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Evaluation on stability process of *Brucella melitensis* - Rev. 1 vaccine in Iran

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ABSTRACT

According to the reports of WHO, stability of Rev.1 vaccine should take more than one year, while the expiring date for the vaccine produced in Iran is 3 to 4 months, therefore any attempt to elongate the stability of this vaccine can solve many problems of the production including the request of Veterinary Organization of Iran in this regard. The objective of this study was to increase the stability of this vaccine using various preserving materials in lyophilisation process. Nine effective preserving materials in two different volumes and different lyophilisation procedures were examined .We found that the best preserving materials which are added to the base formula for Rev.1 vaccine is consisted of bactocasitone %2.5, sucrose %5, L-glutamic acid sodium salt %1. As a result we formulate the most suitable compound in terms of bacterial mass after lyophilisation. The other factor which had to be improved was the duration of liquid form of the vaccine before lyophilisation process which causes reduction of the organism %50 to %70 per dose of the vaccine. This problem was solved by reduction of liquid phase. The most important practical result of this research was finding the optimum condition for the dose of the Rev.1 vaccine as $1\text{-}4\times10^{9}$ CFU and $0.5\text{-}3\times10^{6}$ CFU for the Reduced dose with 1-2% humidity and the vacuum of $1\text{-}2\times10^{-3}$. In these conditions the vaccine can be kept and used for more than 8 months. Hence the expiring date of the present vaccines under these conditions would be increased up to eight months.

Keywords: Sheep brucellosis, Rev.1, Preserving material, Lyophilisation

INTRODUCTION

Rev.1 vaccine is one of the best and most valuable existing vaccines to prevent and control goat and sheep brucellosis suggested by the international organizations such as FAO, OIE, WHO. The efficiency of this vaccine proved by Iranian researchers and cooperation of W.H.O on Iranian goat and sheep that use of the vaccine can decrease the epidemic rate of the disease from %45 to %1.8 since 1963 (Jones *et al* 1964). Two kinds of killed and live attenuated vaccines are used for prevention of brucellosis. Since the killed vaccine inducing short time immunity and a booster dose is needed, it is not applicable as a suitable vaccine. As the disadvantage which has been mentioned does not exist for attenuated vaccine, hence this vaccine recommended. 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

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resistant to streptomycine 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 to prevent brucellosis in sheep and goat (Alton *et al* 1967). After many laboratory experiments and clinical trails the vaccine was recommended by the international organizations such as FAO, OIE and WHO as the best live attenuated vaccine which can be used against brucellosis (WHO Technical report series 1971, 1984, 1997). Early studies about the Rev.1 vaccine production began in Razi Institute in Iran incooperation with WHO in 1963, and it was produced as a domesticated biological product ((Jones *et al* 1964). The aim of this study was to increase the stability of this vaccine using various preserving materials in lyophilisation process.

MATERIALS AND METHODS

The brucella Rev.1 vaccine is prepared in the department of brucellossis in Razi Institute in Iran according to OIE Manual, (2004) and WHO (1967) instruction. It contains 1-4 $\times 10^9$ *B. melitensis* strain Rev.1 per/ml in physiological saline and 1:20000 merthiolate W/V. In this study nine preserving materials consisting of polyssacharide or protein or both were used with the following formula: A. %5 surose, %2.5 bactocasiton, %1 sodium L- glutamate with the formula $C_5H_8N_2NaO_4$, H_2O, solved in 1 litre distilled water. B. %5 sucrose, %2.5 bactocasitone , %1 L-glutamic acid sodium salt with formula of C_2H_2, Na_4, which 147.13 gram of it was solved with 40 gram of NaOH in 1 liter of distilled water. C. %7.5 glucose, %7.5 skim milk which is solved in one liter of distilled water. D. The formula of preserving material (D) composed of %20 sucrose solved in 1 liter of distilled water. E. %2.5 bactocasiton, %10 sucrose, %1 sodium L-glutamate solved in 1 liter of distilled water. F. %2.5 bactocasiton, %10 sucrose, and %1 sodium L-glutamate solved in 1 liter of distilled water. G. %1.5 gelatin %5 sucrose which is solved in 1 liter of distilled water. H. %2 gelatin, %5 sucrose solved in 1 liter of distilled water.

