Antiviral Activity of Scallop-Shell Powder against Avian Influenza Virus and Goose Parvovirus

Misato Tsujimura, Chanathip Thammakarn, Yuki Yamada*, Keisuke Satoh, Tomomi Hasegawa, Sakchai Ruenphet, and Kazuaki Takehara**

Laboratory of Animal Health, Department of Veterinary Medicine, Faculty of Agriculture, Tokyo University of Agriculture and Technology, 3-5-8 Saiwai-cho, Fuchu-shi, Tokyo 183-8509, Japan.
*Laboratory of Zoonoses, School of Veterinary Medicine, Kitasato University, 35-1, Higashi 23 ban-cho, Towada-shi, Aomori 034-8628, Japan.
** Corresponding author: FAX: 81-42-367-5776, e-mail: takehara@cc.tuat.ac.jp

Many infectious animal diseases that could not be controlled well by vaccination, including foot-and-mouth disease (FMD) and highly pathogenic avian influenza (HPAI), have occurred in many countries, especially across Asia. Materials that can enhance biosecurity in farms are important. In Japan, slaked lime has been used as disinfectant in farms, but it has a toxic effect on animals, as well as humans, and is easy to lose antiviral activity by oxidation. The desirable materials should be safe, cost effective, and long life. Aomori prefecture in Japan is very famous for culture of scallops. The scallop-shells after calcination process become powder and the main content of the powder is calcium oxide (CaO) that has bactericidal effects. Here we demonstrated antiviral activity of scallop-shell powder and its long life of antiviral activity against avian influenza virus (AIV) and goose parvovirus (GPV). Scallop-shell powder could reduce viral titers of AIV and GPV more than 10,000 times to undetectable level within 3 minutes. When the powder was spread on containers or on a chicken farm, its antiviral activity lasted more than 8 months. Scallop-shell powder seems to be a good candidate of materials for enhancement of biosecurity in farms.

Key words: avian influenza, biosecurity, calcium oxide, parvovirus, scallop-shell powder.

1. INTRODUCTION

In recent years, foot-and-mouth disease (FMD) and highly pathogenic avian influenza (HPAI), have occurred frequently in Asia [1, 2]. Vaccinations for these diseases can be used to prevent onset of clinical signs but vaccines cannot protect animals from infections [2]. In Egypt, Mexico, and Vietnam where vaccination against HPAI have been used, the causative viruses could not be eradicated [3, 4, 5]. Vaccination is one of the disease control tools but the Office International des Epizootics (OIE) recommends vaccination with monitoring and depopulation. Otherwise, vaccinations cause silent infections, viruses spread over animals and the diseases become enzootic in the area [2, 3, 4, 5].

Slaked lime and other disinfectants have been used widely for enhancement of biosecurity in farms, but slaked lime loses anti-microorganisms activity with rainfall [6] and most disinfectants also lose their activities with organic materials [7].

Aomori prefecture in Japan is very famous for culture of scallops and disposal of large amounts of waste scallop-shells are currently one of the social problem. After calcination process at more than 1,000°C, scallop-shells become powder and the main content of the powder is calcium oxide (CaO). The calcinated calcium made by scallop shells (hereafter referred to as scallop-shell powder: SSP) has been shown to possess anti-bacterial activity [8].

It will be desirable if SSP can be used as one of the materials that can enhance biosecurity in farms. Here we examined antiviral activities of SSP using avian influenza virus (AIV) and goose parvovirus (GPV). HPAI of the subtype H5N1 caused epizootics and human morbidity that are still big problem worldwide. AIV is easy to be
killed with any disinfectant because it has envelope [9].

GPV is one of the most resistant viruses against chemical physical treatments [10]. Antiviral activity of SSP was evaluated not only in laboratory but also under field conditions (in planters and on a farm).

Here we show that SSP can be used as one of the materials that enhance biosecurity in farms.

