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**Original Article**

# Stability of Russell's Viper Venom Toxoid (Lyophilized Form) on Storage

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**SUMMARY**: A previously developed Russell's viper venom toxoid in Myanmar is in a liquid form that shows reversion in the form of a reduced number of formaldehyde linkages and toxicity during storage at 37°C and at room temperature. In order to have a safe, potent and stable toxoid, a lyophilized form was prepared in the present study from the liquid toxoid through the use of a freeze dryer. Both the liquid and lyophilized forms were then stored at 4°C and at room temperature, and in addition to safety and immunogenicity tests, biochemical parameters such as the protein content, the activity of venom enzymes (proteinase, phospholipase A, phosphodiesterase, and arginine esterase), and the released free formalin amounts were assessed at 3-month intervals over a period of 1 year. The results indicate that under both conditions, the lyophilized toxoid shows minimum changes in enzyme activity, a reduced tendency toward formaldehyde linkage, no toxocity, and more immunogenicity in comparison with the respective liquid toxoids. It could therefore be hypothesized that Russell's viper venom toxoid in a lyophilized form is more promising in terms of efficacy and stability for prophylactic use in human immunization than the conventional toxoid in a liquid form.

## INTRODUCTION

Development of a suitable and effective snake venom toxoid for active immunization of people at risk to snake bite is of utmost importance in tropical countries where snake bite is a serious medical problem (1,2). In Myanmar, Russell's viper (*Daboia russelii siamensis*) venom (RVV) toxoid in a liquid form has been successfully produced from RVV by a slow and step-wise formalinization method in the Department of Medical Research (DMR) for 2 decades (3). It has been found to be potent and immunogenic with minimum undesirable side effects in immunized monkeys (4) and human volunteers (5). However, there was a reduction in the number of formaldehyde linkages with an appearance of toxicity and a reduction in the immunogenicity of the toxoid being stored especially at 37°C and at room temperature (RT) (6,7). There is an urgent need to develop a safe, stable form of the toxoid that maintains its potency during storage, as the morbidity and mortality rates, i.e. more than 10,000 snake bite cases per year with a mortality rate of 10%, associated with Russell's viper remain relatively high in Myanmar. Many attempts have been made to improve the stability, safety, and immunogenicity of the DMR toxoid by using only major purified fractions of RVV (8), increasing the concentrations of formalin, and adding formalin binding agent, i.e. sodium bisulfite to the toxoids (9). No promising effects or applicable results have yet been obtained, however.

According to the literature, formalinized Taiwan cobra venom toxoid kept at 37°C shows a reversion to toxicity, whereas the toxoid kept at 4°C as well as freeze-dried (lyophilized) toxoid kept at 37°C remain non-toxic (10,11). In Japan, Habu venom toxoid, which is prepared from the formalinization of two hemorrhagic fractions of the venom, HR-1 and HR-2, followed by lyophilization, has been found to be safe and highly immunogenic for various animals and human beings (12).

In the present study, toxoidation was carried out using the crude RVV by a slow and step-wise formalinization method. Then, the toxoid in a liquid form was treated by a freeze-drying process to obtain a freeze-dried (lyophilized) form. Then, both forms of the toxoid were stored at 4°C or at RT for a 1-year period, during which the toxoids were periodically checked for enzyme activity free formalin released, safety, and immunogenicity, with the ultimate aim of achieving a more safe, potent, and stable toxoid to be used in human immunization.

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## MATERIALS AND METHODS

Desiccated crude RVV and lyophilized anti-snake venom (ASV) were purchased from Myanmar Pharmaceutical Factory (MPF), Yangon, Myanmar. All chemicals used in this experiment were of analytical grade from Sigma Chemical Co., St. Louis, Mo. Adult mice of both sexes, Institute of Cancer Research (ICR) strain, weighing 20 ± 1 g were obtained from the Laboratory Animal Service Division of DMR, Yangon.

**Determination of intramuscular (i.m.) median lethal dose ($LD_{50}$) of RVV in mice**: i.m. $LD_{50}$ was determined by using seven groups of mice, each consisting of six animals envenomed with different concentrations of RVV ranging from 18-600 Ug/0.1 m*l*. Deaths of mice within 24 h were noted, and $LD_{50}$ was calculated by the method of S.Karber (13).