I. phosphate buffer (NaCl, 85 gram, $NaHPo_4$, 5 gram, Na_2HPO_4, 22.5 gram) solved in 1 liter of distilled water. After cultivation of Rev.1 on brucella agar in castaneda container for 120 hours, preparation of the above-mentioned compound were used in the volume of 250 ml of each and was divided by 22 ml and 17 ml in 20 welcome tubes. Then, the vaccine was harvested in Erlenmeyer flasks with volume of 250 ml, separately and CFU of the samples were determined. Microbial suspension was divided into 8 ml and added to tube containing the above compound. Eventually, all of the vials were randomly divided in to 5 groups and each groups were stored in 4°C degree, 0°C degree,-56 degree,-70 degree for 2 hours and then CFU of all the samples was determined. It must be mentioned that lyophilised vials of the brucella strains which were used for production of the vaccine were supplied by Brucellosis Department, Weybridge University, UK.

RESULTS

After the vaccine is mixed with two G and H compounds (preserving materials) at $4^{\circ}C$ in cold room, it turned into two separate layers .The bottom layer became frigid and subsequently the bacterial mass of the vaccine decreased. The vaccine containing of G and H compound which had 1-4×10^9 CFU showed a decrease of 1.9×10^9 CFU / ml after three month. It should be noted that pH of the nine compounds were adjusted to 7.27 and then they were used for collection of the vaccines. The amount of the bacterial mass before and after lyophilisation was determined by colony counting method on brucellosis Agar with ten-fold dilution. The results of the first stage of colony counting before and after lyophilisation are shown in table 1. As it is shown in table 1, the compound B and F

containing vaccine have less reduction than the other preserving media in terms of bacterial mass after lyophilisation.

Table 1. Colony counting of Rev.1 vaccine before and after lyophilization with the nine compounds.

Compound (Preserving Materials)	B.F.D*	A.F.D**	Reduction titre
A	35×10^9CFU	15×10^9CFU	20×10^9CFU
B	26×10^9CFU	13×10^9CFU	13×10^9CFU
C	27×10^9CFU	8.9×10^9CFU	18.1×10^9CFU
D	36×10^9 CFU	15×10^9CFU	21×10^9CFU
E	31×10^9CFU	12×10^9CFU	19×10^9CFU
F	30×10^9CFU	15×10^9CFU	15×10^9CFU
G	36×10^9CFU	10.8×10^9CFU	25.2×10^9CFU
H	32×10^9CFU	12.6×10^9CFU	19.4×10^9CFU
I	30×10^9CFU	10.2×10^9CFU	19.8×10^9CFU

B.F.D* = before freeze drying A.F.D* *= after freeze drying

In another lyophilistion procedure, the vaccines were lyophilised and colony counting was performed on brucella Agar by ten-fold dilution method which the results are shown in table 2.To make a better comparison of the results, the graph of the first stage of colony counting before and after lyophilisation was diagrammed. The results and the differences between these two lyophilisation methods are as follows: The preserving material (A): In the first method, the materials was separated in two layers while in the second method, one of the layer is separated from the flacon and the disc was somehow crystal and had to be changed for lyophilisation process. The second preserving material (B): The results of lyophilisation were formation of lyophilized disc which was like the preserving material (A). The third preserving material (C): In the first method, a one layer disc was formed which easily separated from the flacon, indicating that it had been dried well. In the second method, the product color became brown and well dried without any humidity.

The preserving material (D): In the first method, the product had two layers. The bottom layer was stuck to the flacon and the upper layer was separated. In the second method, the disc had one complete crystal layer.

The preserving material (E): In the first method, the disc had two layers had stuck to each other. In the second method, the disc was one piece but there was adhesion on the flacon and the upper part of the disc was crystal.

The six preserving material (F): In the first method, the disc was one piece containing a very small spongy layer inside. Also in the second method, the disc was again one piece containing a very small spongy layer inside. There is probability that the ingredients could not completely homogenized, therefore, it is better that formulation of the new production to be revised.