2. MATERIALS AND METHODS
2.1 Candidate samples for biosecurity

SSP was kindly supplied by C&C Co. Ltd. (Tokyo, Japan); it was prepared through calcination process of scallop-shells. First, shells were washed, dried, ground and calcinated with electric furnace at 1,700°C. Main content (> 99wt%) of the resulted product is calcium oxide (CaO). Then the product was milled into flour with average particle size of 15µm and named SSP.

Slaked lime (65wt% Ca(OH)₂) was purchased.

2.2 Viruses

A low-pathogenic AIV, A/duck/Aomori/395/04 (H7N1) [11] and goose parvovirus (GPV) strain IHC [12] were used. The AIV was propagated in 10-day-old embryonated chicken eggs and titrated on Madin-Darby canine kidney (MDCK) cells. GPV was propagated and titrated on Muscovy duck embryo fibroblasts (MDEF).

2.3 Determination of antiviral activity

Two hundreds milligram of SSP or slaked lime were measured into a micro-tube, then 100 µl of virus was added to the tube. The tube was incubated for 3 min at room temperature and the remained virus was recovered by adding 900 µl of maintenance medium to the tube and centrifugation. The resulted supernatant that contained 10 times diluted virus was further diluted in serial 10 fold, and titrated on sensitive cells. To evaluate the antiviral activity of samples with contamination of organic materials, 50 µl of fetal bovine serum (FBS) was added to 100 µl of each virus, then mixed with samples and incubated for 3 min. The remaining viruses were recovered with 850 µl of the maintenance medium.

The diluted 100 µl of AIV was inoculated to MDCK cells in 96-well cell-culture plates in Eagle's minimum essential medium (EMEM: Nissui Pharmaceutical Co. Ltd., Tokyo, Japan) with 2 µg/ml trypsin into 4 wells per dilution. At 3 days post inoculation (dpi), hemagglutinin (HA) activity of the culture supernatant was checked with 0.5% chicken red blood cells.

The diluted 100 µl of GPV was inoculated to MDEF cells in 96-well cell-culture plates in EMEM into 4 wells per dilution. Virus-induced cytopathic effect (CPE) was observed and at 7 dpi, MDEF was stained with crystal-violet [13].

Virus titer of 50% tissue culture infectious dose (TCID₅₀)/ml was calculated by the Behrens-Kaarbel’s method [14] with HA results for AIV or with crystal violet staining for GPV.

Neutralizing index (NI) was calculated using the following equation:

\[
NI = tpc - ta, \quad (1)
\]

where tpc is the converted titer of the positive control with no sample treatments, and ta is the titer converted into an index in log₁₀ of the virus recovered from the sample-treated tube. Inactivation of viruses was considered effective when NI > 3.0 as described in references 9 and 15.

2.4 Fields experimental design

Experiment 1: Persistence in field environment. SSP or slaked lime around 500g each was put on compost in a planter (using half areas of the planter, namely 50cm x 20cm: 10 kg/m²), kept in outside, and sampled from the surface every month. Antiviral activity was checked for AIV.

Experiment 2: Persistence in a chicken farm. SSP or slaked lime were scattered on ground around chicken houses (0.5-1.0kg/m²) at a layer chicken farm (Kanagawa, Japan) and kept for 10 weeks. Sample of around 5 g was collected every week and the samples were sent to our laboratory for checking antiviral activity.

3. RESULTS

3.1 Antiviral activity

SSP and slaked lime could reduce viral titers of AIV and GPV more than 10,000 times to undetectable level within 3 min, as shown in Table 1. After recovery of viruses from SSP or slaked lime, 10 times diluted viruses could not be evaluated because of cell death caused by SSP or slaked lime. We could thus observe that the undetectable level was <10⁻⁵₅ TCID₅₀/ml. Antiviral
activities of both samples were not affected by presence of 33% FBS.

Table I. Inactivation of viruses by SSP or slaked lime.