**Preparation of RVV toxoid (liquid form)**: The method used for the toxoidation of RVV (i.e. preparation of RVV toxoid) (liquid form) is a modified method of Kondo et al. (14) described by Aung Khin et al. (15), in which a slow and stepwise formalinization of RVV is performed by increasing the concentrations of formalin by 0.2% at 2-day intervals to obtain a final concentration of 0.8% formalin at day 10. The solution was then dialyzed against M/30 phosphate buffer solution (PBS) to remove excess formalin. The adjuvant adsorption was then carried out by the addition of M/8.45 aluminum phosphate to the toxoid solution at a ratio of 1:1 by volume.

**Preparation of RVV toxoid (lyophilized form)**: Half the venom of the whole toxoid (liquid form) was separated into

1-m*l* samples that were then added to the appropriate glass bottles. The samples were frozen for 48 h followed by a freeze-drying process (i.e. lyophilization) using a freeze-dryer model no. 5, Labconco from Japan, to obtain a lyophilized form. Therefore, at this stage, two types of toxoid, the liquid form and lyophilized form, were obtained. Samples of each form were then divided into two equal parts and stored under two different storage conditions, namely 4°C and RT (i.e. approximately 27°C). Then, 1-m*l* samples of the liquid and lyophilized forms of the toxoid stored at 4°C and at RT were tested for stability every 3 months for 1 year. These tests included the determination of pH and proteins, assays of enzyme activity, namely proteinase, phospholipase A (PLA), phosphodiesterase (PDE), and arginine esterase (AE), tests for chemical reversion, i.e. free formalin released, toxicity, and immunogenicity.

Just before every experiment, each sample of lyophilized toxoid was reconstituted with only 0.65 m*l* of distilled water to compensate for the loss of proteins during the process of lyophilization during which 35% of proteins in the liquid RVV were found to be lost.

**Protein determination and the assay of enzyme activity**: The protein content of each form of stored toxoid was measured by the method of Miller (16). Proteinase activity was determined at pH 8.5 using casein as a substrate by the method of Takahashi and Ohsaka (17). PLA activity was measured according to the method described by Marinetti using phosphatidylcholine (egg yolk lecithin) as a substrate (18). PDE activity was assayed at pH 8.9 according to Kocholaty et al. Using bis-p-nitrophenyl phosphate as a substrate (19). The AE assay was carried out by the method of Tu et al. using Na-p-Tosyl-l-Arginine Methyl Ester (TAME) as a substrate (20).

**Test for chemical reversion**: Determinations of free formaldehyde released from four different samples of stored RVV toxoid were carried out by the method of Greenfield et al. at 3-month intervals (21).

**Toxicity test (safety test)**: One milliliter of each toxoid (containing 5 mg venom protein, i.e. more than 60 $LD_{50}$) stored at different temperatures was injected intramuscularly in an undiluted form to each test group. The groups consisted of five mice, and deaths within 24 h were noted. Toxoids that did not cause any death within 24 h were regarded as safe and used for immunization (14).

**Immunogenicity test**: The immunogenicity of the toxoids was tested according to Sawai and Kawamura (22). Diluted (1:9 with M/30 PBS) 0.1-m*l* samples of the toxoids (containing 0.05 mg of venom protein) were injected subcutaneously to each test group (five mice per group), followed by three booster injections of the same dosage at the 3rd, 4th, and 5th weeks. One week after the last booster dose of toxoid, all mice were sacrificed, serum samples were collected, and circulating venom antibodies were detected by the radioimmunoassay (RIA) method (23).

## RESULTS

Before preparing the RVV toxid, the i.m. $LD_{50}$ of RVV used in the experiment was determined. It was found that $LD_{50}$ and the minimum lethal dose ($LD_{100}$) of RVV in the mice were 2.35 and 4.7 Ug/gm body weight (i.e. 47 and 94 Ug/20 g mice), respectively, as calculated by the S. Karber method (Table 1).

