Enhanced stability of foot-and-mouth disease vaccine antigens with a novel formulation

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Abstract

The structural instability of inactivated Foot-and-mouth disease virus antigen hinders the development of vaccine industry. The use of an inexpensive, biocompatible formulation to slow down the degradation of antigen would address the problem. Here, phosphate-buffered saline (PBS) was showed to be effective in stabilizing 146S and hence determined as basic solution buffer. Excipients such as trehalose, sucrose, arginine, cysteine, calcium chloride, BSA and ascorbic acid were found to protect 146S from massive structural breakdown. Using orthogonal test, we confirmed the novel formulation as a combination of 5% (w/v) trehalose, 5% (w/v) sucrose, 0.05 M arginine, 0.01 M cysteine, 0.01 M calcium chloride, 1% (W/V) BSA and 0.001 M ascorbic acid in PBS. The formulation increased vaccine stabilization, with retention rate of 14% after storage at 4°C for 14 months. Particle size for vaccine was at approximately 220 nm and physicochemical detecting findings were rarely abnormal in morphology and emulsion type. In summary, these results revealed that the novel formulation is beneficial to make the FMD vaccine more stable and effective, reducing the dependence on cold storage and delivery.

1. Introduction

Foot-and-mouth disease (FMD) is an economically and socially severe animal disease that hinders the development of stock raising (Kitching 2005; Porphyre et al. 2018). FMD virus (FMDV) is the cause of this disease, which contains a single-stranded positive-sense RNA and a capsid protein composed of 60 copies of VP1, VP2, VP3, and VP4 (Fry et al. 2005). As an antigenically variable virus, FMDV consists of seven serotypes and a large number of subtypes, resulting in a big challenge to prevent and control FMD effectively (Paton et al. 2009).

Vaccination against FMDV is still an effective measure to block the spread of epidemic in FMD endemic areas. Inactivated vaccine was used extensively and their effectiveness is largely determined by the content of intact viral capsid, which has a sedimentation coefficient of 146S (Spitteler et al. 2019; Li et al. 2021). However, 146S is unstable and prone to dissociation with mild heating, leading to reduced immunogenicity and insufficient maintenance of the antibody, which is responsible for vaccine efficacy loss (Harmsen et al. 2011; 2017; Mansilla et al. 2020). Therefore, it is necessary to develop effective and safe strategies to improve vaccine stability (Cardoso et al. 2017).

Currently, many strategies have been used to enhance vaccine stability (Biswal et al. 2016; Scott et al. 2017). Targeting mutation of viral structural proteins is one of the effective methods, but, it may reduce viral infection, hinder viral propagation, and even alter the antigenicity of the virus (López-Argüello et al. 2019). Instead, a formulation to stabilize FMD vaccine antigens was feasible.

Excipients, known as supplement formulated alongside the active ingredient of a medication, have been demonstrated to be effective to maintain viral structural integrity (Kim et al. 2003; Arakawa et al. 2007; Lin et al. 2021). Several classes of excipients, including carbohydrate, amino acid and metal ion are used as FMD vaccine antigen stabilizers. Carbohydrate can hydrogen bond with FMDV and then prevent dissociation, which could be explained by preferential exclusion theory (Timasheff 1993). For amino acids, such as arginine, exhibited stable effects similar to those of sugars and may therefore be considered to be an alternative excipient (Erorna et al. 2020). The use of metal ion to stabilize viral capsids has been widely reported, and the binding of CaCl$_2$ was observed to enhance the thermostability of FMDV viral capsids (Acharya et al. 1989). The previous study showed that formulation has been applied to several virus vaccines, such as Newcastle disease (Wambura 2011), Marek’s (Colwell et al. 1975) and Classical swine fever (Pachauri et al. 2020). Therefore, minimizing FMDV dissociation can be addressed by selecting appropriate stabilizers for formulation. In this study, we developed a novel formulation of FMDV inactivated vaccine that can provide stabilizing effect for vaccine antigen.

