Development of a stabilizer for lyophilization of an attenuated duck viral hepatitis vaccine

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ABSTRACT The live attenuated vaccine against duck viral hepatitis currently available in Korea requires special freezers for storage and transportation with extra costs involved. The development of a lyophilization stabilizer for live attenuated duck viral hepatitis virus (DHV) vaccines, therefore, has been highly recommended for the wider application of the vaccines. Four conventional vaccine stabilizer formulations containing a disaccharide, such as lactose, trehalose, or sucrose, and new formulations containing sorbitol were tested for their efficacy in stabilizing a new attenuated DHV type 3 vaccine candidate under different storage temperatures, 4 and 37°C. The vaccine virus and each stabilizer formulation were combined and submitted to lyophilization and the viability of the virus was measured in 7-d-old specific-pathogen-free chicken embryos by determining the 50% egg lethal dose. Stabilizer formulations containing 2, 4, or 8% sorbitol preserved the viability of the vaccine virus much better than the other stabilizer formulations and 2% sorbitol was the optimal concentration in a standard stabilizing buffer, phosphate glutamate gelatin (0.0038 *M* KH_2PO_4, 0.0071 *M* K_2HPO_4, 0.0049 *M* monosodium L-glutamate, and 0.5% gelatin). The results demonstrate that the stabilizer formulation containing 2% sorbitol and 0.5% gelatin can be used for convenient storage and transportation of live DHV vaccines.

Key words: stabilizer, lyophilization, duck viral hepatitis virus, vaccine, vaccine storage

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INTRODUCTION

Duck viral hepatitis is a peracute viral infection of young ducklings showing rapid spread, high mortality, and typical hemorrhages in the liver (Woolcock, 2008). Control of duck viral hepatitis mainly involves strict biosecurity procedures and the immunization of young ducklings or breeder ducks with live attenuated vaccines (Woolcock, 2008). In Korea, the occurrence of duck viral hepatitis was first reported in 1985 (Park, 1985), and a live attenuated duck viral hepatitis virus (**DHV**) vaccine was developed and introduced into duckling farms in 2000 (Sung et al., 2000). However, this vaccine requires special freezers for storage and transportation, with extra costs involved because the vaccine virus is not stable with common lyophilization stabilizers.

Lyophilization is often used to stabilize various virus vaccines and a lyophilized product should be stable, efficacious, and safe (Tannock et al., 1987). Therefore, vaccine stabilization requires appropriate stabilizers to prevent inactivation of the vaccine under environmental stress, such as elevated temperature (Monath, 1996). Thermostability of vaccines has been studied with a variety of compounds including carbohydrates, amino acids, and peptides (Bovarnick et al., 1950; Bedu-Addo, 2004). Most common stabilizer formulations include disaccharides such as sucrose, lactose, maltose, and trehalose or sugar alcohols such as sorbitol and mannitol, which are effective in stabilizing lyophilized products (Bovarnick et al., 1950; Bedu-Addo, 2004).

Recently, a variant type of DHV (type 3) has emerged in Korea and has spread into duckling farms (Kim et al., 2009). A new attenuated DHV type 3 vaccine candidate has been developed by the Korean National Veterinary Research and Quarantine Service (Kim et al., 2009). Nevertheless, the wider application of DHV vaccines still requires the development of an appropriate lyophilization stabilizer.

The current study assessed 4 conventional stabilizer formulations that each contained different amounts of lactose, trehalose, or sucrose, which are most commonly used for poultry vaccines in Korea, and also tested additional stabilizer formulations containing sorbitol for their efficacy in stabilizing the developed live at-

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tenuated DHV type 3 vaccine under different storage temperatures.

MATERIALS AND METHODS

Virus and Lyophilization

The previously described live attenuated DHV vaccine candidate, AP-04203P100 (Kim et al., 2009), was used. The original virus stock was composed of allantoic fluid mixed with infected embryos homogenized in allantoic fluid. The titer of the virus stock was measured by determining the 50% egg lethal dose (**ELD_{50}**) as described below, resulting in $10^{5.5}$ ELD_{50}/0.2 mL. The stock was stored at −80°C. Four conventional vaccine stabilizer formulations were prepared according to the recipes obtained from vaccine companies in Korea, and new formulations containing sorbitol were prepared by modifying the composition of a standard vaccine stabilizer, sucrose, phosphate, glutamate, and albumin (**SPGA**; Bovarnick et al., 1950; Table 1). Equal volumes of the vaccine virus and each 2× concentrated stabilizer formulation were combined (1:1 mixture) and lyophilized (Dae Sung Microbiological Labs, Uiwang, Gyeonggi, Korea). The same volume of virus was mixed with each stabilizer formulation and an equal volume of stabilizer was used for each formulation. The lyophilizing steps comprised freezing the live vaccine formulation to about −40°C for about 3 h and subsequently drying the vaccine formulation by gradually increasing the temperature to about 37°C in a VirTis lyophilizer (VirTis, Gardiner, NY) at 60 mTorr for 40 h. The lyophilized samples were stored at 4 or 37°C until testing.

