991; Pasquali et al., 2001).

The role of 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).

Table (4): Production of IFN-γ in stimulated spleen cells from vaccinated or unvaccinated mice challenged with *B. abortus*544 and killed 3, 6, and 10 days after challenge

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Post vaccination</th>
<th>After challenge</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>14 day</td>
<td>28 day</td>
</tr>
<tr>
<td>None + challenge</td>
<td>0.800</td>
<td>0.1670</td>
</tr>
<tr>
<td>SPGA</td>
<td>0.566</td>
<td>0.903</td>
</tr>
<tr>
<td>WHO</td>
<td>0.456</td>
<td>0.850</td>
</tr>
<tr>
<td>Lactose salt urea</td>
<td>0.455</td>
<td>0.790</td>
</tr>
<tr>
<td>WHO + Lactose</td>
<td>0.429</td>
<td>0.822</td>
</tr>
<tr>
<td>Commercial RB51</td>
<td>0.570</td>
<td>0.920</td>
</tr>
</tbody>
</table>

in table (4) By measuring IFN-γ from Spleen cells of injected mice with the prepared vaccines, it was found that animals killed 14 and 28 days after vaccination produced similar amounts of IFN-γ, and the level was noticeably increased in mice killed 42 days after vaccination (*P < 0.05*). Peak induction of IFN-γ in spleen cells from mice killed 42 days after vaccination corresponded to an observed reduction in spleen weight and bacterial counts in these animals. In contrast, IFN-γ production was detectable as early as 3 days after challenge infection in spleen cells from both vaccinated and unvaccinated mice, although levels were significantly higher in vaccinated mice. Levels of IFN-γ in spleen cells from vaccinated, challenged mice showed no differences throughout the experiment, in contrast to spleen cells from unvaccinated, challenged mice which showed increased IFN-γ production that reached levels that can be seen in spleen cells from vaccinated, challenged mice 6 days after challenge.

CONCLUSION

This study suggested that WHO/LS and SPGA stabilizers are the best for maintaining viability of *B. abortus* strain RB51 during storage for up to 1 year at temperatures 4°C. Therefore, we hypothesized that a combination of both WHO and LS & SPGA media may prove superior over either media in maintaining viability during lyophilization and storage. The mice immunized with the locally prepared vaccines *Brucella abortus* RB51 bacteria and subsequently challenged with *B. abortus* 544 were protected from reinfection. After vaccination, the early production of gamma interferon seems to have the prominent role in inducing an immunologically based protection, and the I-ELISA coated plate with the extracted lipopolysaccharide from *B. abortus* strain RB51 indicates that there is a slight increase in titers that can be detected in third month post vaccination, and a decrease from that time until day 360 post vaccination.

CONFLICT OF INTEREST

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

ACKNOWLEDGEMENT

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AUTHOR CONTRIBUTIONS

SM designed and performed the experiments and also wrote the manuscript, NH, MI and AS performed animal treatments, PCR, sample collection, and data analysis. KA, KHA, SM designed experiments and reviewed the manuscript. All authors read and approved the final version.

REFERENCES