2. Materials and methods

2.1. Materials

FMDV O/MYA98/BY/2010 strain provided by China Agricultural Vet .Bio. Science and Technology Co., Ltd., and was adopted as a mode antigen for stabilization study owing to its distinctive structural instability (Kotecha et al. 2015). Sucrose, trehalose, bovine serum albumin (BSA), and amino acids, including arginine, and cysteine were purchased from Sigma (MO, USA). Polyethylene glycol with a molecular weight of 6000 was bought from Sinopharm.
Chemical Reagent Co., Ltd (Beijing, China). All other chemicals were analytical-grade reagents purchased from Beijing Chemical Works (Beijing, China), and all solutions were prepared using Milli-Q-grade water (Millipore, USA).

2.2. High-performance size exclusion chromatography (HPSEC) detection

High-performance size exclusion chromatography (Agilent, USA) was performed to measure 146S content in FMD antigen or vaccine (Supplementary Table) (Yang et al. 2015). In brief, 100 μl samples were injected and eluted at 0.6 ml/min with 50 mM phosphate buffer containing 100 mM Na₂SO₄ at pH 7.2. The peak area at 259 nm was linearly proportional to 146S concentration with an established standard curve. Therefore, the 146S content in testing sample could be calculated in accordance with the peak area.

2.3. Screening of basic buffer solution

In this study, Tris-HCl, phosphate-buffered saline (PBS) and 50% Tris-HCl combined with 50% PBS were screened for basic buffer solution. As the suitable pH for FMDV stabilization is moderate alkaline, all solution buffers were adjusted to this condition (Caridi et al. 2015). Antigen was added to buffer solution and then stored at 37°C for 5 h. After treatment, samples were collected for 146S content determination via the HPSEC method. The initial concentration of the 146S was 20 μg/ml. The buffer solution suitable for protecting 146S against dissociation was used as basic buffer for the formulation.

2.4. Stability study of excipients

The stabilizing effect of several excipients, such as sucrose, trehalose, Tween-20, arginine, cysteine, BSA, calcium chloride, and ascorbic acid, were evaluated in this study. The stock solution of each excipient was prepared in PBS and then diluted further to obtain the final working concentration. Excipient was supplemented into purified 146S with two levels, which was determined in a preliminary study (The details are shown in Table 1). 146S content was examined by HPSEC after incubating at 37°C for 5 h, as described previously. The determined composition was used for subsequent study.

| Excipient         | Concentration (Molarity or % w/v) | Remaining 146S (μg/mL) |
|-------------------|-----------------------------------|------------------------|
| Trehalose         | (Kitching 2005) 3%                 | 10.2 ± 2.1             |
|                   | (Porphyre et al. 2018) 5%          | 12.8 ± 1.7             |
| Sucrose           | (Kitching 2005) 3%                 | 11.5 ± 2.4             |
|                   | (Porphyre et al. 2018) 5%          | 12.4 ± 3.1             |
| Arginine          | (Kitching 2005) 0.01 M             | 10.1 ± 0.1             |
|                   | (Porphyre et al. 2018) 0.05 M      | 11.1 ± 0.1             |
| Cysteine          | (Kitching 2005) 0.01 M             | 7.2 ± 0.2              |
|                   | (Porphyre et al. 2018) 0.05 M      | 9.7 ± 0.3              |
| Calcium chloride  | (Kitching 2005) 0.01 M             | 10.2 ± 2.5             |
|                   | (Porphyre et al. 2018) 0.05 M      | 11.7 ± 1.8             |
| BSA               | (Kitching 2005) 0.5%               | 6.4 ± 0.1              |
|                   | (Porphyre et al. 2018) 1%          | 8.3 ± 0.1              |
| Ascorbic acid     | (Kitching 2005) 0.001 M            | 10.1 ± 2.7             |
|                   | (Porphyre et al. 2018) 0.005 M     | 11.2 ± 3.1             |
| Tween-20          | (Kitching 2005) 0.1%               | 4.2 ± 0.1              |
|                   | (Porphyre et al. 2018) 0.5%        | 6.4 ± 0.1              |
| Control           |                                   | 5.5 ± 0.3              |

Table 1. Efficacy of excipients in stabilizing FMDV antigen at 37°C for 5 h.

2.5. Formulation optimization

After the basic solution buffer and excipients were determined, optimal formulation was analyzed and then determined by Adopt L₉ (2⁷) orthogonal mode. Totally, eight groups formulation were prepared in this study and control one is the sample with no excipients. Antigen was added to formulations respectively and incubated at 37°C for 5 h. The 146S content was measured by HPSEC and the one with more 146S content was regarded to be more effective for 146S stabilization and was determined to be the suitable formulation.