A comparison of the biochemical characteristics of the liquid and lyophilized toxoids (just after reconstitution) is shown in Table 2. No apparent differences in pH, protein content, and the activity of enzymes were observed between the liquid and lyophilized forms of the toxoids. Therefore, the comparison of the potency of liquid and lyophilized toxoids in our study was found to be valid.

Table 1. Determination of intramuscular median lethal dose ($LD_{50}$) of RVV on mice

| Concentrations of RVV (Ug/0.1 m*l*) | Death of mice within 24 h |
|---|---|
| 600 | 6/6 |
| 300 | 6/6 |
| 150 | 6/6 |
| 75 | 5/6 |
| 37 | 2/6 |
| 18 | 0/6 |
| Normal saline | 0/6 |

Numerator indicates number of deaths and denominator indicates number of mice used.
Calculated i.m.$LD_{50}$=47 Ug/0.1 m*l* (i.e., 2.35 Ug/gm body weight)

Table 2. Comparison of pH, protein content and the activity of various enzymes present in the liquid and lyophilized toxoids

| | Liquid | Lyophilized |
|---|---|---|
| pH | 7 | 7 |
| Protein (mg/m*l*) | 6.82 | 6.64 |
| Proteinase (EU/mg protein) | 15.68 | 12.3 |
| PLA (EU/mg protein) | 10.16 | 11.34 |
| PDE (EU/mg protein) | 97.16 | 97.9 |
| AE (EU/mg protein) | 27.38 | 23.64 |

EU=Enzyme Unit

Figure 1 shows the changes in the protein content and the activity of various enzymes present in liquid and lyophilized forms of the RVV toxoid stored at 4°C and at RT measured at 3-month intervals for 1 year. There was no apparent change in the protein content in either form of the toxoid upon storage, especially in toxoids stored at 4°C. However, the enzyme activity was found generally to gradually increase with increases in the time stored. The changes were more apparent in the liquid toxoid than in the lyophilized toxoid, and also in those samples stored at RT compared to those stored at 4°C. Marked changes in the enzyme activity were usually found at 6 months of storage and onwards.

Figure 2 demonstrates the amount of free formalin released from liquid and lyophilized toxoids stored at 4°C and at RT for 1 year. There was little change in the amount of free formalin released from both toxoids stored at 4°C. Although a significant amount of free formalin was released from liquid toxoid stored at RT at 6 months of storage and onwards, only a small amount of free formalin was released from lyophilized toxoid stored at the same temperature.

Data from the safety test of the various stored toxoids are given in Table 3. Only some of those mice injected with liquid toxoid stored at RT for 3 months and onwards died within 24 h after injection. These findings indicate that the liquid toxoid stored at RT becomes toxic at 3 months of storage and onwards, whereas liquid toxoid stored at 4°C and the lyophilized toxoids stored at both 4°C and RT did not show any toxicity for up to 1 year of storage.

Figure 3 illustrates the serum venom antibody levels in mice immunized with liquid and lyophilized toxoids stored at 4°C and at RT. Mice immunized with lyophilized toxoids stored at both 4°C and RT, respectively, showed a significant

