Studies on epidemiology of viral pathogen indicators and biosecurity enhancement on pig farms

豚農場内に蔓延する汚染指標病原体の疫学調査とバイオセキュリティの強化に関する研究

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Contents

Abbreviations

AIV: avian influenza virus

ASTM: American Society for Testing and Materials

BHK-21: baby hamster kidney

bp: base pair

CKC: chicken kidney cell

COVID-19: corona virus disease 2019

CPE: cytopathic effect

DMSO: dimethyl sulfoxide

dpi: days post-inoculation

dW_2: redistilled water

EN: European norm

FAC: free available chlorine

FBS: fetal bovine serum

$FdCa(OH)_2$: food additive grade calcium hydroxide

FMDV: foot-and-mouth disease virus

HOCl: hypochlorous acid

IBV: infectious bronchitis virus

MDCK: Madin-Darby Canine Kidney

MM: maintenance medium

MRV: mammalian orthoreovirus

NaClO: sodium hypochlorite

ND: Newcastle disease

PBS: phosphate-buffered saline

PFU: plaque forming units

PK-15: porcine kidney

ppm: parts per million

PSV: porcine sapelovirus

QAC: quaternary ammonium compound

RF: reduction factors

RT: room temperature

RT-PCR: reverse transcription polymerase chain reaction

SAHW: slightly acidic hypochlorous acid water

SARS-CoV-2: severe acute respiratory syndrome coronavirus 2

SE: standard error

SPF: specific-pathogen-free

$TCID_{50}$: fifty percent tissue culture infectious dose

UTR: untranslated region

Vero: African green monkey kidney

Preface

To date, many preventive measures have been established and utilized to minimize the economic losses due to serious infectious diseases in the domestic livestock industry worldwide. For example, effective disinfection strategies can eliminate many or all the pathogenic microorganisms on surfaces, in liquid and in the air, which is critical to control the spread of infections toward humans and domestic animals (Giraudon et al, 2009; Karl et al, 2022). All in / all out management is applied in the pig industry to prevent infectious diseases in pigs (Beloeil et al, 2004; Farzan et al, 2006). Besides, vaccination of livestock plays an important role in providing animals with immunity against infectious diseases without the risks of contracting diseases (Henning et al, 2013; White et al, 2017). Moreover, changing personal protective equipment (clothes and footwear) reduces the risk of pathogen transmission from infected hosts or from the environment (Kim et al, 2017; Yoo et al, 2022). Although these preventive measures are utilized in the livestock industry, outbreaks of viral infectious diseases in livestock (chickens, bovine, pigs etc.) have caused significant economic losses worldwide, including Japan. Approximately 9.87 million of chickens and ducks were culled during 2020 to 2021 to prevent the further spread of highly pathogenic avian influenza in Japan (MAFF, 2022). In endemic areas of foot-and-mouth disease virus (FMDV), annual costs generated by the production losses and vaccination of FMDV for cattle were estimated at 6.5 to 21 billion dollars (Knight-Jones and Rushton, 2013). When African swine fever epizootic emerged in China and neighboring Asian countries in 2018, an estimated direct economic loss was 55 to 130 billion dollars (Tran et al, 2022). In Japan, an estimated 354 thousand pigs were culled from Sep 2018 to Oct 2022 by the outbreak of classical swine fever (MAFF, 2022). Outbreaks of these infectious diseases indicate that advanced preventive measures need to be established in addition to the conventional countermeasures.

On-farm disinfection is considered as one of the most effective actions to control infections on livestock farms. Effective daily disinfections contribute to reduce harmful pathogens on farms, and enhance the biosecurity at the farm level (FAD PReP/NAHEMS, 2014). Moreover, the reductions of

pathogens at the farm level by disinfection can reduce antimicrobial consumption and the cost of livestock vaccinations (Laanen et al, 2013). In order to select and apply effective disinfectants for routine cleaning on livestock farms, the efficacies of disinfectants toward microorganisms must be accurately evaluated through disinfection tests beforehand. The suspension test is the simplest evaluation test, which is performed by mixing microorganisms and disinfectants. Due to its simplicity, the suspension test has been utilized in many studies (Anderson et al, 2022; Behrendt et al, 2022; Denys et al, 2022; Huang et al, 2022a, 2022b). In the carrier tests, microorganisms on dry surfaces of carrier coupons (e.g. steel, porcelain, glass) are exposed to disinfectants directly or through disinfectant wipes / spray (Caschera et al, 2022; Khalid Ijaz et al, 2022; Song et al, 2022). In both suspension and carrier tests, a reduction of 3 or 4 $\log_{10}$ or more compared to the control titers is required to be considered effective virucidal activity of disinfectant toward viruses (OECD, 2013; Tarka and Nitsch-Osuch, 2021; US EPA, 2018; Walji and Aucoin, 2020). However, existing carrier tests for surface disinfection with mechanical action (wiping tests) are not convenient. As an example of wiping tests, the American Society for Testing and Materials (ASTM) published ASTM E2967-15. In this method, wiperator machine (Wiperator, 2022)) is used to wipe the contaminated carrier and one carrier to test the transfer of pathogens (Cutts et al, 2021; Jacobshagen et al, 2020; Ledwoch et al, 2019). In a case of the European norm (EN) 16615:2015, 4 carriers (one contaminated carrier and three uncontaminated carriers) and the fixed wipe under a granite block with a weight between 2.3 to 2.5 kg are used (Becker et al, 2019; Tyski et al, 2021). These facts mean that the utilization of this method can be difficult for an ordinary laboratory. Moreover, in EN 16615:2015, each carrier is rubbed by nylon swabs to recover the pathogens on the wiped carrier after wiping action. However, disinfectant's activity is not neutralized during this rubbing process, which means that the disinfection time is not accurately controlled through this method. Therefore, more convenient and accurate wiping methods need to be established. Besides, although viral infectious diseases such as seasonal influenza and corona virus disease 2019 (COVID-19) are spread globally through aerosol transmission (Merced-

Morales et al, 2022; WHO, 2022), there are no published studies evaluating disinfectants toward aerosolized microorganisms. Therefore, the virucidal activities of disinfectants toward aerosolized viruses need to be evaluated, so as to prevent aerosol mode infection, in addition to suspension and carrier tests.

To improve the biosecurity level of livestock farms, it is necessary to know the prevalence or frequency of contamination with pathogens that are ubiquitous on their farms. As previously explained, effective strategies contribute to reducing pathogens and enhancing the biosecurity on livestock farms (Hasan et al, 2022; Isomura et al, 2018; Takahashi et al, 2020). For the improvement and assessment of the strategies to enhance biosecurity levels, accurate information on pathogens on the farms needs to be shown (Sasaki et al, 2020). If the prevalence of pathogen indicators on the farm has decreased, it can be concluded that biosecurity has been enhanced. Therefore, data on viral pathogen indicators surveillance is necessary in order to achieve the enhanced biosecurity on farms.

The main objective of this study is to enhance biosecurity in pig farms using effective disinfection strategies. In Chapter 1, convenient and accurate methods for evaluating virucidal activities of disinfectants toward viruses on surfaces were developed and established. In Chapter 2, virucidal activity of slightly acidic hypochlorous acid water (SAHW) toward viruses on surfaces and in the air was evaluated from the perspective of practical application. In Chapter 3, surveillance of viral pathogen indicators on pig farms was conducted, and their disinfectants susceptivity was evaluated.

Chapter 1.
Establishment and utilization of an evaluation system for virucidal activity of disinfectants against a coronavirus

1.1. Introduction

In late 2019, a novel human coronavirus − severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2), a member of *Betacoronaviruses*, − emerged in China and has been declared a pandemic virus since 11 Mar 2020 (WHO, 2022). Human-to-human transmissions through aerosol and fomite are plausible, and probably common, since SARS-CoV-2 can remain viable and infectious in aerosols for hours and on surfaces for days (van Doremalen et al, 2020). Chin et al. also reported the stability of SARS-CoV-2 in the environment (Chin et al, 2020). It has also been suggested that the soles of medical staff shoes may function as carriers of nosocomial infection (Guo et al, 2020). It is essential to disinfect the virus on nonporous surfaces, especially on steel and plastic, because these materials keep the virus infectious for 2 to 4 days (Chin et al, 2020; van Doremalen et al, 2020). In Japan, the Ministry of Health, Labor and Welfare recommends using 70% alcohol for hand washing and sodium hypochlorite (NaClO) of chlorine concentration at 500 to 1,000 parts per million (ppm) for decontaminating the surfaces of substances.

A recent review on inactivation of human and animal coronaviruses on inanimate carriers with biocidal agents (Kampf et al, 2020), presents several papers showing that NaClO of chlorine concentration at 1,000 ppm or 2,100 ppm could inactivate coronaviruses within 1 min on the carriers (Dellanno et al, 2009; Sattar et al, 1989).

It has been reported that decontamination of surfaces with dried inoculum is invariably more difficult, as compared to contaminated suspension (Mosteller and Bishop, 1993; Springthorpe and Sattar, 2005). In some experiments, it was also shown that pathogens in aqueous phase could be inactivated with disinfectants in short periods, such as 5 sec; however, pathogens on abiotic carriers or in feces could survive disinfectant treatments for considerably longer periods, namely, minutes or hours (Alam et al, 2018a, 2018b; Hakim et al, 2017; Sangsriratanakul et al, 2018; Thammakarn et al, 2015). Particularly, the bactericidal activities of NaClO diminish in the presence of organic material loads (Toyofuku et al, 2017). Besides, it was elucidated that an alkaline agent, namely food additive grade calcium hydroxide

($FdCa(OH)_2$) has synergistic effects together with NaClO or quaternary ammonium compound (QAC), in terms of capacity for inactivating microorganisms in the presence of organic material contamination (Alam et al, 2018a; Ito et al, 2018; Toyofuku et al, 2017).

Here an avian coronavirus (infectious bronchitis virus: IBV) was used instead of the pandemic SARS-CoV-2, so as to demonstrate an equivalent evaluation system for certain disinfectants applied through two techniques, assuming its applicability to both viruses. For the carrier tests, dropping and wiping disinfection techniques were established and compared. This evaluation system seems to be important and workable for resultful selection of the tested disinfectants against the pertinent viruses on surfaces, particularly (yet not solely) on plastic fomite.

In Chapter 1, suspension and carrier tests contaminated with organic materials were conducted to compare virucidal activities of disinfectants. For the carrier experiments, IBV was deposited on a plastic carrier, and the two following methods were compared to evaluate virucidal efficacy of disinfectants toward the virus on the carrier. 1) 500 μL of each disinfectant was dropped to the virus on the carrier; hereafter dropping technique. 2) a rayon sheet containing 500 μL of each disinfectant was used for wiping the carrier; hereafter wiping technique.