2.6. Vaccine preparation

Vaccines were prepared by emulsification of 75 ml FMDV antigen (20 μg/ml 146S) with 50% (w/w; 88 ml) ISA 206 adjuvant. Both warmed up at 32°C for 30 min, the antigenic media was added into the adjuvant beaker slowly and then maintained the agitation at 10000 rpm for 3 min. The vaccine was cooled at 20°C overnight and used for subsequent experiments.

2.7. Vaccine demulsification

Vaccine sample should be demulsificated before detecting. 1-pentanol was a common emulsion agent for demulsification. In brief, vaccine was mixed with 1-pentanol in a 10 ml centrifuge tube at a ratio of 9:1. The mixture was shaken fully to break the emulsion. After being placed at 4°C for 1 h, the aqueous phase at the bottom was absorbed slowly for 146S determination.

2.8. Thermal stability evaluation

Monitoring the changes of 146S content in formulated antigen and vaccine were performed to evaluate thermal stabilizing effect of the formulation. The control one is sample prepared with PBS. Vaccine was prepared by emulsification of formulated antigen with ISA 206 adjuvant. All samples were incubated at 4, 25 and 37°C respectively.

2.9. Physical characterization analysis

Particle size is a crucial parameter to evaluate emulsion stability. In this study, vaccine samples were diluted at 1:10 and then 1 ml of diluted sample was used for average size detection by dynamic light scattering automatically (Beckman, USA). In addition, dispersion assay was carried out for determining emulsion type when dropping 1 ml vaccine into water quickly.

2.10. Vaccine stability on storage

Increased shelf life in cold storage is crucial when deriving vaccine formulation. Monitoring 146S concentration could reflect vaccine stability (Yang et al. 2017). Vaccine was collected and tested at set time points: 0, 2, 4, 6, 8, 10, 12, and 14 months. After demulsification, 146S concentration was determined by the HPSEC method, as described previously.

2.11. Statistical analysis

The means and standard deviations (n = 3) of all values were analyzed with descriptive statistics. The statistically analysis of excipients was performed with t-test, and differences between samples were considered statistically significant at p < 0.05.
3. Results

3.1. High performance size-exclusion chromatography measurement

For quantitative analysis of 146S concentration, we first established a standard curve by plotting the peak area against 146S standard concentration. As shown in Figure 1(A), the standard curve had a strong linear correlation ($R^2 = 0.9986$), and the corresponding linear regression equation with x slope and y intercept was $y = 63038x - 56725$ (Figure 1(A)). From the typical changes shown in Figure 1(B), the 146S content can be calculated in accordance with the peak area appearing at 12.5 min.

3.2. Screening of basic buffer solution

PBS and Tris-HCl are a commonly used buffer for a protein treatment. Thus, to quickly determine the potential basic buffer solution, we assessed the stable effect of PBS, Tris-HCl, and a combination of PBS and Tris-HCl (1:1) on FMDV antigen. The antigens prepared with three types of buffer solution were incubated at 37°C for 120 h and the 146S content in each buffer solution were measured by the method established above. The results showed that during the incubation process, the concentration of 146S decreased more slowly in PBS than in Tris-HCl and the
combination solution. At the end of 120 h incubation, the 146S content in PBS was 10.2 µg/mL, much higher than that of Tris-HCl (2.8 µg/ml) and the combination (4.7 µg/ml) (Figure 2). Therefore, PBS is more conductive to the stability of 146S and is determined as the basic buffer solution.

3.3. Effect of excipient on thermal stability

Results summarized in Table 1 suggested that excipients have the capability of inhibiting 146S dissociation. Among them, 5% trehalose and sucrose were identified as effective, with 146S content of 12 µg/ml, obviously higher than that of control (5.5 µg/ml). About half of 146S was protected with addition of 0.05 M arginine, 0.05 M calcium chloride and 0.05 M ascorbic acid. BSA and cysteine showed almost similar protection efficiency, with 146S content of 8.3 and 9.7 µg/ml respectively. The protection efficiency for Tween-20 is similar to that of the control. Taken together, sucrose, arginine, calcium chloride, BSA, cysteine, trehalose and ascorbic acid were determined as the composition for formulation.