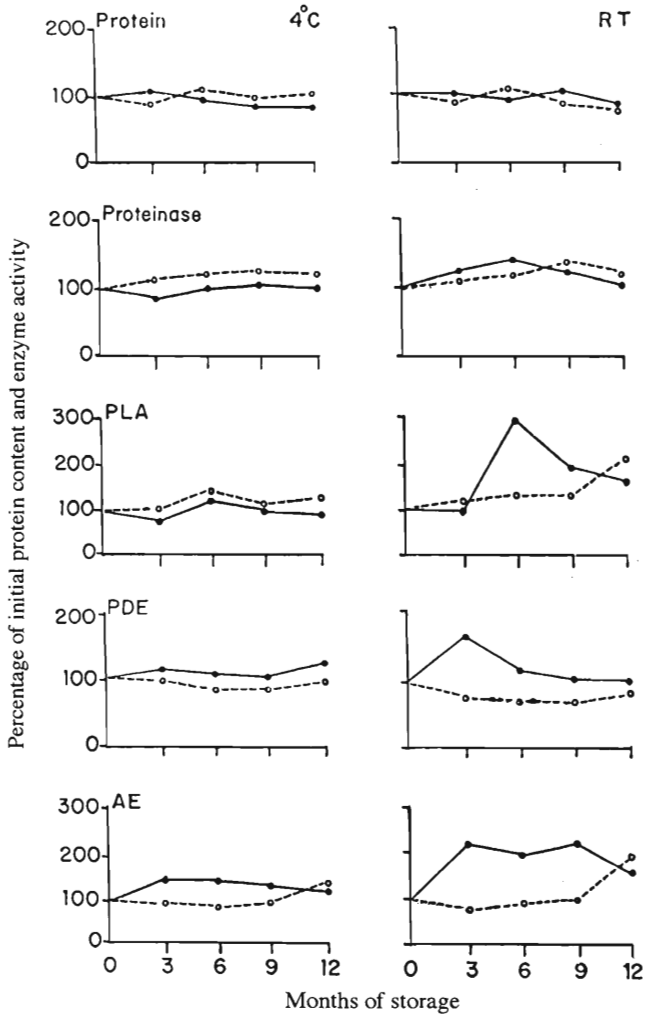


Fig. 1. Changes in the protein content and the activity of various enzymes in liquid and lyophilized toxoids during storage at 4°C and RT for 1 year.
•—•—•Liquid o—o—o Lyophilized

rise in serum antibody levels compared to those induced by immunization with liquid toxoids at corresponding intervals throughout the 1-year storage period. In addition, a more significant rise in antibody levels was found in mice immunized with the lyophilized toxoid stored at 4°C than with the same toxoid stored at RT.

## DISCUSSION

It is well known that freeze-dried biologicals have an

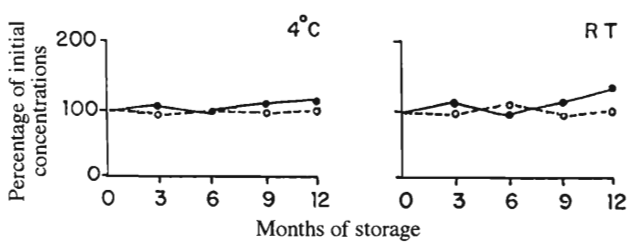


Fig. 2. Changes in the amounts of free formalin released from liquid and lyophilized toxoids during storage at 4°C and RT for 1 year.
•—•—•Liquid o—o—o Lyophilized

Table 3. Safety test of the various stored toxoids on immunized experimental mice

| Months of storage | Death of mice within 24 h | | | |
|---|---|---|---|---|
| | Liquid* | | Lyophilized* | |
| | 4°C | RT | 4°C | RT |
| 0 | 0/5 | 0/5 | 0/5 | 0/5 |
| 3 | 0/5 | 2/5 | 0/5 | 0/5 |
| 6 | 0/5 | 1/5 | 0/5 | 0/5 |
| 9 | 0/5 | 1/5 | 0/5 | 0/5 |
| 12 | 0/5 | 1/5 | 0/5 | 0/5 |

Numerator indicates number of deaths and denominator indicates number of mice used.
*Dose of toxoid administered=1 m*l* (containing more than 60 $LD_{50}$ of RVV)

advantage over liquid products in that they can be stored at ordinary RT for a long period without deterioration (24). In this study, lyophilized RVV toxoid was developed from crude RVV by detoxification and polymerization of venom proteins induced by formaldehyde. As a result, the RVV formed methylene bridges with amino acid side chains to form large molecular weight protein complexes. These polymerized large molecules are non-toxic but immunogenic and can be used as a snake venom toxoid (25). Therefore, the stability of a toxoid totally depends on the stability of the formaldehyde linkages formed in the toxoid molecule. If there is a dissociation of these formaldehyde linkages free formaldehyde will be released from the polymer and can be detected in the toxoids.