1.2. Materials and methods

1.2.1. Virus

Coronaviridae, Gammacoronavirus, IBV strain M41, kindly supplied by National Institute of Animal Health (Tsukuba, Ibaraki, Japan), was propagated in primary chicken kidney cell (CKC) cultures, and titrated in plaque assay on CKC monolayers as described (Takehara et al, 1991). For virus growth, Eagle's minimum essential medium containing 0.3% tryptose phosphate broth, without fetal bovine serum (FBS), was used. For evaluating decontamination in the presence of organic material, 0.5% or 5% FBS was added to the virus medium; these percentages were selected as the representative of normal human secretions, namely, 0.5% of bovine mucin (Sattar et al, 1989), alongside with 5% as the imitation

of field organic contamination (Alam et al, 2018a). Plaques were counted at 3 days post-inoculation (dpi). The titer was calculated as plaque forming units (PFU)/mL. Each test was carried out in triplicate; the titers are shown as mean ± standard error (SE). Inactivation was considered to be effective, if more than 1,000 times reduced virus titer was obtained (Alam et al, 2018a; Lombardi et al, 2008).

1.2.2. Disinfectants and blocking solution

$FdCa(OH)_2$ powder (Fine Co, Ltd, Tokyo, Japan), NaClO solution containing chlorine at 13% (Fujifilm Wako pure chemical Co, Ltd, Osaka, Japan) and QAC (Rontect®, Scientific Feed Laboratory Co, Ltd, Tokyo, Japan) were purchased. For making 0.17% $FdCa(OH)_2$ solution, 1.7 g of $FdCa(OH)_2$ powder was added to 1,000 mL of redistilled water (dW_2) and then centrifuged at 1,750 × g for 10 min at 4°C. The resulting supernatants were used as 0.17% $FdCa(OH)_2$ solution. NaClO solutions at 1,000 ppm or 500 ppm of total chlorine (NaClO-1000 or NaClO-500, respectively) were prepared in dW_2, whereas for the mixed solution (NaClO-500 and 0.17% $FdCa(OH)_2$: Mixed-500), NaClO was diluted at 500 ppm of total chlorine in 0.17% $FdCa(OH)_2$. The resultant solutions containing NaClO, namely NaClO-1000, NaClO-500 and Mixed-500, were each used for the experiments within 30 min after preparation. QAC was diluted 1:500 (QAC-500) with dW_2 to obtain a final concentration of 200 ppm didecyl-dimethylammonium chloride, as recommended by the manufacturer.

A blocking solution containing 30% FBS in 0.7 M 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (pH 7.2) was prepared for neutralizing the virus inactivation reaction by the disinfectants as described (Alam et al, 2018a; Ito et al, 2018).

1.2.3. Experimental designs

1.2.3.1. Suspension tests for evaluating the virucidal activities of the solutions against IBV in the aqueous phase

Suspension tests were performed at room temperature (RT: 25 ± 2°C) as previously described (Alam

et al, 2018a; Ito et al, 2018). Briefly, 400 µL of 0.17% $FdCa(OH)_2$, NaClO-1000, NaClO-500, Mixed-500 or QAC-500 were mixed with 100 µL of IBV containing 5% FBS in a microtube, respectively, then incubated for 30 sec. Following incubation, the virus inactivation was immediately stopped by adding 500 µL of the blocking solution. To ascertain the effect of the blocking solution, the test solutions were mixed with the blocking solution before the addition of virus (considered as 0 sec treatment and contact time). For the water control, 100 µL of IBV was inoculated in 400 µL of dW_2 and at 30 sec, 500 µL of the blocking solution was added. Then, the remaining virus was titrated after making serial 10-fold dilutions.

1.2.3.2. Evaluating the virucidal activities of the test solutions toward IBV on contaminated carriers through the dropping technique

Carrier test with the dropping method was performed at RT as described previously (Alam et al, 2018a). In brief, an amount of 100 µL of IBV strain M41 containing 0.5% FBS was spotted on a plastic carrier coupon (around 5.0 cm × 5.0 cm) and subsequently spread by sterile glass spreader onto the carriers and air dried for 60 min inside the biological safety cabinet at RT (Fig. 1.1). Then 500 µL of each solution including dW_2 as the water control was dropped on each carrier and incubated for 1 min (Fig. 1.2). After incubation, the virucidal activities of the test solutions were blocked by placing the carrier into stomacher bags (size 100 × 150 × 0.09 mm, capacity 80 mL; iwatsuki Corporation, Tokyo, Japan) containing 2 mL of the blocking solution. Subsequently, each carrier surface was rubbed vigorously by finger over the stomacher bag to dislodge the virus from the carrier surfaces into fluids. The remaining viable virus in each sample, including the dW_2 control, was titrated on CKC cultures (PFU/mL).

1.2.3.3. Evaluating the virucidal activities of the test solutions toward IBV on contaminated carriers

through the wiping technique

In the carrier test with the wiping method, the plastic plates with the spotted virus, as shown in the dropping technique, were wiped with a rayon-polyester sheets (5 cm × 5 cm, Alphase® 5, Iwatsuki Co, Ltd, Tokyo, Japan) folded into four pieces (2.5 cm × 2.5 cm) (Fig. 1.1) containing 500 μL of each disinfectant for 30 sec (Fig. 1.2). Each sheet was thereafter transferred into a stomacher bag containing 2.0 mL of blocking solution. The wiped plastic carrier was immediately put into a stomacher bag containing 2.5 mL of the blocking solution. Subsequently, each carrier was rubbed vigorously, as shown in the dropping technique. Rayon sheets were treated with a BagMixer (MiniMix 100 W CC, Practical Japan Inc, Chiba, Japan) to remove viruses from the sheets. Then, the recovered virus was titrated on CKC cultures.

1.3. Results

1.3.1. Suspension test for evaluating the virucidal activities of the solutions against IBV in the aqueous phase

As shown in Table 1.1, 0.17% $FdCa(OH)_2$ solution, NaClO-1000, NaClO-500, Mixed-500 and QAC-500 could inactivate IBV to undetectable level within 30 sec, even in the presence of 5% FBS. When the blocking solution was added before the addition of the virus (0 sec), the viral titer was similar to the positive control. Thus, the blocking solution could stop the virucidal activity of the tested solutions, when mixed together, at equal volume to the reaction medium.

1.3.2. Evaluating the virucidal activities of the test solutions toward IBV on the contaminated carriers with the dropping and wiping techniques

As shown in Table 1.2, 0.17% $FdCa(OH)_2$ solution resulted in more than 1,000 times reduced virus titer through the dropping technique, within 1 min. NaClO-1000 and QAC-500 inactivated IBV to the undetectable level. NaClO-500 could not inactivate the virus higher than 1,000 times reduction, whereas

Mixed-500 did inactivate the virus to undetectable level. With the dropping method, the virus titer in the dW_2 was $10^{6.00}$ PFU/mL, and the original viral titer was $10^{6.47}$ PFU/mL (Table 1.2). The recovery ratio of IBV from the carrier in dW_2 was around 30%.

With the wiping method, no virus was detected within the sheets after wiping with 0.17% $FdCa(OH)_2$ solution, NaClO-1000, Mixed-500, or QAC-500. These data mean that 0.17% $FdCa(OH)_2$ solution, NaClO-1000, Mixed-500 and QAC-500 were capable of inactivating IBV within 30 sec to undetectable level. The infectious virus remained in NaClO-500 sheet (Table 1.2), but the virus titer underwent higher than 1,000 times reduction, and was even though considered effective (Alam et al, 2018a; Lombardi et al, 2008). When the carrier was wiped with dW_2 sheet, the virus was detected within the sheet at $10^{5.45}$ PFU/mL. The recovery ratio of the virus from the sheet with dW_2 was around 20%.

Subsequent to wiping with disinfectant solutions including NaClO-500, no virus was detected on the carriers. With dW_2 wiping, the virus at $10^{2.88}$ PFU/mL was thereafter detected on the carriers. This means that wiping with dW_2 could remove the virus up to more than 99.9% from the contaminated carrier via a mechanical action, although the sheet contained large amount of infectious virus (around 20% of the original viral load).

1.4. Discussion

As shown in Table 1.1, at 0 sec, 500 μL of blocking solution was added to the disinfectants before adding the virus, and the viral titer was similar to the virus control for each disinfectant, showing that an equal amount of blocking solution was capable of stopping the virucidal effect of the disinfectants. This means that the 4 times volumes of the blocking solution halted disinfectant activity in the carrier tests.

All disinfectants could inactivate IBV in the suspension tests within 30 sec. Such a suspension test should be used as the first step for screening of disinfectants, while the second screening is required by means of carrier disinfection tests for field usage (Rabenau et al, 2014; Sattar and Maillard, 2013;

Springthorpe and Sattar, 2005). Sattar et al. used the concentration of 0.5% of bovine mucin, which is representative of the level of human mucin found in normal human secretions (Sattar et al, 1989). In the present study, 0.5% FBS was used to imitate mucin for the carrier tests.

Normally, pathogens will not be recovered from towels used for decontamination, because relatively large volumes of a neutralizer are required to properly immerse the entire towel for microbial recovery (Sattar and Maillard, 2013). Becker et al. found that the wipes contained viruses after use, yet they did not use blocking solution to stop the virucidal activities of the disinfectants, and detected cytotoxicity of the disinfectants toward the host cells (Becker et al, 2019). In the present study, the rayon-polyester sheet was used for wiping, and it was then transferred into the blocking solution immediately after wiping. The virus was recovered from the sheet to confirm the inactivation of the virus. Thus, 60 mm dishes were used for the plaque assay that allowed low detection limit as $10^{1.40}$ PFU/mL, without cytotoxicity of the disinfectants in the suspension tests (Table 1.1). For the carrier tests, 500 μL of disinfectant solutions and 2 mL of the blocking solution allowed detection limit as low as $10^{1.80}$ PFU/mL (Table 1.2).

It was previously demonstrated that pathogens on abiotic carriers or in feces are more resistant to disinfectants, as compared with the aqueous phase contamination (Alam et al, 2018a, 2018b; Hakim et al, 2017; Sangsriratanakul et al, 2018; Thammakarn et al, 2015). When the disinfectant efficacies toward pathogens found on a table or a button of an elevator are appraised, the carrier test should be performed. It has been suggested that carrier tests which simulate practical conditions are required to evaluate disinfectants (Rabenau et al, 2014; Springthorpe and Sattar, 2005). Otherwise, the proper decisions concerning the effectiveness and usability of disinfectants toward pathogens deposited on fomites may not be made.