3.4. Formulation determination

To determine an optimal formulation for FMD vaccine, an orthogonal test was performed to further evaluate the stabilizing function of different excipients combinations for FMDV 146S antigen. As shown in Table 2, experimental group 2 exhibited the best effect on 146S stability. Ascorbic acid, cysteine and calcium chloride had the highest 146S content at concentration/level 1, while BSA, trehalose, arginine and sucrose had the highest 146S content at concentration/level 2. The optimal combination of excipients was A1B1C1D2E2F2G2 (Table 2).

Table 2. Orthogonal test and visual analysis of efficiency of novel solution buffer.

| Groups | A   | B   | C   | D   | E   | F   | G   | Remaining 146S (µg/mL) | Mean value |
|--------|-----|-----|-----|-----|-----|-----|-----|------------------------|------------|
| 1      | 1   | 1   | 1   | 1   | 1   | 1   | 1   | 14.203                 | 14.140     |
| 2      | 1   | 1   | 2   | 2   | 2   | 2   | 2   | 14.566                 | 14.108     |
| 3      | 1   | 2   | 1   | 1   | 2   | 1   | 2   | 14.152                 | 14.059     |
| 4      | 1   | 2   | 2   | 2   | 1   | 2   | 2   | 14.210                 | 14.161     |
| 5      | 2   | 1   | 2   | 1   | 2   | 2   | 1   | 14.222                 | 14.125     |
| 6      | 2   | 1   | 2   | 2   | 1   | 2   | 2   | 14.077                 | 14.157     |
| 7      | 2   | 2   | 1   | 1   | 2   | 1   | 2   | 14.157                 | 14.125     |
| 8      | 2   | 1   | 2   | 2   | 1   | 2   | 1   | 14.407                 | 14.105     |
| 9 (control) | 0   | 0   | 0   | 0   | 0   | 0   | 0   | 11.406                 | 11.406     |
| K1     | 14.140 | 14.136 | 14.189 | 14.086 | 14.065 | 14.128 | 14.052 | 14.032                 |            |
| K2     | 14.108 | 14.112 | 14.059 | 14.161 | 14.182 | 14.12 | 14.196 | 14.161                 |            |
| R      | 0.032 | 0.024 | 0.130 | 0.075 | 0.117 | 0.008 | 0.114 | 0.105                 |            |

Annotation: column A represents ascorbic acid. B is cysteine. C is calcium chloride. D is BSA. E is trehalose. F is arginine. G is sucrose.

Figure 3. Thermal stability of antigen and vaccine at three temperatures. Antigen and vaccine were prepared with PBS and formulation respectively at 146S concentration of 20 µg/mL. Samples were incubated at 4 °C (A), 25 °C (B), and 37 °C (C) at indicative time points. After demulsification and extraction, the antigens in vaccine were obtained and detected via HPSEC, while the antigens were measured directly with no treatment.
Moreover, the effect of excipients on 146S stability was calcium chloride > trehalose > sucrose > BSA > ascorbic acid > cysteine > arginine (Table 2). Therefore, the optimal concentrations of the supplemented excipients were 5% (w/v) trehalose, 5% sucrose, 0.05 M arginine, 0.01 M cysteine, 0.01 M calcium chloride, 1% BSA and 0.001 M ascorbic acid.

3.5. Thermal stability study

The thermal stability of the antigen and vaccine prepared with above determined optimal excipients combination were verified by measuring the 146S content during stored at 4, 25 and 37°C respectively. As shown in Figure 3 antigen in PBS showed the most drastic reduction after stored at 25°C, 37°C for 120 h and

![Graph showing 146S content (%)](image)

![Graph showing particle size distribution](image)

**Figure 4.** Shelf life and particle size of vaccine. (A) Monitoring the 146S content in vaccine and antigen during storage at 4°C. (B) Measurement and comparison of particle size of vaccine droplets with and without formulation. The red curve indicated normal vaccine (without formulation), and the green curve denoted formulated vaccine.
48 h, while more than 45% of formulated antigen preserved until 48 h at 37 °C. Vaccine in formulation exhibited dramatic improvement as it displayed only 40% 146S was lost at 37 °C for 24 h, however, the equal lost in 146S content was observed after 12 h in normal antigen and only 10% was maintained until 48 h. Compared to antigen, the vaccine was instability, with 54% 146S maintained after 1 month at 25 °C.