The preserving material (G): In the first method, the disc was complete but had two spongy layers adhered to sides. In the second method, the preserving material of the vaccine was a complete and white disc, easily separated from the glass. The preserving material (H): In the first method, the disc was a complete layer, stuck to the wall of the flacon. In the second method, the preserving material was a complete and the dry disc, easily removed from the wall. The preserving material (I): In both methods, the preserving material was complete and dry. Generally, the obtained results showed that in the first method, the third preserving material was better lyophilized than the other material and in the second method; the seventh preserving material was better lyophilized than others. Some of the first preserving material has been lyophilised with another program with a Chinese l. The amount of its humidity was 1.877 which is good for the vaccines preserving material. In colony counting method on brucella Agar, the bacterial number before lyophilisation in one ml was 1.9×10^9 while after drying, it decreased to 8×10^9. The stability of the vaccine under this

humidity was optimum but after four months, the amount of the bacterial mass was not acceptable. The representative of the Chinese manufacturer suggested the use of preserving materials having sugar component which is suitable for drying with this machine. The results showed that in the case of a quick and urgent vaccination, the (G) and (H) preserving materials can be used for the stability of the vaccine. This vaccine has enough bacterial mass for 3 months. It is obvious from the obtained results that the preserving materials (A) and (B) which is recommended by the international centers such as OIE and WHO, are capable of reducing the bacterial mass periodically. Although the vaccine in the of form of lyophilising as well as in liquid state in cold room, turns into two layers, but due to rigidity of the bacteria, the above- mentioned preserving materials may be recommended after checking their harmlessness.

DISCUSSION

Additives provide a better environment for the preserving of the bacteria such as Brucella. The preserving materials or additives keep the bacteria, during the drying period, in a way that crystals of water molecules are formed separately among the frozen molecules and transmitted in to the condenser by vacuum. With regard to references, preservation of the vaccine for more than 6 months is possible (Blasco 1997). To preserve Brucella, different materials are used, the best of them is bactocasiton which includes %2.5 bactocasitone, %5 sucrose , %1 sodium L-glutamate. Results from bactocasitone during the drying were satisfactory reactions and stability of the product (Ferry 1995). Process are indicative of According to protocol of WHO, lyophilised Rev.1 vaccine can be preserved for more than one year. But using of the vaccine is forbidden after 18 months. In 1997, Thomas discovered the important function of the ingredients in preserving materials of a product within lyophilisation. Accordingly, some products can be preserved for 20 years with lyophilisation. They also showed the role of heat, vacuum and pressure on the product (Thomas *et al* 1997).

Table 2. Results of colony counting before and after lyophilisation by other lyophilisation procedure

Preserving Material	B.F.D*	A.F.D**	Reduction titre
A	30×10^9CFU	15×10^9CFU	15×10^9CFU
B	28×10^9CFU	10×10^9CFU	18×10^9CFU
C	32×10^9CFU	8×10^9CFU	24×10^9CFU
D	36×10^9CFU	10×10^9CFU	26×10^9CFU
E	26×10^9CFU	5×10^9CFU	21×10^9CFU
F	$21\times10^9$1CFU	4×10^9CFU	17×10^9CFU
G	18×10^9CFU	5×10^9CFU	13×10^9CFU
H	22×10^9CFU	6×10^9CFU	16×10^9CFU
I	22×10^9CFU	5×10^9CFU	17×10^9CFU

B.F.D* =before freeze drying A.F.D **=after freeze dryin

Experiences of Ferry (1995) showed that the best rate of sugar is %5 to %10. If a protein material is added to the sugar, its stability will increase in a way that, after 42 months, %15 of bacteria will remain alive (Ferry 1995). According to Greaves (1964) sucrose could give the best result for stability in such a way that the result is good up to %50, but the least stability was obtained between percent10 to %20. Also carbohydrate used with gelatin, milk serum and haemoglobin but the best results were obtained with combination of serum, cow milk, globulin with sugar. In sensitive organisms, adding three parts of serum to the compound of %7.5

glucose and broth have given satisfactory output. The carbohydrates cause the minimum humidity remain in the product, so that the remaining humidity (%1 - %2) is highly effective on stability of the product. It's increase or decrease has reverse effect on the duration of preservation (Alton *et al* 1988, Lodato 1999) obtained the best result on trehalose and maltodextrin by %61 trehalose and %32 maltodextrin and also from %100 trehalose. The method of lyophilisation is important for the quality, specially stability and preservation of biologic materials. In recent years, research about lyophilisation has increased, in a way that through changing of time, pressure and temperature, the stability of Brucella vaccine may be increased (Ferry 1995, Thomas *et al* 1997, John 1995).