In Chapter 1, dropping and wiping techniques were also compared to evaluate the efficacy of virucidal activities of disinfectants. To inactivate the virus on the carrier with the dropping method, more time or higher concentration than with the wiping method was required. The hypothesis is that during wiping

the virus particles were removed from the carrier into the disinfectant solution in the rayon-polyester sheets, and that this wiping step was essential for inactivation, because the wiping allowed virus particles on the carrier moving to the aqueous phase (Fig. 1.2).

Even under organic contamination, namely with 5% FBS, the 0.17% $FdCa(OH)_2$ solution was capable of inactivating the virus in the aqueous phase within 30 sec. This fact means that it is safe to reuse the towel wiped on a surface and rinse it with a bucket of 0.17% $FdCa(OH)_2$ solution, in order to wipe a new area. NaClO-1000, NaClO-500 and QAC-500 could also inactivate IBV inoculum containing 5% FBS, but it is widely known that NaClO and QAC lose their microbicidal activities in the presence of organic materials (Bloomfield et al, 1991; Gerba, 2015).

At the level of field usage, one wipe (towel or sheet) should be used for a wide surface, while the accumulation of organic materials in the wipe must be considered. Notably, the 0.17% $FdCa(OH)_2$ solution showed stable virucidal efficacy for more than 3 months after preparation.

1.5. Tables and figures

Table 1.1. Virucidal efficacy of the tested solutions toward infectious bronchitis virus (IBV) in aqueous phase containing 5% fetal bovine serum (FBS).

Tested solutions	Viral titer [$\log_{10}$ (PFU/mL)] at different contact times		
	IBV control	0 sec	30 sec
$FdCa(OH)_2$[a]	6.01 ± 0.03	6.01 ± 0.03	< 1.40 ± 0.00
NaClO-1000[b]		5.42 ± 0.03	< 1.40 ± 0.00
NaClO-500[c]		5.39 ± 0.03	< 1.40 ± 0.00
Mixed-500[d]		5.47 ± 0.07	< 1.40 ± 0.00
QAC-500[e]		5.93 ± 0.02	< 1.40 ± 0.00

[a] Food additive grade calcium hydroxide at 0.17% solution. [b] Sodium hypochlorite solution at 1,000 ppm of total chlorine. [c] Sodium hypochlorite solution at 500 ppm of total chlorine. [d] Sodium hypochlorite at 500 ppm of total chlorine in 0.17% $FdCa(OH)_2$. [e] Quaternary ammonium compound diluted 500-fold in redistilled water. Figures are shown as mean ± standard error (SE) from replicated 3 times. Viral titer < 1.40 $\log_{10}$ plaque forming units (PFU)/mL indicates the virus was inactivated to undetectable level.

Table 1.2. Virucidal efficacies of the tested solutions toward infectious bronchitis virus (IBV) containing 0.5% fetal bovine serum (FBS) on a plastic carrier with the dropping and the wiping techniques.

Tested solutions	Viral titer [$\log_{10}$ (PFU/mL)] on the carrier or in the sheet			
	IBV control	Dropping-carrier	Wiping-sheet	Wiping-carrier
$FdCa(OH)_2$[a]		3.03 ± 0.04*	< 1.80 ± 0.00	< 1.80 ± 0.00
NaClO-1000[b]		< 1.80 ± 0.00	< 1.80 ± 0.00	< 1.80 ± 0.00
NaClO-500[c]	6.47 ± 0.04	4.78 ± 0.05	2.52 ± 0.15*	< 1.80 ± 0.00
Mixed-500[d]		2.08 ± 0.23*	< 1.80 ± 0.00	< 1.80 ± 0.00
QAC-500[e]		< 1.80 ± 0.00	< 1.80 ± 0.00	< 1.80 ± 0.00
dW_2		6.00 ± 0.04	5.45 ± 0.05	2.88 ± 0.22*

[a] Food additive grade calcium hydroxide at 0.17% solution. [b] Sodium hypochlorite solution at 1,000 ppm of total chlorine. [c] Sodium hypochlorite solution at 500 ppm of total chlorine. [d] Sodium hypochlorite at 500 ppm of total chlorine in 0.17% $FdCa(OH)_2$. [e] Quaternary ammonium compound diluted 500-fold in redistilled water. Figures are shown as mean ± standard error (SE) from replicated 3 times. * Single asterisk indicates effective viral reduction (≥ 3 $\log_{10}$ plaque forming units (PFU) /mL). Viral titer < 1.80 $\log_{10}$ PFU/mL indicates the virus was inactivated to undetectable level.

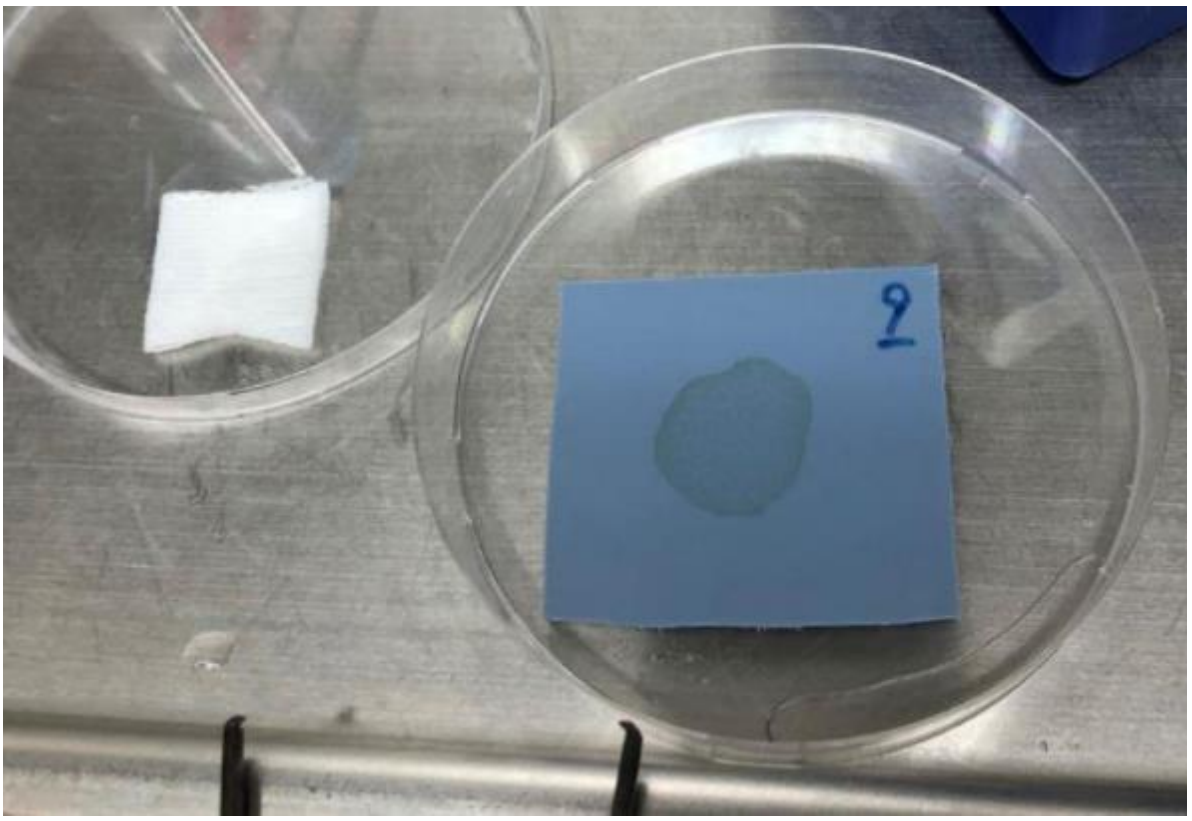


Fig.1.1. Deposition of infectious bronchitis virus (IBV) for the carrier tests.

One hundred microliter of IBV containing 0.5% fetal bovine serum (FBS) was spotted on a plastic carrier coupon (around 5.0 cm x 5.0 cm) and subsequently spread by sterile glass spreader onto the carriers and air dried for 60 min inside the biological safety cabinet at room temperature (RT). A rayon polyester sheet folded into four pieces (2.5 cm x 2.5 cm) is shown in the left.

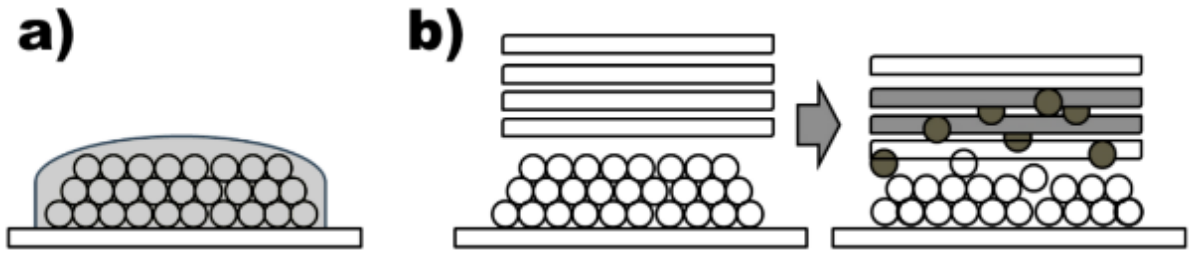


Fig. 1.2. Schematic presentation of the hypothesis relating to the virucidal mechanism difference between dropping and wiping techniques.

a) Dropping method. The disinfectant quantified at 500 μL was dropped on the carrier to cover the spotted virus inoculum (infectious bronchitis virus: IBV). After 1 min incubation, the carrier was transferred to the stomacher bag containing the blocking solution.

b) Wiping method. The carrier spotted with IBV was wiped during 30 sec by means of a rayon-polyester sheet folded into four pieces (2.5 cm x 2.5 cm) containing 500 μL of each disinfectant, and the sheet was transferred into a stomacher bag containing 2.0 mL of blocking solution. The wiped plastic carrier then immediately put into a stomacher bag containing 2.5 mL of the blocking solution.

Chapter 2.

Evaluation of virucidal activity of slightly acidic hypochlorous acid water toward influenza virus and coronavirus with the tests simulating practical usage

2.1. Introduction

In Chapter 1, convenient and accurate carrier tests to evaluate the virucidal activities of disinfectants toward viruses were established and utilized. Moreover, the difference of efficacies of disinfectants between the suspension test and the carrier test was shown. In Chapter 2, the virucidal activity of slightly acidic hypochlorous acid water (SAHW) toward viruses were evaluated simulating practical usage in livestock farms.

