



Journal homepage:
<http://www.bsu.edu.eg/bsujournals/JVMR.aspx>
Online ISSN: 2357-0520 Print ISSN: 2357-0512



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 the 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. 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, *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.

ARTICLE INFO

Article history:

Received: 15 October 2016

Accepted: 20 November 2016

Available Online: 27 August 2017

Keywords:

REV 1 vaccine, *Brucella melitensis*, stabilizer stability, PCR

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1. 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; 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 $\times 10^9$ CFU/dose or the living attenuated *Brucella abortus* RB51 vaccines (rough strain) with the dose of 1-3.4 $\times 10^{10}$ 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 $\times 10^9$ CFU/dose (Wareth et al., 2014).

One of the best attenuated vaccines is Rev.1 obtained by passing through *Brucella mellitensis* 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.

2. Materials 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 Alton et al. (1988) and OIE (2012).

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 polysaccharides 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 (Angus, 1984).

Culture 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; 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; Kamaraj et al., 2010) using roux bottle method. Vaccine batches were adjusted to contain 6.5×10^9 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 and Smith, 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; 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).

3. Results

Master seed was identified morphologically, biochemically (Fig. 1) and serologically as a typical *Brucella melitensis* biovar 1 (Rev-1) and also BRUCE-LADDER (Fig. 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 on using acriflavine test (Fig. 3). Physicochemical inspection 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 presence 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.

In the current 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. Vaccine was adjusted to contain 6.5×10^9 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 X10⁹, 2.3 X10⁹ and 4.4 X10⁹ respectively when tested directly after lyophilization as shown in (Table 2 and Fig. 4). 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 (Table 3 and Fig. 5).

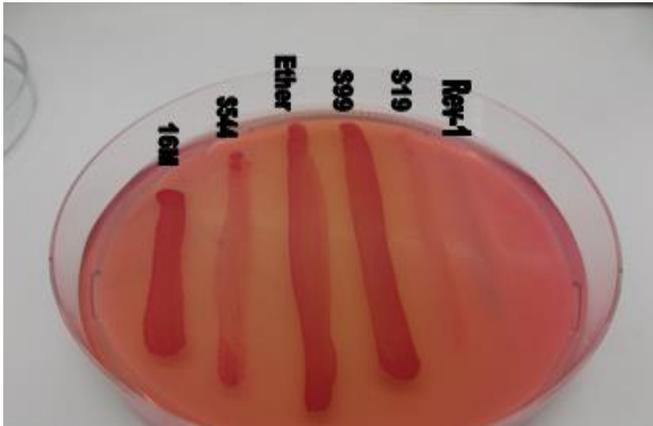


Fig. 1. Growth of *Brucella melitensis* biovar 1 (Rev-1), *B. abortus* (S19), *Brucella abortus* biovar 1 (S99), *Brucella melitensis* biovar 3 (ETHER), *Brucella abortus* biovar 1 (S544) and *Brucella melitensis* biovar 1 (16M) on TSA media containing 20ug/ ml of basic fuchsin.

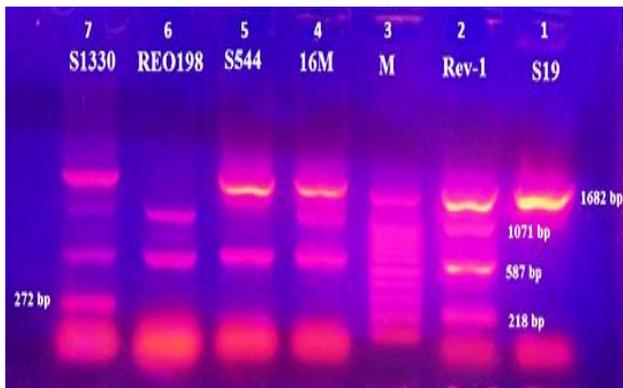


Fig. 2. multiplex PCR (BRUCE-LADDER), Lane 1: *Brucella abortus* (S19), Lane 2, *Brucella melitensis* biovar 1 (Rev-1), Lane 3: marker (GenLadder 100 bp + 1.5 kbp, GENAXXON bioscience), Lane 4: *Brucella melitensis* biovar 1 (16M), Lane 5: *Brucella abortus* biovar 1 (S544), Lane 6: *Brucella ovis* (REO198)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