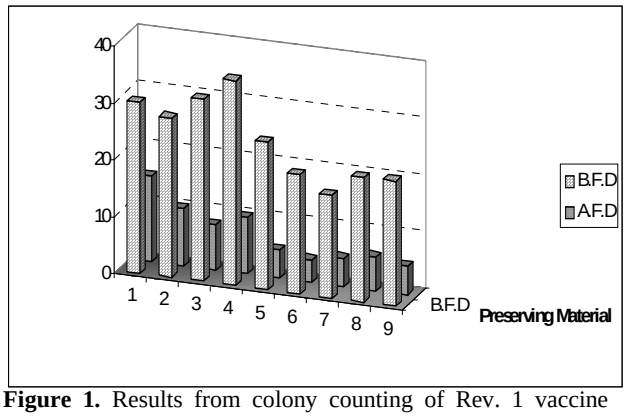

Figure 1. Results from colony counting of Rev. 1 vaccine before and after lyophilisation with 9 preserving materials.

On the basis of the above reported results, in this research nine effective preserving materials in two different lyophilisation procedures were examined. We found that the best preserving material for Rev.1 vaccine was consisted of bactokasitone %2.5, sucrose %5 and L-glutamic acid sodium salt %5. Another factor which has to be improved was the problem with lyophilisation. Reduction of the organisms per dose of vaccine in lyophilisation process is between %50 to %70. On the other hand the longer time of preservation of vaccine in liquid form at $4^{\circ}C$ is adversely correlated with the maintaining period of the vaccine after lyophilisation. The most important applied results of this research for the Rev.1 vaccine is that a complete dose of $1\text{-}4\times10^{9}$ CFU and for the reduced dose of $0.5\text{-}\ 3\times\ 10^{6}$ CFU with 1-2% humidity which can be kept and used for more than 8 months. The analysis of this research showed that no improvement was obtained to increase preservation time in spite of using protein or sugar or both of them and even applying the first and second preserving materials recommended by WHO(. FAO/ WHO 1986). The suggestion to improve the vaccine stability is first to reduce the gap between harvesting of the vaccine and its lyophilisation. Secondly, to increase the mass of bacteria in unit of volume, it must be set in a way that after drying by lyophilisation, vial of the vaccines have the highest amount of bacterial mass in unit of volume. So that at the end of expiring date of vaccine the mass of bacteria must not be less than the minimum standard for each dose. The remaining humidity of the vaccine according to the international experts and protocols must not be more than %1 to %2. Unfortunately, in the present research most of the 15 different samples of vaccine vials, dried by the lyophilizator of our center, had humidity between %2.361 - %5.35 and the mean humidity was %3.74. The effect of low humidity and high mass on the batch of Rev.1 vaccine which had the mass of about two milliards and had low humidity was synchronically controlled by different groups. Stability of the vaccine was found to be more than six months. After six months, the remained mass in each dose was more than one milliard. According to above-mentioned results, it is suggested that:

i. Since sugar based preservatives for brucellosis vaccine precipitate quickly, the cooling must be created as quick a possible.

ii. Preservatives A and B are suitable for preserving the vaccine if lyophilisator could produce $-40^{\circ}C$ in at most two hours and the condenser could reach to $-50^{\circ}C$.

iii. The optimal humidity of the product must not be

more than %1-%2, hence, %90 of the humidity must be removed from the product under drying condition.

iv. The vacuum for the vaccine vial must be optimazied to 1-2 × 10^{-3} since the environmental oxygen reduces stability of the product.

v. The interval time between the harvesting and the lyophilization process must be as short as possible.

vi. Lyophilisation of the vaccine which has been included two preservatives (A and B) must be repeated by new lyophilisator machines which are able to instruct the process within less than one hour.

vii. The potency and the safety of the produced vaccine by preservatives (G) and (H) must be compared with the current method to ensure its safety, then it can be used in liquid form in a short time.

iix. The vaccines which consist of about two milliards alive bacteria, humidity less than %2 and the amount of vacuum 1-2×10^{-3} have stability of more than six months , however the vaccines consisting of two and a half milliard to three milliards of alive bacteria with the standard humidity and vacuum, have stability of more than 8 months. Hence the expiring date of the present vaccines having these conditions would be increased up to six to eight months.

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