SAHW is a type of hypochlorous acid (HOCl) solution and a powerful oxidizing agent, which contains free available chlorine (FAC). The concentration of FAC in the solution is shown as ppm. SAHW solution, made from NaClO and purified water using ion-exchange resin, contains a large amount of FAC, but it does not cause metallic corrosion, because of the absence of sodium. HOCl solutions, including SAHW, show the virucidal activity toward some viruses in the aqueous phase: SARS-CoV-2 (Takeda et al, 2020), duck hepatitis B virus (Tagawa et al, 2000), FMDV (Bui et al, 2017), avian influenza virus (AIV, H5N1 and H9N2) (Tamaki et al, 2014), porcine reproductive and respiratory syndrome virus and pseudorabies virus (Hao et al, 2013b). On the other hand, its effect on viruses with tests simulating practical usage, such as a virus-carrier test, has been rarely reported. Chapter 1 has shown the difference of efficacies of disinfectants between the suspension tests and the carrier tests. Park et al. (2007) showed that fog-based of HOCl solution had virucidal activity against human norovirus on non-porous surfaces (as a carrier) (Park et al, 2007). Recently, it was demonstrated that sprayed SAHW have the inactivation effects on AIV and bacteria (*Salmonella* Infantis and *Escherichia* coli) on surfaces (Hakim et al, 2015a, 2016).

Dispersing aerosols through sneezing and coughing is one of the multiple routes of human-to-human transmissions for airborne infectious viruses, such as SARS-CoV-2 and influenza viruses (CDC, 2020; Cowling et al, 2013). To prevent human-to-human transmissions through aerosols, the inactivation of viruses in the air by sterilizing/disinfecting agents seems to be an effective way. Although there are no established standardized methods for assessing the inactivation of aerosolized microorganisms by

sterilizing agents, HOCl solutions were used for experimental aerosol disinfection in some studies (Hao et al, 2013a; Kurahashi et al, 2021; Zhao et al, 2014). These studies maintained that HOCl solutions may be a way to deal with infection via spatial spraying for a long period of time (more than 20 min) in a space where humans or animals are present. Alongside, though, it has also been concluded that peak exhalation speeds of droplets from a human sneeze can reach up to 10 to 30 m per second, creating a cloud that can span approximately 7 to 8 m (Bourouiba, 2020). The data in this research are of great value to prove that aerosols can move in the air at high speed and shortly reach humans or animals. Therefore, it would be essential to evaluate the virucidal activity of sterilizing agents toward aerosolized microorganisms for an abrupt duration. A previous report evaluated the virucidal activity of SAHWs toward aerosolized Newcastle disease (ND) live vaccine (B1 strain) for a few seconds, using three-day-old conventional chicks *in vivo* experiments (Hakim et al, 2015b). Thereupon no clinical signs were observed, and no virus was isolated from the group of chicks exposed to SAHW containing FAC at the rate of 100 ppm. On the other hand, little is known about the virucidal activity of SAHWs toward aerosolized viruses for a short time through a quantitative evaluation test.

In Chapter 2, the virucidal activity of SAHW toward AIV (H7N1) and IBV on surfaces were evaluated through the established carrier tests in Chapter 1 and further developed carrier tests. Additionally, the virucidal activities of sprayed SAHWs during a few seconds were evaluated toward aerosolized IBV in simulated human-dispersed droplets in reference to the field usage of SAHW. These results suggest that SAHW, through the wiping technique, could remove/inactivate viruses on contaminated carriers. In contrast, viruses remained on the wiping rayon sheets. Besides, the virucidal activity was observed via the direct spray of SAHW, indicating that a small volume of SAHW in spray form could kill the viruses on the rayon sheets to undetectable level. Moreover, a method was provided to measure the inactivation of airborne viruses and prove that SAHW is capable of inactivating viruses in the air within a few seconds. These results strongly support the field usage of SAHW for daily disinfecting in the application area.

2.2. Materials and Methods

2.2.1. Test viruses

For the suspension and carrier tests, a low pathogenic AIV, A/duck/Aomori/395/04 (H7N1), isolated from wild ducks, was prepared in CK cells. IBV strain M41, kindly provided by National Institute of Animal Health, was also prepared in CK cells as described in Chapter 1. These virus cultures were centrifuged at 1,750 x g for 15 min, taken supernatants, aliquoted and then kept at −80 °C until use. The virus stock titers of AIV and IBV were around 10^7 fifty percent tissue culture infectious dose ($TCID_{50}$)/mL and around 10^7 PFU/mL, respectively. For the aerosol disinfection test, the virus suspension was prepared in phosphate-buffered saline (PBS, pH 7.4) supplemented with 0.5% FBS, in order to simulate human droplets, as previously described (Sattar et al, 1989).

2.2.2. Hypochlorous acid water and blocking solution

For the suspension and carrier tests, SAHW containing different concentrations of FAC, namely 62, 119, 220, 300 and 540 ppm (SAHW-62, -119, -220, -300 and -540, respectively) were kindly supplied by Nanoscale Co, Ltd. (Tokyo, Japan), kept sheltered from sunlight at RT, and used within 3 months after preparation. For the aerosol disinfection test, SAHWs containing FAC at the rates of 100, 200, 300, were used. A blocking solution was prepared for stopping inactivation reactions, as used in Chapter 1 and previously described (Alam et al, 2018a; Ito et al, 2018; Komura et al, 2019).

2.2.3. Experimental design

2.2.3.1. Evaluating the virucidal activity of SAHW toward AIV and IBV with the suspension test

To fifty microliters of each viral solution containing 0.5 % or 5% FBS, 450 µL of SAHW-62 was added to give 500 µL of virus-SAHW mixture, and incubated for 30 sec at RT. Then, 500 µL of the blocking solution was immediately added to each mixture to stop virus inactivation. In parallel, 450 µL of SAHW-62 was mixed with 500 µL of the blocking solution in a microtube and then was added 50 µL

of each viral solution containing 0.5 % or 5% FBS to the microtube, in order to evaluate the blocking solution's effect (shown as 0-sec treatment and contact time). For field application, incubation period of 30 sec was selected as described previously, and the suspension test does not simulate the practical use; only the lowest concentration, at 62 ppm, was used.

2.2.3.2. Carrier tests for evaluating the virucidal activity of SAHW toward AIV and IBV on porous and non-porous carriers by means of the dropping technique

Two types of carriers were chosen: plastic carrier coupons (around 5.0 cm × 5.0 cm), as the non-porous substrate, and rayon-polyester sheets folded into four pieces (2.5 cm × 2.5 cm), as the porous substrate. For the non-porous carrier test, 100 μL of viral solutions containing 0.5 % FBS were pipetted on the plastic carrier coupons, and subsequently distributed with sterile glass spreader onto the plastic carriers. The inoculum on plastic carrier was dried for 60 min inside the biological safety cabinet at RT. For the porous carrier test, ten 10-μL of viral solutions containing 0.5 % FBS were spotted onto the rayon sheets in order to deliver viruses onto a wide area of the sheets. These contaminated carriers were tested for the virucidal activity of SAHW. Thereby, 500 μL of each concentration of SAHW was dropped on each carrier to cover all spotted area and incubated for 1 min at RT. Then, the plastic carriers and the rayon sheets were placed into stomacher bags containing 2 mL of the blocking solution, respectively. Each plastic carrier surface was rubbed vigorously by hand over the bag to dislodge the remaining viruses from the plastic carriers into the blocking solution. Rayon sheets were treated with a BagMixer as described above in Chapter 1.

2.2.3.3. Carrier test for evaluating the virucidal activity of SAHW toward AIV and IBV on plastic carrier surface by means of the wiping technique

The carrier test's performance is described in Chapter 1 for measuring the virucidal efficacies of disinfectants toward IBV, using the wiping technique. Briefly, 100 μL of viral solutions containing 0.5 %

FBS were pipetted on plastic carriers and dried, as described above. Then the carriers were wiped with rayon-polyester sheets folded into four pieces (2.5cm × 2.5cm) containing 500 μL of each concentration of SAHW for 30 sec at RT. Immediately after wiping, these sheets and wiped plastic carriers were transferred into the stomacher bags containing 2.0 mL and 2.5 mL of the blocking solution, respectively. Each carrier surface was rubbed by hand, and the wiping rayon sheets were treated with a BagMixer as described above, respectively, to remove the remaining viruses from the wiped plastic carriers and the wiping rayon sheets into fluids.

2.2.3.4. Evaluating the virucidal activity of SAHW toward AIV and IBV on rayon sheets by means of the direct spray test

For the direct spray test, an aerosol sprayer that has ability to release water at 60 mL/hour with small aerosol particles (average size 12 μm in diameter) was kindly prepared by Nanoscale Co, Ltd. Plastic boxes measuring W440 x D740 x H230 mm were purchased from a local market. In the initial set of experiments, the volume of sprayed SAHW was measured onto a rayon sheet at a distance of 30 cm between the spray nozzle and the rayon sheet for 10 min and 60 min at RT in triplicate. In the 60 min spray, because the sheets and glass plates got much wet with around 200 μL of SAHW per glass plate (5cm × 5cm), the spraying periods of 10 and 20 min were determined for the field usage.

In the direct spray test, in order to deliver viruses onto a wide area of the rayon sheets, ten 1-μL of viral solutions containing 0.5 % FBS were spotted onto rayon sheets folded into four pieces (2.5 cm × 2.5 cm) placed onto a 5 × 5 cm glass plate inside a 90-mm petri dish without a lid and transferred into the plastic box at a distance of 30 cm between the spray nozzle and the rayon sheets. The inoculated ten 1-μL solutions of the viruses on the rayon sheets were used to simulate droplets (Lin and Marr, 2020; Yang et al, 2012), rather than smaller aerosols (Duguid, 1946). Then, SAHW was sprayed by the aerosol sprayer for 10 min or 20 min at RT. The respective rayon sheet with the glass plate was transferred into the stomacher bag containing 1 mL of blocking solution in order to stop virus inactivation, and then,

after removal of the glass plate, treated with a BagMixer, as described above, to remove the viruses from the rayon sheets into the blocking solutions.