In the present study, we examined the effects of different storage temperatures (4°C and RT) and different storage forms (liquid and lyophilized) on the stability of the RVV toxoid. Regarding the storage temperature, both liquid and lyophilized toxoids stored at 4°C showed little changes in protein content, a reduced increase in enzyme activity, minimal chemical

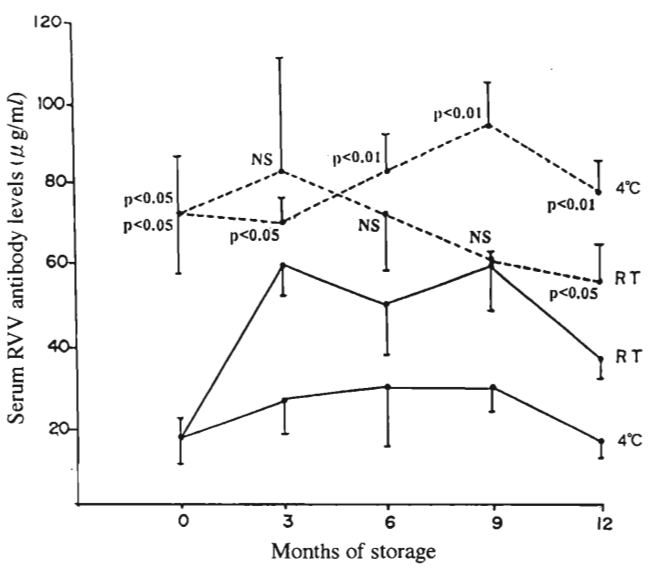


Fig. 3. Serum RVV antibody levels in mice immunized with RVV toxoids; liquid and lyophilized forms stored at 4°C and RT for 1 year.
Each value represents mean ± SEm (n=5)
Statistical evalutions were performed between the antibody levels induced by the lyophilized toxoids and those of the respective liquid toxoids, using the Student's unpaired "t" test.
NS means not significant at the 5 % probability level.
•—•—•Liquid •—•—• Lyophilized

reversion, no toxicity, and greater immunogenic properties compared to the respective toxoids stored at RT throughout the study period of 1 year. With storage at higher temperatures, there was an apparent chemical reversion, with a release of free formalin detected at 3 months of storage and onwards, indicating the release of more enzyme molecules that had been previously linked in the molecular mass of the toxoid, resulting in increased enzyme activity. This increase may also have been due to the autolysis of proteins stored at high temperatures for a long period. At 4°C, there was lower incidence of formaldehyde-linkage reversion in both forms of the toxoid, resulting in the stable polymerized molecule being retained with persistent antigenic properties and no toxicity. Therefore, there was a greater immunogenic response with no lethality in mice injected with either toxoid stored at low temperature.

Regarding the storage forms of the toxoid, lyophilized toxoid stored at both 4°C and RT was also found to have a lower incidence of formaldehyde-linkage reversion. As a result, the protein content was more stable, as was the enzyme activity, and the toxoid therefore remained safe and elicited a stronger immune response, compared to the respective liquid toxoids, stored at both conditions, over a period of 1 year. Therefore, it is apparent that the lyophilized toxoid is more potent and more stable than the corresponding liquid toxoids at any storage temperature. Although the lyophilized toxoids stored at 4°C and at RT showed little difference in potency, safety, and immunogenicity, the stability of the lyophilized toxoid stored at 4°C was slightly superior to that stored at RT, at which temperature there was slight chemical reversion with an appearance of free formaldehyde at 6 months of storage and onwards. Hence, the most potent form of the stored toxoid possessing no toxicity and high immunogenicity was the lyophilized toxoid stored at 4°C. This was followed by lyophilized toxoid stored at RT and liquid toxoid stored at 4°C. The least potent and the most dangerous toxoid was liquid toxoid stored at RT.

In conclusion, it is evident that lyophilized toxoids stored at 4°C and at RT are totally safe (i.e. non-toxic) and are also potent enough to induce an immunoprophylactic antibody response in experimental mice. In addition, the potency of the lyophilized toxoid is apparently stable for 1 year, even when stored at RT. Therefore, the prophylactic use of lyophilized toxoid for human immunization in the rural areas of Myanmar, where people are at risk of snake bite and proper cold storage facilities are lacking, could be considered in the near future.

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