Virus Assay and Stability Test

The viability of the virus was measured in 7-d-old specific-pathogen-free chicken embryos by determining the ELD_{50}. Briefly, serial 10-fold dilutions of the virus resuspended in PBS were inoculated into the allantoic cavities of chicken embryos and 5 embryos per each virus dilution (at least 30 eggs per sample) were used. They were incubated at 37°C and candled twice a day for 5 d. Embryos that died within 24 h were discarded. The ELD_{50} was calculated as described previously (Reed and Muench, 1938). The long-term stability test for the type 3 DHV vaccine was conducted at 4°C over a storage period of 11 mo. At 3, 6, and 11 mo after lyophilizaton, samples were taken and their titers were determined as described above.

RESULTS AND DISCUSSION

Stabilizer formulations containing sorbitol (2, 4, or 8%) and 0.5% gelatin preserved the viability of the vaccine virus stored at 4°C for 2 wk or at 37°C for 1 wk much better than the other common stabilizer formulations that were tested (Table 2). The optimal concentration of sorbitol in the stabilizing buffer was determined, resulting in a stable lyophilized formulation containing 2% sorbitol that showed the least loss of virus viability from $10^{3.3}$ to $10^{3.2}$ ELD_{50}/0.2 mL at 4°C or $10^{2.6}$ ELD_{50}/0.2 mL at 37°C (Table 2). A stable lyophilized formulation for the developed DHV vaccine candidate was composed of 0.0038 *M* KH_2PO_4, 0.0071 *M* K_2HPO_4, 0.0049 *M* monosodium L-glutamate, 2% sorbitol, and 0.5% gelatin (Table 1). The long-term stability test conducted with the above formulation revealed a slight loss of virus viability from $10^{5.2}$ to $10^{4.1}$ ELD_{50}/0.2 mL after 3 mo of storage. Subsequently, viability loss was minimal until 11 mo of storage ($10^{3.5}$ ELD_{50}/0.2 mL; Table 3).

Four conventional stabilizer formulations containing a disaccharide assessed in this study did not help to stabilize the DHV during the lyophilization process. One percent BSA combined with 0.2 *M* sucrose (i.e., SPGA) is one of the most widely used lyophilization stabilizers (Bovarnick et al., 1950). In the presence of SPGA, lyophilized viruses can be more stably stored and transported without undue losses in potency (Tannock et al., 1987). One-half percent gelatin combined with 10% lactose or trehalose is also commonly used as

Table 1. Compositions of stabilizers for lyophilization of the duck viral hepatitis virus tested in this study

Component	SPGA,[1] g (*M*)	LPGG 1,[2] g	LPGG 2, g	TPGG,[3] g	Sorbitol-PGG 1,[4] g	Sorbitol-PGG 2, g	Sorbitol-PGG 3, g
KH_2PO_4	0.52 (0.0038)[5]	0.52	0.52	0.52	0.52	0.52	0.52
K_2HPO_4	1.25 (0.0071)	0.854	1.25	0.854	1.25	1.25	1.25
Monosodium L-glutamate	0.829 (0.0049)	0.829	2	10	0.829	0.829	0.829
Lactose		100	68				
Trehalose				100			
Sucrose	74.62 (0.218)						
Sorbitol					20	40	80
Gelatin		5	4	5	5	5	5
BSA	10						

[1]Sucrose phosphate glutamate albumin.

[2]Lactose phosphate glutamate gelatin.

[3]Trehalose phosphate glutamate gelatin.

[4]Phosphate glutamate gelatin.

[5]The molar concentration of the component is shown in parentheses. The amount of component of each formulation is for 1 L of the final virus vaccine formulation for lyophilization.

Table 2. Changes in viability of the lyophilized duck viral hepatitis virus at different storage temperatures

	Virus titer[2]		
		Lyophilized	
Stabilizer[1]	Original[3]	After 2 wk at 4°C	After 1 wk at 37°C
SPGA	3.3	1	0.2
LPGG 1		<[4]	<
LPGG 2		<	<
TPGG		1.5	<
Sorbitol (2%)-PGG		3.2	2.6
Sorbitol (4%)-PGG		3	1.8
Sorbitol (8%)-PGG		2.5	1.7

[1]SPGA = sucrose phosphate glutamate albumin; LPGG = lactose phosphate glutamate gelatin; TPGG = trehalose phosphate glutamate gelatin; PGG = phosphate glutamate gelatin.