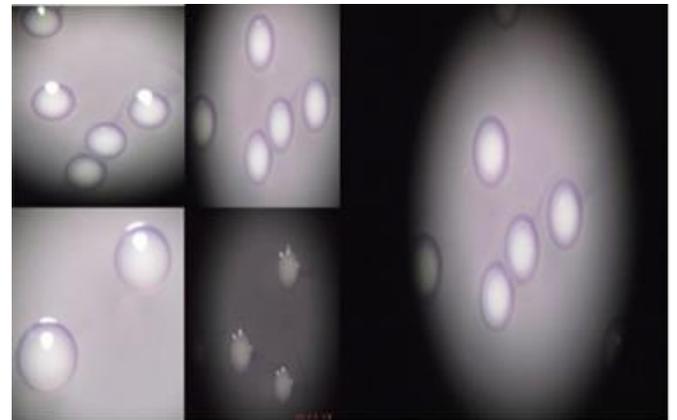


Fig. 3. *Brucella melitensis* biovar 1 (Rev-1) colonies stained with crystal violet (colonies did not take up the stain, smooth).

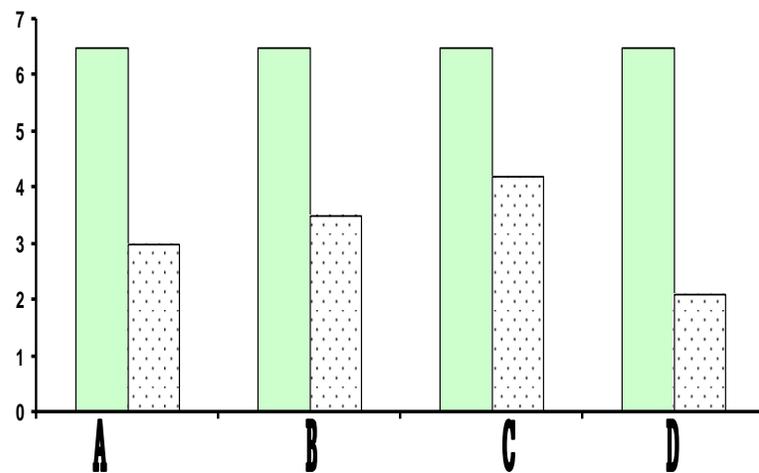


Fig. 4. Colony counting of Rev.1 vaccine before and after lyophilization with A, B, C and D stabilizers.

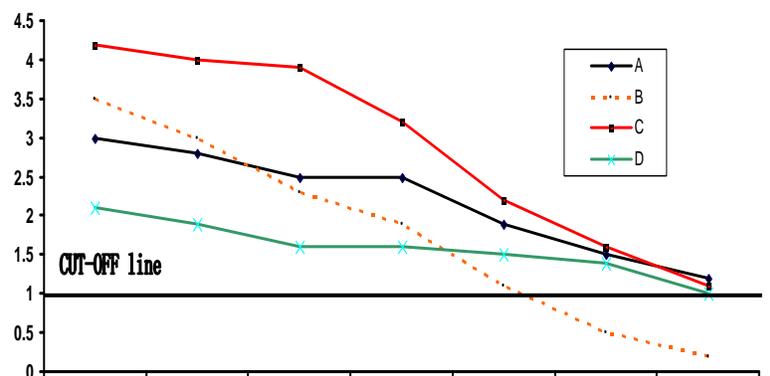


Fig. 5. Viable colony count (CFU/dose) of Rev-1 *Brucella* vaccines with A, B, C and D stabilizers in 12 months long-stability study.