2.2.3.5. Evaluating the virucidal activity of SAHW toward aerosolized IBV for a few seconds

2.2.3.5.1. Experimental set up of a chamber

A virus in simulated human-dispersed droplets was aerosolized and exposed to SAHW in a closed chamber. Fig. 2.1A depicts a scheme of the experimental chamber used in this study to evaluate the virucidal activity of SAHWs toward aerosolized IBV in simulated human droplets for a few seconds. As a closed chamber, a plastic box as used in the direct spray tests was placed in a biosafety level II cabinet to ensure the safety of the operator in case of leakage. The temperature in the biosafety level II cabinet was kept at RT. The closed chamber consisted of three areas, intended for: (1) aerosolization of IBV, (2) nebulization of SAHWs, and (3) aerosol sampling. The layout in the chamber and each distance from sampling area is presented in Fig. 2.1B. The first area included a sprayer, Aerial Mist, kindly prepared by Aelph Co, Ltd (Tokyo, Japan) and designed at the liquid aerosolization of 40 mL/hour with small aerosol particles (average size 26 μm in diameter; range 11 to 42 μm). The sprayer was connected to the chamber at a distance of 53 cm from an air sampler. The virus suspension containing 0.5% FBS was aerosolized by the sprayer, and the aerosol was let into the chamber. The second area contained two nebulizers (Omron Corp, Kyoto, Japan) that can release water at the liquid aerosolization form of 0.3 mL/min, each with small aerosol particles (size 5 μm in diameter), to inject SAHW into the chamber. Each nebulizer was placed on both sides faces of the chamber, at a distance of 30 cm from an air sampler, respectively. SAHW was released from each nebulizer and let into the chamber for virus exposure. The third area includes an air sampler consisted of a Rayon-polyester sheet (100 mm x 100 mm), air bypass tubes, a bottle filled with a sterilizing agent and a suction pump (SP40, MARKOS-MEFAR, Bovezzo, Italy) equipped with HEPA filter. As a sterilizing agent, a mixture of QAC diluted 500 times with 0.17% FdCa$(OH)_2$ solution (namely Mix-500) was used, for

preventing contamination by bioaerosols toward a suction pump, which passed air samples; its virucidal effect has been previously described (Ito et al, 2018; Kabir et al, 2021). The bottle filled with the mixture was connected to the suction pump providing an airflow rate of 35 L/min via an air bypass tube, without interrupting the airflow through the chamber.

In order to prove that a sterilizing agent inactivated virus in the air, yet not on the air sampler, it is required to neutralize at once the virucidal activity of the sterilizing agent on the air sampler. In the present study, FBS treatment was performed toward the air sampler to neutralize the virucidal activity of SAHWs before the aerosol experiments. Based on results from the preliminary test (data not shown), the sampler was treated with FBS, described as follows. Firstly, the rayon sheet was soaked in 2 mL of FBS, and sterilized in a microwave oven for 3 min. The rayon sheet coating with FBS was subsequently connected to the bottle filled with the mixture via an air bypass tube without interrupting the sampling airflow by the suction pump, and then placed at the aerosol sampling area in the chamber. Secondly, three mL of FBS was released from the sprayer onto the sampler in the chamber, aspirated, and then captured on the sampler completely by means of sampling airflow for 3 min, which is sufficient to empty the chamber.

2.2.3.5.2. Aerosol disinfection test

Fig. 2.2A shows a scheme of the spraying duration of IBV and SAHW in the aerosol experiments. Two nebulizers were run in parallel at the liquid aerosolization of 0.3 mL/min each for 3 min simultaneously, so as to equilibrate SAHW concentration in the chamber. After equilibration of SAHW, a volume of one mL of virus prepared in PBS supplemented with 0.5% FBS was aerosolized and sprayed completely for 3.5 min from the sprayer into the chamber. At the same time, the suction pump was run for sampling airflow to collect the aerosolized virus on the air sampler. Concurrently with the virus aerosolization for 3.5 min, SAHW was also released from the two nebulizers, aiming to be in contact with the aerosolized virus across airflow in the chamber. After complete viral aerosolization, SHAW

was sprayed for 3 min, thus having contact with the virus-containing air in the chamber. The remained virus was thereby exposed to SAHW, and then captured on the sampler by means of sampling airflow. The bioaerosols that passed the sampler were collected in the bottle filled with the sterilizing mixture via air bypass tube in order to disinfect the virus, and the sterilized air was exhausted through HEPA filter to avoid any contamination of general space in the laboratory room.

After incubation, each rayon sheet was transferred into 2.0 mL of blocking solution in a stomacher bag. The captured viruses on the sheets were extracted, using a BagMixer for 1 min, and the supernatant was decanted into a clean 1.5 mL microtube for infectivity assay.

2.2.4. Controls in the experiments

In each test, two controls were prepared during this study. As a first control, dW_2 was used instead of SAHWs in the test (dW_2 control). All the settings were the same as with the test, except for dW_2. A second control was prepared to measure the viral titer of the viruses in each experiment. The virus suspension with 0.5 or 5 % FBS was kept at RT in parallel along each experiment, and the concentrations of the viruses were measured (viral control).

In the aerosol disinfection test, a third control was also prepared to verify whether SAHWs were neutralized immediately on the air sampler treated with FBS, while a scheme of the spraying duration of IBV and of SAHW for the control experiments is presented in Fig. 2.2B, as follows. Briefly, experimental chamber setting was performed as described above. Then, SAHW was injected into the chamber by each nebulizer for 9.5 min, captured on the sampler treated with FBS through sampling airflow for 3 min, which is sufficient to empty the chamber. Following this, one mL of virus suspension prepared in PBS containing 0.5% FBS was sprayed by the sprayer and captured on the sampler through sampling airflow for 3.5 min. After complete viral aerosolization, additional sampling for 3 min was conducted to collect the virus remaining in the air within the chamber. This third control served as “the samples pre-exposed to SAHWs”. Virus extraction from the sampler and treatment of bioaerosol which

passed the sampler were performed as well, as described above.

2.2.5. Infectivity assays

All experiments were performed in triplicate. Each treated sample was serially 10-fold diluted with the maintenance medium (MM) as previously used (Komura et al, 2019; Takehara et al, 1991). Controls were also diluted with MM. For AIV, the dilution was inoculated onto Madin-Darby Canine Kidney (MDCK) cells in MM containing a final concentration of 2 μg/mL of bovine pancreatic trypsin (Sigma, St. Louis, MO, USA) in a 96-well cell-culture plate and incubated at 37 °C in a 5% CO2 incubator. At three dpi, cytopathic effect (CPE) was observed, and the hemagglutinin activity of the inoculated supernatant was assayed using 0.5 % chicken red blood cells. Finally, virus titration was performed by $TCID_{50}$, based on assay by Spearman-Käerber method (Villegas, 2008). For IBV, the treated viruses were inoculated and titrated on CK cells, as previously described (Takehara et al, 1991). These viral titers are reported as $\log_{10}TCID_{50}$/mL or $\log_{10}$PFU/mL and shown as mean ± SE in triplicates.

In the suspension and carrier tests, when the treated viral titer was reduced by more than 1000 times compared with the control virus, the virucidal activity was regarded as effective, as shown by previous studies (OECD, 2013; Springthorpe et al, 1986, US EPA, 2018). In the aerosol disinfection test using IBV, to investigate the virucidal activity toward the aerosolized virus in the air, yet not on the sampler, reduction factors (RF) derived from the difference between the viral titer of pre-exposed and exposed to SAHWs were calculated, and presented as $\log_{10}$ PFU/mL.

2.2.6. Statistical analysis

In the aerosol disinfection test, one-way analysis of variance and post-hoc Tukey-Kramer tests were performed for comparison of samples (the controls and the samples exposed to SAHWs) to each other. P values < 0.05 were considered to be statistically significant.

2.3. Results

2.3.1. The virucidal activity of SAHW-62 toward AIV and IBV in the suspension test

Table 2.1 shows the virucidal activity of SAHW-62 toward AIV and IBV in the suspension test. At 0 sec, when the blocking solution was mixed with SAHW-62 before adding the viruses, the viral titer was similar to the controls. Therefore, virus inactivation by SAHW was immediately stopped with the blocking solution. SAHW-62 showed the virucidal activity toward both AIV and IBV in the presence of 0.5 % FBS since the infectious viral titers were reduced to the effective level within 30 sec. In the presence of 5% FBS, SAHW-62 inactivated IBV at a rate of more than 1,000 times reduction within 30 sec, whereas SAHW-62 could not inactivate AIV to the effective level within 30 sec.

2.3.2. The virucidal activity of SAHW toward AIV and IBV on carriers with the dropping technique

Table 2.2 shows the virucidal activity of SAHW toward AIV and IBV containing 0.5 % FBS on the inoculated carriers with the dropping technique. The recovery ratio of the viruses with dW_2 from plastic carrier surface after drying for 1 hour was measured: 4% for AIV and 30 % for IBV, respectively. The dropped SAHW-62 achieved a three $\log_{10}$ reduction of AIV containing 0.5 % FBS on the plastic carrier within 1 min. In contrast, IBV on the plastic carrier was not inactivated to the effective level by dropped even with SAHW-220, within 1 min. With the dropping technique on the porous carrier, SAHW-119 for AIV and SAHW-220 for IBV could not reduce the viral titers on the rayon sheets to the effective level within 1 min.

2.3.3. The virucidal activity of SAHW toward AIV and IBV on plastic carriers with the wiping technique

Table 2.3 shows the virucidal activity of SAHW toward AIV and IBV on the inoculated plastic carriers with the wiping technique. SAHW-220 showed highly virucidal activity toward AIV and IBV containing 0.5 % FBS on the plastic carriers, as the titers of the infectious viruses on the wiped carriers decreased to the undetectable levels (AIV: < 1.90 $\log_{10}TCID_{50}$/mL, IBV: < 2.10 $\log_{10}PFU$/mL). SAHW-220 could

inactivate AIV in the wiping rayon sheet at a rate of more than 10,000 times reduction within 30 sec, but IBV titer in the wiping rayon sheet was not inactivated to the effective level. With dW_2 wiping, the virus titers on the wiped carriers were reduced by more than 99.9 %. However, many infectious viruses were transferred from the carriers to the wiping rayon sheets and remained viable with dW_2 (AIV; 5.08 $\log_{10}TCID_{50}$/mL, IBV; 5.61 $\log_{10}PFU$/mL).

2.3.4. The virucidal activity of SAHW toward AIV and IBV on rayon sheets with the direct spray test

Table 2.4 shows the result of the direct spray test. In the initial set of experiments, when the volume of sprayed SAHW was measured on a rayon sheet on a glass plate inside a Petri dish without a lid placed at a distance of 30 cm from the spray nozzle in triplicate, around 5 μL of SAHW was obtained on the rayon sheet on an average, for an exposure time of 10 min. When ten 1-μL of viral solutions were inoculated on the rayon sheets and then sprayed at a distance of 30 cm from the spray nozzle, AIV was inactivated by SAHW-300 to below the detection limit (< 1.50 $\log_{10}TCID_{50}$/mL) after 10 min. SAHW-540 could reduce IBV by more than 99.9 % within 10 min and inactivate to below the detection limit (< 2.50 $\log_{10}PFU$/mL) within 20 min, but SAHW-300 could not.

