Original Research Article

The use of different stabilizers for improving integrity of the locally prepared lyophilized *Brucella melitensis* Rev 1 vaccine

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**ABSTRACT**

Stability study of biological products especially living bacterial vaccines plays an important role for determination of product changes in maintenance period and ensures safety, efficacy and maintenance of biological properties of the vaccines. So, the objective of this study was to establish stability and keeping quality of the local *Brucella melitensis* Rev-1 vaccine using different types of stabilizers in lyophilization process. A long-term stability study was carried out for four batches of reduced-dose *Brucella melitensis* Rev-1 vaccine manufactured by veterinary serum and vaccine research institute using four different stabilizers. These stabilizers were (A) sucrose and skimmed milk, (B and C) different concentrations of sucrose, sodium glutamate and gelatin, and (D) casein, sucrose and sodium glutamate. The quality control tests including colony forming unit, purity, dissociation and physicochemical tests on all batches until 12 months post-production were performed. The obtained results indicated that, in spite of collapse (shrinkage) of lyophilized cake in a number of bottles in batches prepared using stabilizer A, the brucella vaccine batches were stable and met the specification recommended by OIE 2012 for 12 months post-production in vaccine batches with stabilizers A and D.

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Introduction

Brucellosis is a major bacterial zoonotic and an emerging disease of global importance caused by bacteria of the genus Brucella affecting a range of different mammals including man, cattle, sheep, goats, swine, rodents and marine mammals worldwide. Three species only of brucella are of zoonotic importance, these species are *Brucella melitensis* (main host is sheep and goats), *Brucella abortus* (main host is cattle and buffaloes) and *Brucella suis* (main host is pigs) (Corbel, 2006 and Mohamed et al., 2010). The bacterial pathogen is classified by the CDC as a category (B) pathogen that has potential for production of a bio-weapon. *Brucella* spp. is considered as the most common laboratory-acquired pathogens (Mohamed et al., 2010).

Although the efforts and works of Egyptian veterinary services to overcome brucellosis started from more than 30 years, brucellosis is still endemic in both domestic ruminants and humans and represents one of public health hazard in Egypt. National brucella committee representing veterinary laboratories, veterinary universities and General Organization of Veterinary Services (GOVS) establishes brucellosis control programs which depend mainly on the test and slaughter policy and vaccination programs. The vaccination programs recommended by OIE depends mainly on immunization of calves (6-8 months of age) with living attenuated *Brucella abortus*, S19 vaccine (smooth strain) with the dose of 3-10 X10⁹ CFU/dose or the living attenuated *Brucella abortus* RB51 vaccines (rough strain) with the dose of 1-3.4 X10¹⁰ CFU/dose and inoculation of small ruminants (3-6 months of age) with living attenuated *Brucella melitensis* Rev 1 vaccine (smooth strain) with the dose of 1-3 X10⁹ CFU/dose (Gamal et al., 2014).

One of the best attenuated vaccines is Rev.1 obtained by passing through *Brucella melitensis* wild strain 6056 serotype 1 on the media containing streptomycine, resulting in a mutant resistant to streptomycin called strain Rev.1 (Blasco 1997). This mutant was discovered by Elberg in the University of California in 1957 for the first time and used in the vaccine production to prevent brucellosis in sheep and goat (Alton et al., 1967). Knowledge about the stability of a vaccine especially the rate at which it loses viability (colony count) and in consequences loses of its potency at a recommended storage temperature, can be helpful in determination of vaccine shelf life. So, Stability of vaccines has a major impact on the success and effectiveness of vaccination programs worldwide and may be responsible for vaccine failures with the results of corruption of whole vaccination programs (Knezevic, 2009). According to the reports of World Health Organization “WHO”, stability of Rev.1 vaccine should take at least one year, therefore attempts to establish the stability of this local vaccine can solve many problems in regard to exportation of this vaccine and to meet the specifications and the requests of General Organization of Veterinary Services (GOVS). So, the objective of this study was to establish long term stability and keeping quality of the local *Brucella melitensis* Rev-1 vaccine produced by VSVRI with regard of the most suitable stabilizer used in lyophilization process and also to standardize a method for production of other live attenuated brucella vaccines.