<table>
<thead>
<tr>
<th>Samples</th>
<th>Viruses</th>
<th>FBS</th>
<th>Treated NI</th>
<th>Control NI</th>
</tr>
</thead>
<tbody>
<tr>
<td>SSP</td>
<td>AIV -</td>
<td>&lt;2.5</td>
<td>7.3</td>
<td>&gt;4.8</td>
</tr>
<tr>
<td></td>
<td>GPV -</td>
<td>&lt;2.5</td>
<td>7.0</td>
<td>&gt;4.5</td>
</tr>
<tr>
<td></td>
<td>AIV +</td>
<td>&lt;2.5</td>
<td>6.8</td>
<td>&gt;4.3</td>
</tr>
<tr>
<td></td>
<td>GPV +</td>
<td>&lt;2.5</td>
<td>7.0</td>
<td>&gt;4.5</td>
</tr>
<tr>
<td>Slaked lime</td>
<td>AIV -</td>
<td>&lt;2.5</td>
<td>7.3</td>
<td>&gt;4.8</td>
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<tr>
<td></td>
<td>GPV -</td>
<td>&lt;2.5</td>
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<td>7.0</td>
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</table>

Virus titers are shown in log10 TCID<sub>50</sub>/ml.

3.2 Field experiments

When SSP or slaked lime were put on compost in the planters, anti-AIV activities of SSP or slaked lime were gradually decreased over the course of time, but AIV titer was reduced more than 1000 times by SSP or slaked lime when harvested at 8 months post-scattering (Fig. 1).

![Fig. 1. Antiviral activities of samples in the planter experiments. Antiviral activities of SSP and slaked lime were shown as NI.](image)

After rain, especially at 3 months post-scattering or later, slaked lime became hardened and it was difficult to take samples from the surface of the lime. The slaked lime which hardened in the form of a board is shown in Fig. 2. However, this phenomenon was not observed in SSP. At the end of the experiment (8 months post-scattering), SSP was still soft and it was easy to take samples from the surface.

At the chicken layer farm, except at 5 weeks post-scattering, SSP and slaked lime showed anti-AIV activities (NI > 3) for 10 weeks (Fig. 3).

![Fig. 2. Slaked lime hardened in the shape of a board in the experiment 1. An arrow shows a board of lime.](image)

![Fig. 3. Antiviral activities of samples in the farm experiments. Antiviral activities of SSP and slaked lime were shown as NI.](image)

4. DISCUSSION

Parvovirus is known as one of the most physic-chemical resistant viruses in the world. GPV required heating at 80°C for 30 min or incubation with chlorine at 4000 ppm for 30 min to become inactivated [10]. Normally most disinfectants lose their anti-microbial activities in the presence of organic materials [7]. To evaluate new samples for enhancing biosecurity in poultry farms, we established an evaluation system with AIV and GPV as reference viruses and also examined samples with presence of FBS at 33% concentration. Antiviral activities of SSP and slaked lime were shown in Table 1. Both SSP and slaked lime could kill AIV and GPV to undetectable level even with organic material contamination.

To mimic the field condition, SSP or slaked lime were placed on compost in planter (10kg/m<sup>2</sup>). For a longitudinal study, the antiviral activity within each
sample was being checked during 8 months. In contrast with the previous report [6], anti-AIV activity of slaked lime lasted long. Because in the present study, slaked lime was scattered in 10kg/m² and it was 10 times more than the previous report using 0.5-1kg/m². Slaked lime tended to be harden especially after rain (Fig. 2), and it was difficult to take samples from the surface. This means that under field conditions, micro-organisms seem not to be trapped well on slaked lime. On the farm experiment, samples were scattered in a proportion of 0.5-1.0kg/m², as reported previously [6]. Here slaked lime also tended to harden, while, SSP did not.

In Japan, SSP has licensed as a food additive, which means if domestic animals eat SSP by accident, it will be safe.

5. CONCLUSION

Here we demonstrated that SSP has anti-viral activity and the activity lasts long in the field conditions. These data suggest that SSP can be used for the material that is intended to enhance bio-security on farms.

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REFERENCES


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