2.3.5. The virucidal activity of SAHW toward IBV in the air

The virucidal activities of SAHWs at various concentrations of FAC (100 to 500 ppm) against aerosolized IBV in simulated human droplets are presented in Table 2.5 and Fig. 2.3. The aerosolized IBV was exposed to each SAHW for a few seconds in the closed chamber. The dW_2 control was used under the same condition as SAHWs. The mean viral titers and SE for aerosolized IBV picked up by the air sampler were the following: 3.33 ± 0.11, 3.02 ± 0.35, 2.87 ± 0.13 and 1.97 ± 0.26 ($\log_{10}$ PFU/mL), when exposed to SAHW-100, -200, -300 and -500, respectively. The variations were not significantly different in viral titer exposed to SAHW-100 and -200 compared to controls (samples pre-exposed to SAHWs and dW_2 control), whereas there were statistically significant differences in relation to the

samples exposed to SAHW-300 and -500, as compared to the controls ($P < 0.05$).

Pre-exposed controls represent the effects of each SAHW toward virus captured within the sampler treated with FBS, so as to apply a correction for the effects of SAHW toward virus in the air. The mean titers and SE for aerosolized IBV in the samples pre-exposed to SAHWs were the following: 3.54 ± 0.23, 3.82 ± 0.10, 4.03 ± 0.27 and 3.64 ± 0.27, when utilizing SAHW-100, -200, -300 and -500, respectively. The viral titers of samples pre-exposed to SAHWs were close to those of dW_2 control; statistically, the difference in the titer among these controls was not significant ($P > 0.05$).

The log RFs for aerosolized IBV between the titers of exposed and pre-exposed to SAHWs were the following: 0.21, 0.80, 1.16 and 1.67, when utilizing SAHW-100, -200, -300 and -500, respectively.

2.4. Discussion

For the evaluation of disinfectants within the context of practical use, tests simulating practical use provide rather precise information (Becker et al, 2019), which is helpful when a disinfectant is chosen for the field application. It has been reported that the capacity of chlorine-based disinfectants was interfered with by organic materials such as FBS (Takeda et al, 2020). Thus, in the present study, FBS (as bovine mucin in a previous study) was selected, so as to perform the experiments under practice conditions, and added FBS to the viruses. In the suspension test, 0.5 % or 5% FBS was added to the viral solutions as the representative of normal human secretions, namely, 5 mg/mL bovine mucin (Sattar et al, 1989), and 5% as the imitation of field organic contamination (Alam et al, 2018b; Kunanusont et al, 2020). FBS at 0.5 % was used to imitate mucin as the level of contamination in carrier and direct spray tests. In the previous study, HOCl solutions (50, 100, 200 ppm) lost their efficacy toward AIV containing 5% FBS in the aqueous phase (Hakim et al, 2015a). Similar results were obtained in a suspension test (Table 2.1). SAHW-62 appreciably lost sufficient virucidal activity when mixed with AIV in 5% FBS, yet inactivated IBV even in the presence of 5% FBS.

In the carrier test, the stability of the two test viruses was examined after drying process and the

dropping technique. Hirose et al. (2020) found that the survival time of viruses on human skin was significantly longer for SARS-CoV-2 - a coronavirus family member, as IBV - than for influenza A virus (Hirose et al, 2021). The result showed the recovery ratio of IBV was higher than that of AIV. Therefore, IBV might be more stable during the drying process than AIV. Although the drying process reduced the AIV titer, the virucidal activity of SAHW toward AIV could be measured as a three $\log_{10}$ reduction being the effective level. In Chapter 1, it has been shown that the virucidal activity of disinfectants toward IBV was influenced by the system applied, either in liquid or on the surface. This study showed also that IBV was reduced by more than three $\log_{10}$PFU/mL with SAHW-62 within 30 sec in the aqueous phase, whereas dropped SAHW-220 could not reduce IBV on the carriers to the effective level within 1 min, thus indicating that IBV on abiotic carrier is more resistant to SAHW than the contamination in liquid.

It was previously demonstrated that viable viruses on non-porous surfaces could remain infectious for significantly longer periods than those on porous surfaces (Riddell et al, 2020; Thompson and Bennett, 2017). In the results of the dropping technique on two types of carriers (a plastic carrier coupon and a rayon sheet), IBV on each carrier was not reduced to the effective level with SAHW even at 300 ppm, whereas SAHW reduced AIV on the plastic carrier to the sufficient level but not on the rayon sheet. These data indicate that porous carrier reduces the virucidal activity of SAHW toward AIV compared with non-porous carrier despite the weakened stability of virus on the porous carrier. With the wiping technique, dW_2 could reduce both AIV and IBV from the contaminated plastic carriers by more than 99.9 %, but viruses remained in high titer in the wiping rayon sheets. Thus, mechanical action could remove the viruses from plastic carriers as shown in Chapter 1. Although SAHW could inactivate the viruses on the wiped carriers to undetectable level with SAHW-220 for IBV and SAHW-119 for AIV, IBV remained in the wiping rayon sheets with SAHW-220 in high titer. These findings suggest that there is the potential risk of virus spreading to other surfaces while using the wiped rayon sheet with SAHW. To investigate this risk, further study is needed.

For the direct spray tests, in the preliminary test, ten spots of 10 μL of viruses (total 100 μL of AIV and IBV) containing 0.5 % FBS on the rayon sheets were not inactivated to the effective level with sprayed SAHW-300 within 10 min at a distance of 60 cm between the spray nozzle and the inoculated rayon sheets. AIV on the rayon sheet was inactivated to undetectable level (< 1.50 $\log_{10}TCID_{50}$/mL) by SAHW-300 sprayed for 60 min (data not shown). However, spraying SAHW for 60 min is not recommended for daily cleaning, since the floor gets pretty wet. In the next preliminary test, considering the pipetted viral concentration and the interference effect of organic materials, ten 10-μL of IBV ten times diluted with PBS were inoculated onto a rayon sheet and then sprayed with SAHW-540 for 10 min at a distance of 60 cm between the spray nozzle and IBV on the rayon sheet. As a result, the IBV titer after spraying SAHW-540 decreased within 10 min by 0.8 $\log_{10}$ PFU/mL, as compared to spraying dW_2 for 10 min (data not shown). Thus, it was supposed that the effect of SAHW in spray form on viruses is influenced by the inoculated viral volume or the distance from the spray nozzle. This hypothesis was confirmed since AIV with SAHW-300 for 10 min as well as IBV with SAHW-540 for 20 min, were reduced to the detection limit, respectively, when ten 1-μL of the viral solutions on rayon sheets were sprayed with SAHW at a distance of 30 cm. In these reactions, around 5 μL of SAHW was obtained on the rayon sheet on a glass plate inside a Petri dish without a lid placed at a distance of 30 cm from the spray nozzle, for an exposure time of 10 min (10 μL for 20 min). Thus, a small volume of SAHW could inactivate AIV and IBV on the rayon sheets to the detection limit.

Aerosols released through sneezing and coughing move in the air at high speed, and would shortly reach humans or animals (Bourouiba, 2020), whereas it is not clear what the virucidal activity of sterilizing agents is attained toward aerosolized virus during a few seconds – which might be insufficient for virus exposure – through a quantitative evaluation test. To the knowledge, this is the first study evaluating the virucidal activity of a sterilizing agent toward aerosolized viruses containing 0.5% FBS in simulated human droplets for a few seconds. It was obtained that SAHW could cause a reduction of 0.21 $\log_{10}$ PFU/mL and 0.80 $\log_{10}$ PFU/mL due to virus exposures to SAHW-100 and -200, respectively,

compared with that of pre-exposed to SAHW-100 and -200, respectively. Also, exposures to SAHW-300 and -500 inactivated the aerosolized virus by 1.16 $\log_{10}$ PFU/mL and 1.67 $\log_{10}$ PFU/mL, respectively. This study provides new information on utilizing SAHWs in a space where humans are present on practical usage, in order to prevent human-to-human transmissions by aerosols.

Besides studies using HOCl products, recent investigations evaluated the virucidal activity of UV light, and of ozone gas toward airborne viruses, for establishing aerosol disinfection strategies (Dubuis et al, 2021, 2020; Walker and Ko, 2007). Users need to understand that each aerosol disinfection strategy has advantages and disadvantages. For example, UV light and ozone gas are hazardous for humans, which make it difficult to utilize those measures for practical usage in a space where humans are present. HOCl products, including SAHWs, revert to water after used (Adam et al, 2002), which minimizes the damage to human health and causes no environmental pollution. SAHW used in the present study – produced by ion-exchange resin using NaClO and purified water – is different from the method of mixing an acid with sodium hypochlorite. Thus, since this solution contains no sodium, it causes no metallic corrosion (Hakim et al, 2016), hence it can be utilized in a space where a metallic substance is present. From these points of view, the practical usage of SAHWs for efficient and safe aerosol disinfection is expected.

In the aerosol disinfection test, FBS was used to simulate human droplets in a real-life scenario (Sattar et al, 1989) and neutralize immediately the effects of SAHWs toward aerosolized viruses on the air sampler. Firstly, the rayon sheet, as the air sampler was soaked in 2 mL of FBS, sterilized in a microwave oven for 3 min, and then placed in the aerosol sampling area. Secondly, three mL of FBS was released from the sprayer to the sampler in the chamber, aspirated, and then completely captured via the sampler, through sampling airflow for 3 min. In the preliminary test, after treatment of the rayon sheet by soaking in 2 mL of FBS and sterilizing in a microwave oven for 3 min, the IBV was aerosolized and exposed to SAHW-500 in the chamber. Accordingly, the IBV titer exposed to SAHW-500 decreased by 2.09 $\log_{10}$ PFU/mL, as compared to that of dW_2, which was close to the results in the present study (a reduction of

2.17 $\log_{10}$ PFU/mL). On the other hand, the mean viral titer and SE in the samples pre-exposed to SAHW-500 were 3.24 ± 0.14 ($\log_{10}$ PFU/mL) in the preliminary test (data not shown), which was lower than the result in the present study (3.64 ± 0.27 $\log_{10}$ PFU/mL). These results demonstrate that the captured IBV was reduced by the effect of SAHW-500 on the sampler in the preliminary test, indicating that the method of treatment with FBS toward the sampler in the preliminary test was not effective in neutralizing the effects of SAHWs immediately on the sampler. Moreover, it was shown that the effects of SAHWs toward aerosolized virus in the air were not interfered by FBS in this study after FBS in the air was aspirated completely through airflow and captured on the sampler. Additionally, in this study, the statistical difference in the titer among controls (the samples pre-exposed to SAHWs and dW_2 control) was not significant ($P > 0.05$); there were statistically significant differences among the samples exposed to SAHW-300 and -500 to the controls ($P < 0.05$). Taken together, it is reasonable to conclude that aerosolized IBV was inactivated by SAHWs in the air, within a few seconds, which was thus found to be sufficient to reach the virus from the sprayer to the sampler.