Material and methods

Bacterial strains

Reference *Brucella melitensis* Rev-1 (vaccinal strain), *Brucella melitensis* biovar 1 (16-M), *Brucella melitensis* biovar 3 (ETHER), *Brucella abortus* S19 (vaccinal strain), *Brucella abortus* biovar 1 (S544), *Brucella ovis* (REO198) and *Brucella suis* 1330 obtained from Centro de Investigación y Tecnología Agroalimentaria de Aragón (CITA), Spain (Dr Jose Maria Blasco) as well as *Brucella abortus* biovar 1 (S99) supplied by VSVRI were subjected for conventional identification according to the methods described by Alton et al., (1988) as well as multiplex PCR (Bruce-ladder assay)
according to López–Goni et al., (2008) and OIE, (2012) (Table 1). *Brucella melitensis* Rev-1 strain was used as a seed culture for production of *Brucella melitensis* Rev-1 vaccine according to standard procedures as described by OIE, (2012) and Alton et al., (1988).

### Table 1. Primer sets for Bruce-ladder multiplex PCR

<table>
<thead>
<tr>
<th>Primer</th>
<th>Sequence (5’–3’)</th>
<th>Amplicon size (bp)</th>
<th>DNA targets</th>
<th>Source of genetic difference</th>
</tr>
</thead>
<tbody>
<tr>
<td>BMEI0998F</td>
<td>ATC-CTA-TTG-CCC-CGA-TAA-GG</td>
<td></td>
<td></td>
<td>S711 insertion in BMEI0998 in B. abortus RB51 and deletion of 15,079 bp in BMEI0993-</td>
</tr>
<tr>
<td>BMEI0997R</td>
<td>GCT-TCG-CAT-TTT-CAC-TGT-AGC</td>
<td>1682</td>
<td>Glycosyltransferase, gene <em>wboA</em></td>
<td>BMEI1012 in <em>B. ovis</em></td>
</tr>
<tr>
<td>BMEII0843F</td>
<td>TTT-ACA-CAG-GCA-ATC-CAG-CA</td>
<td>1071</td>
<td>Outer membrane protein, gene <em>omp31</em></td>
<td>deletion of 25,061 bp in BMEI826-</td>
</tr>
<tr>
<td>BMEII0844R</td>
<td>GCG-TCC-AGT-TGT-TGT-TGA-TG</td>
<td></td>
<td></td>
<td>BMEI0850 in <em>B. abortus</em></td>
</tr>
<tr>
<td>BMEII0428F</td>
<td>GCC-GCT-ATT-ATG-TGG-ACT-GG</td>
<td>587</td>
<td>Erythritol catabolism, gene <em>eryC</em> (D-erythrulose-1-phosphate dehydrogenase)</td>
<td>deletion of 702 bp in BMEI0427-</td>
</tr>
<tr>
<td>BMEII0428R</td>
<td>AAT-GAC-TTC-ACG-GTC-GTT-CG</td>
<td></td>
<td></td>
<td>BMEI0428 in <em>B. abortus</em> S19</td>
</tr>
<tr>
<td>BR0953F</td>
<td>GGA-ACA-CTA-CGC-CAC-CTT-GT</td>
<td>272</td>
<td>ABC transporter binding protein</td>
<td>deletion of 2653 bp in BR0951 BR0955 in <em>B. melitensis</em> and <em>B. abortus</em></td>
</tr>
<tr>
<td>BR0953R</td>
<td>GAT-GGA-GCA-ACG-GCT-GAA-G</td>
<td></td>
<td></td>
<td><em>B. abortus</em></td>
</tr>
<tr>
<td>BMEI0752F</td>
<td>CAG-GCA-AAC-CCT-CAG-AAG-C</td>
<td>218</td>
<td>Ribosomal protein S12, gene <em>rpsL</em></td>
<td>point mutation in BMEI0752 in <em>B. melitensis</em> Rev.1</td>
</tr>
<tr>
<td>BMEI0752R</td>
<td>GAT-GTG-GTA-ACG-CAC-ACC-AA</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
Freeze drying stabilizer medium