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

Primer	Sequence (5'-3')	Amplicon size (bp)	DNA targets	Source of genetic difference
BMEI0998F	ATC-CTA-TTG-CCC- CGA-TAA-GG	1682	Glycosyltransferase, gene <i>wboA</i>	S711 insertion in BMEI0998 in <i>B. abortus</i> RB51, and deletion of 15,079 bp in BMEI0993- BMEI1012 in <i>B. ovis</i>
BMEI0997R	GCT-TCG-CAT-TTT- CAC-TGT-AGC			
BMEII0843F	TTT-ACA-CAG-GCA- ATC-CAG-CA	1071	Outer membrane protein, gene <i>omp31</i>	deletion of 25,061 bp in BMEII826-BMEII0850 in <i>B. abortus</i>
BMEII0844R	GCG-TCC-AGT-TGT- TGT-TGA-TG			
BMEII0428F	GCC-GCT-ATT-ATG- TGG-ACT-GG	587	Erythritol catabolism, gene <i>eryC</i> (D- erythrose-1- phosphate dehydrogenase)	deletion of 702 bp in BMEII0427-BMEII0428 in <i>B. abortus</i> S19
BMEII0428R	AAT-GAC-TTC-ACG- GTC-GTT-CG			
BR0953F	GGA-ACA-CTA-CGC- CAC-CTT-GT	272	ABC transporter binding protein	deletion of 2653 bp in BR0951 BR0955 in <i>B. melitensis</i> and <i>B. abortus</i>
BR0953R	GAT-GGA-GCA-AAC- GCT-GAA-G			
BMEI0752F	CAG-GCA-AAC-CCT- CAG-AAG-C	218	Ribosomal protein S12, gene <i>rpsL</i>	point mutation in BMEI0752 in <i>B.</i> <i>melitensis</i> Rev.1
BMEI0752R	GAT-GTG-GTA-ACG- CAC-ACC-AA			

Table 2. Colony counting of Rev.1 vaccine before and after lyophilization with A, B, C and D stabilizers.

Stabilizers	Before freeze drying	After freeze drying	Reduction titer	
			CFU	%
A	6.5 X10 ⁹	3 X10 ⁹	3.5 X10 ⁹	53.48
B		3.5 X10 ⁹	3 X10 ⁹	46.15
C		4.2 X10 ⁹	2.3 X10 ⁹	35.38
D		2.1 X10 ⁹	4.4 X10 ⁹	67.69

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

Stabilizers	Months after production (final products)								Mean loss		Valid vaccine titer
	0	2 nd	4 th	6 th	8 th	10 th	12 th	CFU	%		
A	3 X10 ⁹	2.8 X10 ⁹	2.5 X10 ⁹	2.5 X10 ⁹	1.9 X10 ⁹	1.5 X10 ⁹	1.2 X10 ⁹	1.8 X10 ⁹	60		
B	3.5 X10 ⁹	3 X10 ⁹	2.3 X10 ⁹	1.9 X10 ⁹	1.1 X10 ⁹	0.5 X10 ⁹	0.2 X10 ⁹	3.3 X10 ⁹	94.28	1-3 X10 ⁹	
C	4.2 X10 ⁹	4 X10 ⁹	3.9 X10 ⁹	3.2 X10 ⁹	2.2 X10 ⁹	1.6 X10 ⁹	1.1 X10 ⁹	2.9 X10 ⁹	69.04		
D	2.1 X10 ⁹	1.9 X10 ⁹	1.6 X10 ⁹	1.6 X10 ⁹	1.5 X10 ⁹	1.4 X10 ⁹	1 X10 ⁹	1.1 X10 ⁹	52.38		

4. Discussion

Vaccines are combination 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; Schofield 2009).

Many factors effect stability of vaccines such as, stabilizer, heavy water, process and equipment used in production, lyophilization process, the cold chain used for maintenance and transportation of vaccines and vials or tubes of vaccines (Wang et al., 2000). In this study, Long-term stability was performed, after production of vaccine batches using roux bottles methods (Alton et al., 1988; OIE, 2012), 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 (Ferry, 1995; Behroozikhah et al., 2009). 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 X10⁹ to 2.1 X10⁹ CFU/ml i.e. 67.69%. *Brucella melitensis* Rev-1 Vaccine to be standard must not loss more than 2 X10⁹ CFU/ml/12 months (66.66%) when colony count of tested vaccine is 3 X10⁹ 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 (4.2×10^9 CFU/ml), colony count was 2.9×10^9 CFU/ml at the end of the study which mean that the reduction titers in colony count was more than 2×10^9 CFU/ml. Stabilizer B was the least one in maintaining viability of *Brucella* vaccine with reduction titers rates of 94.28%.

5. Conclusion

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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