As an index to verify whether the inactivation of microorganisms by sterilizing agents is to be considered effective, log RF was used for the evaluation test of the agents. The guidelines (Anonymous, 2018; OECD, 2013, US EPA, 2018) specify the effective level at least a 3 or 4 -log RF, based on the results of the surface tests. On the other hand, there are no established standardized methods for assessing the inactivation of aerosolized microorganisms by sterilizing agents, which means that no index can be found to verify whether their inactivation by the aerosol disinfection is to be considered effective. As shown in Table 2.5, the log RF was increased with the increase in the concentration of FAC: more than 1 log reduction of viral titer exposed to SAHW-300, compared with that of pre-exposed to SAHW-300. Although several points bear on the effective level for aerosol disinfection, 1 log reduction of viral titer in the air may be considered to be effective.

Compared with the viral titer pre-exposed to SAHW-100, 38% of the aerosolized IBV was reduced by exposure to SAHW-100 for a few seconds. A previous report showed that the virucidal activity of

SAHWs toward aerosolized ND vaccine strain for a few seconds using three-day-old conventional chicks *in vivo* experiments was evaluated (Hakim et al, 2015b). Briefly, in the aerosol infection experiment, in the group of chicks exposed to 10 doses of aerosolized ND vaccine, two chicks of 5 were infected, whereas, in the group exposed to 25 doses of the vaccine, all 5 chicks were infected. Moreover, in the aerosol disinfection test with SAHWs, no virus was isolated from the SAHW-100 treatment group exposed to the aerosolized ND vaccine at 25 doses. These results show that the aerosolized ND vaccine exposed to SAHW-100 for a few seconds was reduced to less than 10 doses from 25 doses (more than 60% reduction). The reduction ratio with SAHW-100 in the previous study was higher than that in the present study (38%), which is considered to associate with the presence of FBS in this study and innate differences between the tested viruses (ND vaccine and IBV). Admittedly, a 38% reduction of virus in the air due to SAHW-100 exposure seems to be incomplete to prevent transmission by aerosols, nonetheless, the exposure of SAHW-100 would help reduce the aerosol infection risk by appreciably decreasing the number of viral particles and aerial contagion.

2.5. Tables and figures

Table 2.1. Virucidal efficacy of slightly acidic hypochlorous acid water (SAHW) at 62 parts per million (ppm) toward avian influenza virus (AIV) and infectious bronchitis virus (IBV) in aqueous phase containing fetal bovine serum (FBS).

SAHW	Virus	FBS (%)	Viral titer at different contact times		
			Virus control	0 sec	30 sec
62 ppm[a]	AIV	0.5	7.30 ± 0.12 ($log_{10}TCID_{50}$/mL)	7.55 ± 0.24	2.30 ± 0.20*
		5.0		7.47 ± 0.18	> 5.80 ± 0.00
	IBV	0.5	6.63 ± 0.01 (log_{10}PFU/mL)	6.36 ± 0.15	2.52 ± 0.31*
		5.0		6.27 ± 0.12	2.66 ± 0.42*

[a] Slightly acidic hypochlorous acid water (SAHW) at 62 ppm chlorine; figures are shown as mean ± standard error (SE) from replicated 3 times. Viral titer measured for avian influenza virus (AIV) as log_{10} 50% tissue culture infective dose ($TCID_{50}$)/mL, and for infectious bronchitis virus (IBV) as log_{10} plaque forming units (PFU)/mL. * Single asterisk indicates effective viral reduction (≥ 3 log_{10} $TCID_{50}$/mL or PFU/mL).

Table 2.2. Virucidal activity of slightly acidic hypochlorous acid water (SAHW) toward avian influenza virus (AIV) and infectious bronchitis virus (IBV) containing 0.5% fetal bovine serum (FBS) on carriers with the dropping technique.

Tested solutions	Virus	Viral titer on the carrier or in the sheet		
		Control	Dropping-carrier	Dropping-sheet
SAHW-62[a]	AIV	6.31 ± 0.13 ($log_{10}TCID_{50}$/mL)	2.44 ± 0.19*	5.65 ± 0.02
SAHW-119[b]			2.06 ± 0.11*	5.07 ± 0.07
dW_2			4.90 ± 0.19	6.15 ± 0.12
SAHW-62	IBV	6.40 ± 0.04 (log_{10}PFU/mL)	5.51 ± 0.03	6.02 ± 0.05
SAHW-119			5.29 ± 0.11	5.81 ± 0.08
SAHW-220[c]			4.86 ± 0.10	5.76 ± 0.03
dW_2			5.87 ± 0.08	6.20 ± 0.14

[a] Slightly acidic hypochlorous acid water (SAHW) at 62 ppm chlorine. [b] SAHW at 119 ppm chlorine. [c] SAHW at 220 ppm chlorine. Figures are shown as mean ± SE from replicated 3 times. Viral titer measured for AIV as log_{10} $TCID_{50}$/mL, and for IBV as log_{10} PFU/mL. * Single asterisk indicates effective viral reduction (≥ 3 log_{10} $TCID_{50}$/mL).

Table 2.3. Virucidal activity of slightly acidic hypochlorous acid water (SAHW) toward avian influenza virus (AIV) and infectious bronchitis virus (IBV) containing 0.5% fetal bovine serum (FBS) on plastic carriers with the wiping technique.

Tested solutions	Virus	Viral titer on the carrier or in the sheet		
		Virus control	Wiping-sheet	Wiped-carrier
SAHW-62	AIV	6.31 ± 0.13 ($log_{10}TCID_{50}$/mL)	2.82 ± 0.06*	1.98 ± 0.06*
SAHW-119			2.07 ± 0.13*	< 1.90 ± 0.00
SAHW-220			2.23 ± 0.24*	< 1.90 ± 0.00
dW_2			5.09 ± 0.40	2.53 ± 0.2*
SAHW-62	IBV	6.40 ± 0.04 (log_{10}PFU/mL)	4.71 ± 0.40	2.81 ± 0.3*
SAHW-119			4.93 ± 0.10	2.56 ± 0.21*
SAHW-220			3.62 ± 0.13	< 2.10 ± 0.00
dW_2			5.61 ± 0.06	3.38 ± 0.10*

Figures are shown as mean ± SE from replicated 3 times. Viral titer measured for AIV as $log_{10}TCID_{50}$/mL, and for IBV as log_{10} PFU/mL. * Single asterisk indicates effective viral reduction (≥ 3 log_{10} $TCID_{50}$/mL or log_{10}PFU/mL). Viral titer < 1.90 log_{10} $TCID_{50}$/mL or < 2.10 log_{10} PFU/mL indicate the viruses were inactivated to undetectable level.

Table 2.4. Virucidal activity of slightly acidic hypochlorous acid water (SAHW) toward avian influenza virus (AIV) and infectious bronchitis virus (IBV) containing 0.5% fetal bovine serum (FBS) on rayon sheets in the direct spray form.

Tested solutions	Virus	Viral titer on the rayon sheet		
		Control	10 min	20 min
SAHW-300[a]	AIV	6.83 ± 0.18 ($log_{10}TCID_{50}$/mL)	< 2.50 ± 0.00	< 2.50 ± 0.00
SAHW-540[b]			< 2.50 ± 0.00	< 2.50 ± 0.00
dW_2			5.83 ± 0.30	5.83 ± 0.14
SAHW-300	IBV	6.87 ± 0.03 (log_{10}PFU/mL)	4.62 ± 0.11	4.44 ± 0.10
SAHW-540			3.44 ± 0.21*	< 2.70 ± 0.00
dW_2			6.11 ± 0.10	6.00 ± 0.05

[a] Slightly acidic hypochlorous acid water at 300 ppm chlorine. [b] Slightly acidic hypochlorous acid water at 540 ppm chlorine. Figures are shown as mean ± SE from replicated 3 times. Viral titer measured for AIV as $log_{10}TCID_{50}$/mL, and for IBV as log_{10} PFU/mL. * Single asterisk indicates effective viral reduction (≥ 3 log_{10} $TCID_{50}$/mL). Viral titer < 2.50 log_{10} $TCID_{50}$/mL or < 2.70 log_{10} PFU/mL indicate the viruses were inactivated to undetectable level.

Table 2.5. Virucidal activity of slightly acidic hypochlorous acid water (SAHW) toward aerosolized infectious bronchitis virus (IBV) containing 0.5% fetal bovine serum (FBS) for a few seconds.

Tested solutions	Viral titer in the air				
	Virus	Control [d]	Exposed [e]	Pre-exposed [f]	RF (%)[g]
SAHW-100[a]	IBV	5.93 ± 0.16 (log_{10}PFU/mL)	3.33 ± 0.11	3.54 ± 0.23	0.21 (38)
SAHW-200[b]			3.02 ± 0.35	3.82 ± 0.10	0.80 (84)
SAHW-300			2.87 ± 0.13	4.03 ± 0.27	1.16 (93)
SAHW-500[c]			1.97 ± 0.26	3.64 ± 0.27	1.67 (98)
dW_2			4.14 ± 0.11	NT [h]	NC [i]

[a] Slightly acidic hypochlorous acid water (SAHW) at 100 ppm chlorine. [b] SAHW at 200 ppm chlorine. [c] SAHW at 500 ppm chlorine. [d] Viral titer of 1 mL virus sprayed into a chamber in each experiment. [e] Aerosolized IBV was exposed to SAHW (100, 200, 300 and 500 ppm) or dW_2. [f] Each tested solution was sprayed in a chamber and aspirated completely; the same space was then sprayed with IBV. [g] Log reduction factor between the exposed and pre-exposed viral titer. [h] Not tested. [i] Not calculated.