In this study four stabilizers (additives preserving materials) were prepared and used in production of Brucella melitensis Rev-1 vaccine. These additives are consisting of protein or polyssacharides or both were used with the following formula:

A. 7% sucrose and 7.5% skimmed milk (Modified Behroozikhah et al. 2009).
B. 5% sucrose, 1.5% sodium glutamate and 1.5% gelatin (Modified Behroozikhah et al. 2009).
C. 7% sucrose, 2% gelatin and 1.5% sodium glutamate (Modified Behroozikhah et al. 2009).
D. 2.5% enzymatic digest of casein, 5% sucrose and 1% sodium glutamate.

(Cultural medium)

Potato infusion agar, tryptose soya agar and Soyabean casein digest medium (SCDM) are the reference media used for culturing and preparation of seed culture and production of vaccines (Alton et al., 1988 and OIE, 2012). Media used for purity test (OIE, 2012), testing of Performance, validation and verification (Keeping quality) of culture media used in production of brucella vaccines and purity test were tested according to (ISO17025 and ISO11133-2014).

Preparation of master seed, working seed and vaccine batches:

Master seed, working seed and vaccine batches were prepared according to (Alton et al., 1988 and Govindasamy et al., 2010) using roux bottle method. Vaccine batches were adjusted to contain 6.5 X 10^9 CFU/ml then they were labeled and kept at 4 °C during all period of study (12 months).

Long-term stability study

The vaccines were tested seven times (every two months) in 0, 2, 4, 6, 8, 10 and 12 months after production for long-term stability. In each period all of the quality control tests were performed as follow:

A. Colony Forming Unit.

In each period of the study, viability was determined for each vial according to the OIE protocol (OIE, 2012). After 3-5 days incubation at 37 °C, CFU per each dose of vaccine was enumerated (Larry et al., 1998).

B. Purity test.

The purity test was performed according to the OIE manual (2012).

C. Dissociation test.

After counting of colonies, the cultures were tested for smoothness and roughness by using crystal violet and acriflavine test according to (white & Wilsons staining method, (Alton et al., 1988 and OIE, 2012). The rough colonies take up the stain (blue colonies), but the smooth colonies not absorb the stain (white colonies).

D. Physicochemical tests.

Physicochemical tests were carried out according to British Pharmacopoeia 2012. In each period, physicochemical tests including appearance, negative pressure (vacuum), and solubility, and extraneous agents, vacuum and labeling test were performed for all of the samples. Color, consistency, form of lyophilized vaccine and any visible particle after reconstitution were considered for appearance and extraneous agents tests (Hasannia et al., 2015).

Results

Master seed was identified morphologically, biochemically (photo 1) and serologically as a typical Brucella melitensis biovar 1 (Rev-1) and also BRUCE-LADDER (photo 2) assay was performed as a confirmatory test using different Brucella reference strains as control positives.
Seed culture and all prepared vaccine batches were free from bacterial (aerobic and anaerobic), fungal and Mycoplasma contaminations along the entire period of the study and all colonies of seed culture and tested batches during the period of the study were 100% smooth when stained by crystal violet and also when acriflavine test was carried out according to (Alton et al., 1988 and OIE, 2008), (photo 3). Physicochemical inspection according to (Hasannia et al., 2015) revealed that all batches met the specifications (lyophilized cream color, free of any visible particles after reconstitution in appearance, readable and stable label in label test, good soluble in saline in solubility test and prescience of negative pressure (vacuum) in each period of long-term stability study. pH of buffers, used for collection and harvesting of Brucella cultures during production and evaluation of Brucella vaccine samples, diluents and additives, used as stabilizers, were adjusted to 7.2 (Behroozikhah et al., 2009).