[2]Virus titers were determined in eggs and are expressed as the $\log_{10}$ 50% egg lethal dose (ELD_{50})/0.2 mL. The original titer was measured with the final dilution of the original virus stock in PBS prepared for the required dose (10^3 ELD_{50}/0.2 mL) and the lyophilized vaccine titers were measured with final dilutions of lyophilized viruses in PBS prepared as the original.

[3]The original value (3.3) applies to all of the stabilizers.

[4]< = the titer was below the limit of detection (<0.2 $\log_{10}$ ELD_{50}/0.2 mL).

a lyophilization stabilizer for poultry vaccines in Korea. Nevertheless, the stabilizers containing disaccharides could not be used as stabilizers for the lyophilization of the type 3 DHV vaccine. Only stabilizer formulations containing a sugar alcohol, sorbitol, were found to stabilize the lyophilized DHV. Increased concentrations of sorbitol may result in comparable increases in the thermal stability of some viruses, such as measles virus (de Rizzo et al., 1989). However, an increase in sorbitol concentrations had an adverse effect on the stability of DHV. The initial loss of the virus viability shown in the long-term stability test indicates that the lyophilization process with the stabilizer formulation containing 2% sorbitol may also initially destabilize the virus, probably due to freezing or drying damage. Nevertheless, after the initial loss, the virus titers were constant until the end of the experiment, indicating the long-term stability of the lyophilized virus.

Sorbitol-gelatin is an effective stabilizer in the preparation of freeze-dried suspensions of measles virus (de Rizzo et al., 1989) and rinderpest virus (Mariner et al., 1990). The addition of sorbitol as a vaccine stabilizer to a Japanese encephalitis vaccine is also effective in maintaining long-term immunogenicity (Toriniwa and Komiya, 2008). Albumin and gelatin are critical factors for long-term maintenance of the activity of lyophilized products (Kumagai et al., 1997; Marth and Kleinhappl, 2001). In particular, the gelling characteristics of gelatin prevent inactivation of viral vaccines from environmental changes such as temperature; therefore, gelatin has been widely used as one of the major components of stabilizers in many vaccines including live attenuated virus vaccines (Chun et al., 2004). Therefore, 0.5% gelatin was substituted for 1% albumin in modifications of SPGA containing sorbitol in this study. It is not clear why the stabilizing effect of sorbitol on the DHV is better than that of the disaccharides. These results may be specific to the lyophilization condition used in this study and also the different formulations may perform better than reported here if used under different conditions. Nevertheless, sorbitol-gelatin can be a good choice in stabilizing the lyophilized virus vaccine.

We have demonstrated that the stabilizer formulation containing 2% sorbitol and 0.5% gelatin is useful

Table 3. Long-term stability of the lyophilized duck viral hepatitis virus stored at 4°C

	Virus titer[1]			
		Lyophilized		
Stabilizer	Original[2]	After 3 mo at 4°C	After 6 mo at 4°C	After 11 mo at 4°C
Sorbitol (2%)-PGG[3]	5.2	4.1	3.8	3.5

[1]Virus titers were determined in eggs and are expressed as the $\log_{10}$ 50% egg lethal dose (ELD_{50})/0.2 mL. Titers were measured with lyophilized viruses resuspended in PBS prepared for the original volume before lyophilization.

[2]The original virus titer represents half of the titer ($10^{5.5}$ ELD_{50}/0.2 mL) of the original virus stock before mixing with stabilizer formulations because each lyophilized virus stock was mixed with the same volume of a stabilizer formulation.

[3]PGG = phosphate glutamate gelatin.

for convenient storage and transportation of the type 3 DHV vaccine. Because of the poor stabilization of the virus with the conventional stabilizer formulations, the current commercial DHV vaccine is provided as frozen vials that require −80°C freezers or liquid nitrogen and thus greatly increases the logistical and infrastructural costs associated with DHV vaccination. The stabilizer formulation containing 2% sorbitol and 0.5% gelatin developed in this study now allows the lyophilization of at least the DHV type 3 vaccine candidate. Availability of the lyophilized DHV vaccine may facilitate application of the vaccine to duckling farms to prevent the disease because the vaccine can be more conveniently handled in the field where freezers are impractical.

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