3.6. Stability evaluation for long-term storage

The 146S content in formulated vaccine and antigen were monitored during the 14 months of storage at 4 °C. As indicated in Figure 4(A), the 146S content in both antigen and vaccine decreased on a time-dependent manner, but a relatively slow degradation were found in formulated vaccine and antigen, with retention rate of 14% and 53% respectively, higher than that of the control one.

3.7. Assessment of vaccine physical properties

Particle size was examined to investigate the effect of the formulation on vaccine physical properties. Results revealed that the particle size was at approximately 220 nm, similar to that of the control (Figure 4(B)). Moreover, The vaccine droplets disperse in water completely, no significant difference to the control group (not shown).

4. Discussion

The structural instability of inactivated FMD vaccine antigen is one of the biggest challenges affecting vaccine quality. It is important to adopt effective measures to improve vaccine stability and then prolonging vaccine shelf life. Formulation composed with excipients could enhance vaccine stability. Therefore, considering safe, reliable application for large-scale production, a formulation may be employed.

Initially, three types buffer were screened as the basic buffer solution. Mild acidic pH could stimulate histidine protonation, inducing 146S dissociation easily (Yuan et al. 2017, 2020), thus, all buffers in this study were adjusted to suitable condition (Caridi et al. 2015). Excipients are also known to stabilize proteins through a variety of mechanisms such as salt-bridge establishment, inhibition of conformational changes and buffering (Prestrelski et al. 1993; Pikal-Cleland et al. 2002; Van Eldijk et al. 2016). In this study, the combination of 5% trehalose, 5% sucrose, 0.05 M arginine, 0.01 M cysteine, 0.01 M calcium chloride, 1% BSA and 0.001 M ascorbic acid exhibited the best stabilizing effect on 146S. Previous study reported that the 30% sucrose combined with 1% bovine serum albumin was effective for FMDV stabilization (Harmsen et al. 2015). However, such high concentration, in turn, could increase the viscosity of the vaccine, hardly absorbed by animal. Our studies showed that 5% sucrose combined with other excipients could also play a vital role in maintaining structural integrity, no impact on vaccine viscosity. BSA showed different stabilizing effect when used alone or combined with other excipients. Sucrose and BSA addition could reduce 146S dissociation in a synergistic manner for all FMDV strains (Yang et al. 2015). Similarly, in this study, BSA in formulation showed improved stabilizing effect, superior to ascorbic acid, cysteine and arginine (Table 2).

Thermal stability study showed that the formulation could both improve the stability of FMD antigen and vaccine at all three temperature. Of note, in our study, the vaccine has characteristic instability compared to antigen. This result was consistent with that of previous studies, which demonstrated that emulsification with adjuvant could influence structural stability of lysozyme (Van de Weert et al. 2000). Additionally, inactivation of protein antigen may also occur during encapsulation, the adopted antigen loading modes of particulate adjuvants, resulting in antigen instability (Van der Walle et al. 2009). However, the mechanism of poor stability in vaccine was not clear.

The changes of 146S content in FMD vaccine was monitored to further confirm the stable effect of formulation on vaccine during long-term storage at 4 °C. The results revealed that the degradation rate in vaccine decreased significantly, suggesting the enhanced stability of inactivated vaccine. Meanwhile, particle size and emulsion type were not changed in formulated vaccine, indicating no effect on vaccine physical properties, available for large-scale production.

Adjuvant, a key component, has an important effect on protein stability (Harmsen et al. 2015). This study showed the stability of vaccine prepared with ISA206, while the situation about other adjuvants, such as ISA201, was not clear. Thus, future investigation should be required to assess vaccine stability prepared with ISA201. In addition, antibody titer is an important parameter to evaluate vaccine quality. The problems of whether the formulation could influence the immunogenicity and whether stabilized vaccine contributes to antibody titer are the two considerable aspects to be fully considered. Therefore, further study should also take immune response in animals into account. Overall, these studies could provide a new insight into the improvement of the stability of inactivated vaccine.

In conclusion, the novel formulation could enhance vaccine stability and prolong the shelf life of vaccine, suggesting the widely utilize in FMD vaccine manufacturing facilities.

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Disclosure statement

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