In this study, the amount of the bacterial mass before and after lyophilization process was tested by colony counting method on Trypticase Soya Agar with ten-fold dilution according to (Alton et al., 1988 and OIE, 2012). Vaccine was adjusted to contain 6.5 X10⁹ CFU/ml for the four batches before freeze drying process. The four batches were subjected to lyophilization process at the same time to establish the condition of freeze drying and the judgment. The vaccine containing stabilizers A, B, C and D compound showed a decrease in CFU/ml of 3.5 X10⁹, 3 X10⁸, 2.3 X10⁸ and 4.4 X10⁹ respectively when tested directly after lyophilization as shown in (table 2 and figure 1).

The results of Long-term stability study showed a mean loss of activity of 60, 94.28, 69.04 and 52.38 % for vaccines with A, B, C, and D stabilizers respectively at the end of 12 months after vaccine production as shown in (table 3 and figure 2).

Photo 1: Growth of Brucella melitensis biovar 1 (Rev-1), B. abortus (S19), Brucella abortus biovar 1 (S99), Brucella melitensis biovar 3 (ETHER), Brucella abortus biovar 1 (S544) and Brucella melitensis biovar 1 (16M) on TSA media containing 20ug/ ml of Basic fuchsin.

Photo 2: multiplex PCR (BRUCE-LADDER), Lane 1: Brucella abortus (S19), Lane 2, Brucella melitensis biovar 1 (Rev-1), Lane 3: marker (GenLadder 100 bp + 1.5 kbp, GENAXXON bioscience), Lane 4: Brucella melitensis biovar 1 (16M), Lane 5: Brucella abortus biovar 1 (S544), Lane 6: Brucella ovis (REO198) and Lane 7:Brucella suis (S1330).

Photo 3: Brucella melitensis biovar 1 (Rev-1) colonies stained with crystal violet (colonies did not take up the stain, smooth)
Table 2. Colony counting of Rev.1 vaccine before and after lyophilization with A, B, C and D stabilizers

<table>
<thead>
<tr>
<th>Stabilizers</th>
<th>Before freeze drying</th>
<th>After freeze drying</th>
<th>Reduction titer</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>CFU</td>
<td>%</td>
<td></td>
</tr>
<tr>
<td>A</td>
<td>3 X10⁹</td>
<td>3.5 X10⁹</td>
<td>53.48</td>
</tr>
<tr>
<td>B</td>
<td>6.5 X10⁹</td>
<td>3.5 X10⁹</td>
<td>46.15</td>
</tr>
<tr>
<td>C</td>
<td>4.2 X10⁹</td>
<td>2.3 X10⁹</td>
<td>35.38</td>
</tr>
<tr>
<td>D</td>
<td>2.1 X10⁹</td>
<td>4.4 X10⁹</td>
<td>67.69</td>
</tr>
</tbody>
</table>

Figure 1. Colony counting of Rev.1 vaccine before and after lyophilization with A, B, C and D stabilizers
Table 3. Viable colony count (CFU/dose) of Rev-1 brucella vaccines with A, B, C and D stabilizers for 12 months

<table>
<thead>
<tr>
<th>Stabilizers</th>
<th>Months after production (final products)</th>
<th>Mean loss</th>
<th>Valid vaccine titer</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>0</td>
<td>2nd</td>
<td>4th</td>
</tr>
<tr>
<td>A</td>
<td>3 X10^9</td>
<td>2.8 X10^9</td>
<td>2.5 X10^9</td>
</tr>
<tr>
<td>B</td>
<td>3.5 X10^9</td>
<td>3 X10^9</td>
<td>2.3 X10^9</td>
</tr>
<tr>
<td>C</td>
<td>4.2 X10^9</td>
<td>4 X10^9</td>
<td>3.9 X10^9</td>
</tr>
<tr>
<td>D</td>
<td>2.1 X10^9</td>
<td>1.9 X10^9</td>
<td>1.6 X10^9</td>
</tr>
</tbody>
</table>

Figure 2. Viable colony count (CFU/dose) of Rev-1 brucella vaccines with A, B, C and D stabilizers in 12 months long-stability study.
4. Discussion:

Vaccines are combinations of components (biological and non-biological) that are sensitive to environmental factors and changes in non-biological ingredients of vaccines by different factors. So, biological changes may be occurred especially in live vaccine as decrease in CFU of live bacterial vaccines which consequently affects potency of vaccine. So, to determine product changes in maintenance period and ensure safety and efficacy of vaccines, stability study of biological products is needed. Stability is the ability of a vaccine to retain its chemical, physical, microbiological and biological properties within specified limits throughout its shelf life (Galazk et al., 1998 and Schofield 2009).

Many factors effect stability of vaccines such as, stabilizer, heavy water, process and equipments used in production, lyophilization process, the cold chain used for maintenance and transportation of vaccines and vials or tubes of vaccines (Der yuan et al., 2000). In this study, Long-term stability was performed, after production of vaccine batches using roux bottles methods (OIE, 2012 and Alton et al., 1988), to recommend storage conditions and to establish the shelf life and the release specifications (Knezevic, 2009).

CFU results of Rev-1 brucella vaccines with different stabilizers in period of the 12 months long-term stability study revealed that, all of the vaccine samples of stabilizers A, C and D passed the specifications of OIE until the end of 12 months post-Production in spite of a number of vaccine samples of stabilizers A showed a collapse or shrinkage of lyophilized cake (disc) which may be due to moisture increase which may be explained by many reasons as low quality rubber used (Hasannia et al., 2015), a lyophilization program not compactable with stabilizer or may be due to low proteins or sugar content of stabilizer (Behroozikah et al., 2009 and Ferry, 1995). Anyhow, the results indicated that the increasing of moisture content do not reduce the potency of vaccine under the specifications or not greatly affect potency of vaccine and this agrees with (Hasannia et al., 2015).

Stabilizer D was the best stabilizer that maintains the viability of brucella vaccine along the 12 months post-production and showing the least titers reduction (52.38%) but during the dry freezing process, it showed dramatically reduction in titers in comparison with other three batches. This titers reduction was from 6.5 X10^9 to 2.1 X10^9 CFU/ml i.e. 67.69%. Brucella melitensis Rev-1 Vaccine to be standard must not loss more than 2 X10^9 CFU/ml/12 months (66.66%) when colony count of tested vaccine is 3 X10^9 CFU/ml (maximum limit of OIE specification of Rev-1 vaccine) directly after dry freeze process. So, although vaccine samples prepared with stabilizer C show reduction in colony count of 35.38% after lyophilization process and met OIE specification at the end of 12 months post production, but it was not considered as a good stabilizer in maintaining the viability of brucella along the entire period of the test as the colony count average in vaccine samples with stabilizer C was beyond the standard specification at zero day post production (4.2 X10^9 CFU/ml), colony count was 2.9. X10^9 CFU/ml at the end of the study which mean that the reduction titers in colony count was more than 2X10^9 CFU/ml. Stabilizer B was the least one in maintaining viability of brucella vaccine with reduction titers rates of 94.28%.

Conclusion

From all above data obtained it could be concluded that stabilizers D and A were the most suitable stabilizers to be used in production of brucella Rev-1 vaccine. All vaccines especially living bacterial vaccines should be stored at temperatures recommended by national immunization programs and manufacturers.
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