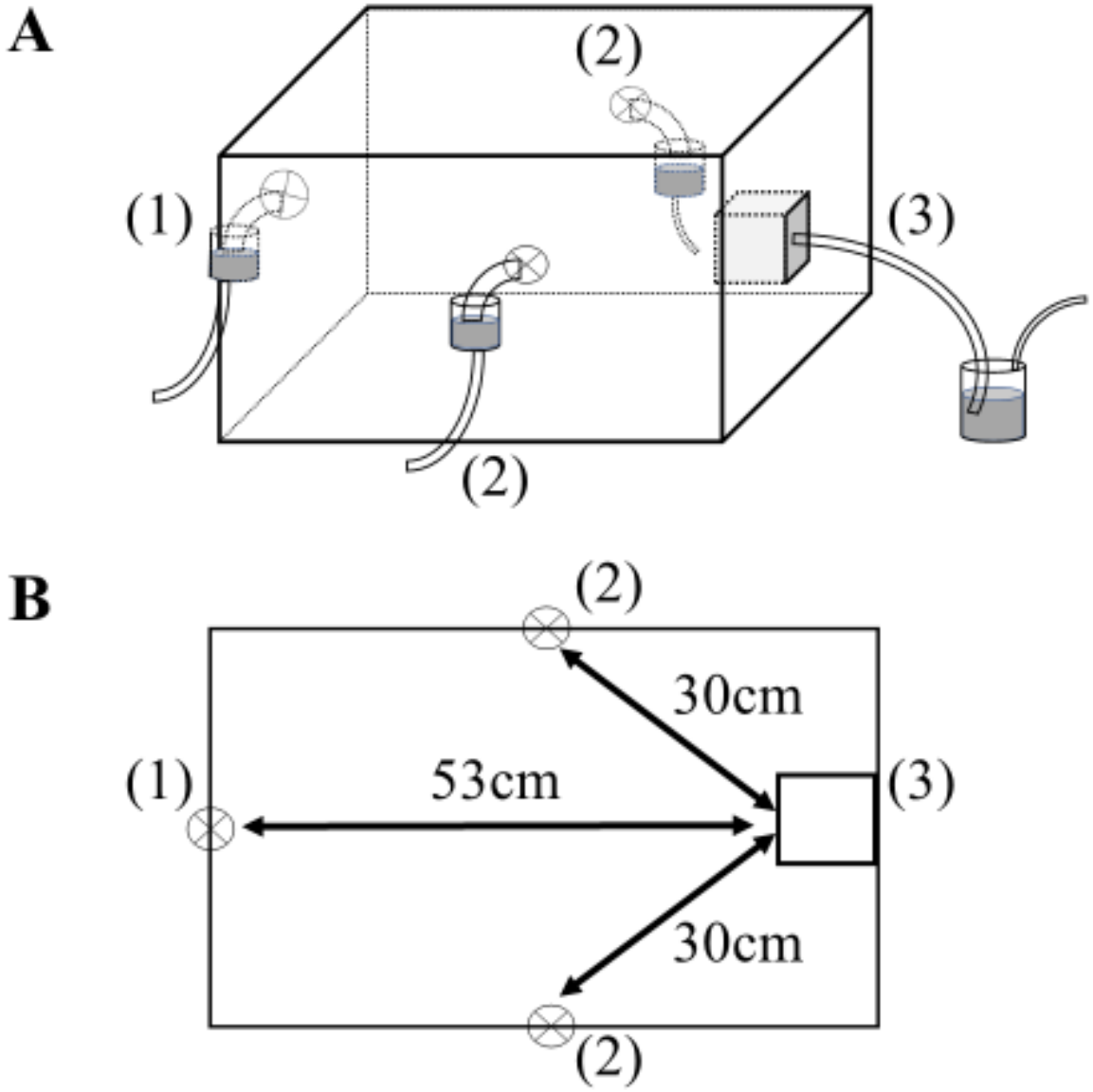


Fig. 2.1. A schematic diagram of the experimental chamber setting, as intended for utilizing three areas: (1) sprayer for infectious bronchitis virus (IBV), (2) nebulizers for slightly acidic hypochlorous acid waters (SAHWs) and (3) air sampler.

(A) The evaluation system of the virucidal activity of SAHWs toward aerosolized virus in simulated human droplets for a few seconds. The virus was injected into the chamber via the sprayer, exposed to SAHWs coming from the nebulizers, and then collected through sampling airflow using the air sampler. (B) The layout in the chamber. The arrow indicates each distance from the air sampler.

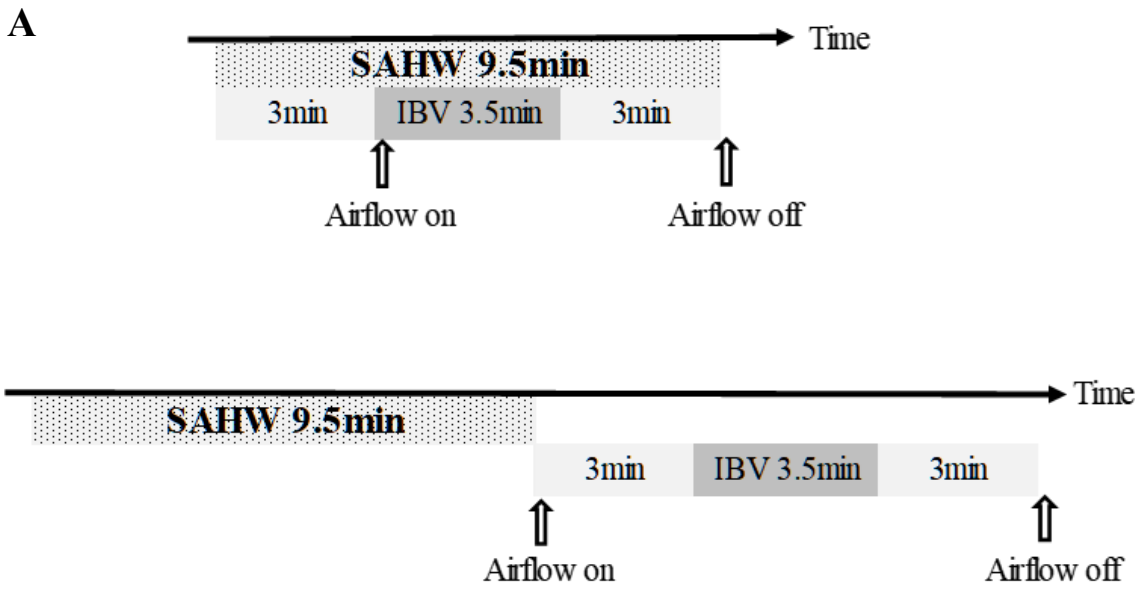


Fig. 2.2. A scheme of the spraying duration of infectious bronchitis virus (IBV) and of slightly acidic hypochlorous acid water (SAHW).

(A) A scheme of the spraying duration of IBV and of SAHW in the aerosol experiments. SAHWs were sprayed for 9.5 min (3 min before and 3 min after IBV spraying) in a chamber. Within 3.5 min, one mL volume of IBV was sprayed and exposed to SAHWs in the same space. (B) A scheme of the spraying duration of IBV and of SAHW in the control experiments. SAHWs were sprayed for 9.5 min (3 min before and 3 min after IBV spraying) in a chamber and aspirated completely; the same space was then sprayed with one mL volume of IBV.

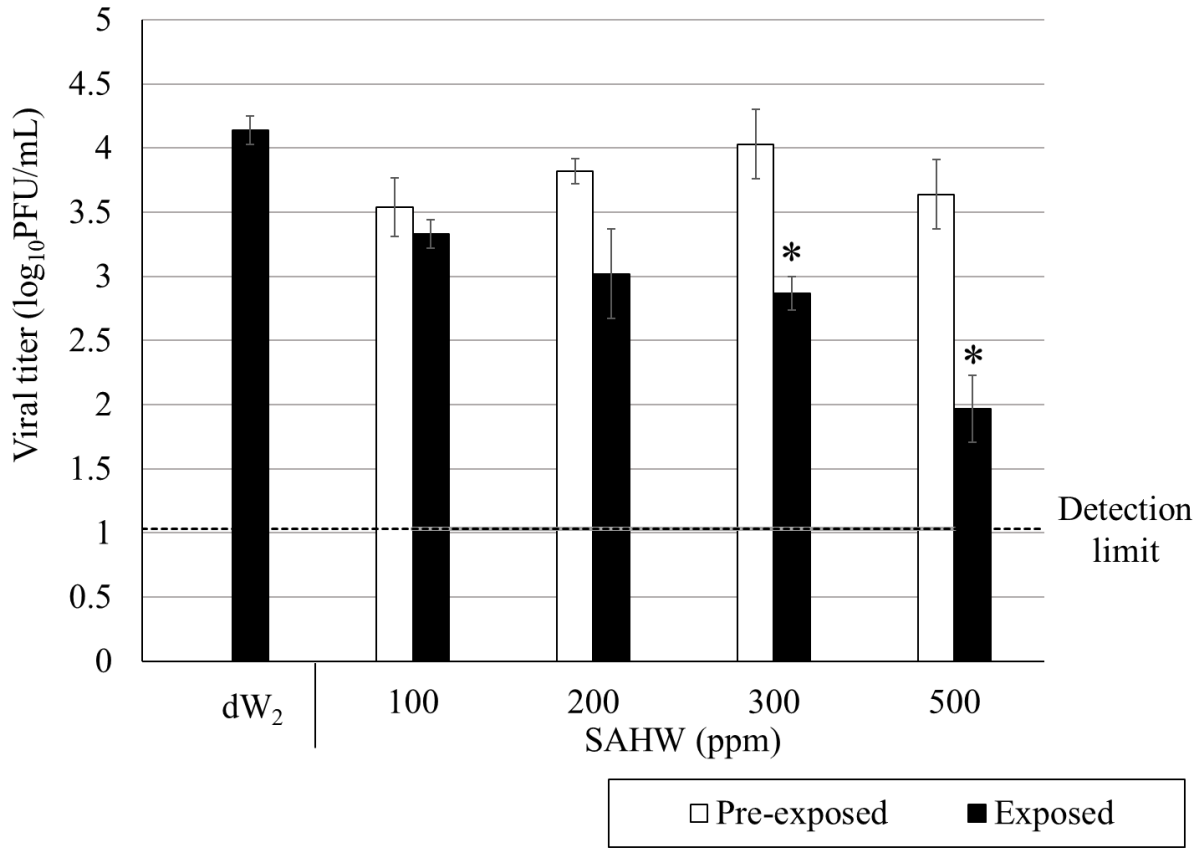


Fig. 2.3. Virucidal activity of slightly acidic hypochlorous acid water (SAHW) toward aerosolized infectious bronchitis virus (IBV) containing 0.5% fetal bovine serum (FBS) for a few seconds.

* Single asterisk indicates that values are significantly different between respective pre-exposed and exposed viral titers. Dot line indicates detection limit (1.031 log_{10} PFU/mL) in the present study.

Chapter 3.
Isolation, molecular characterization, and disinfectants susceptibility of porcine-derived mammalian orthoreoviruses and sapelovirus in Japan in 2020-2022

3.1. Introduction

Biosecurity enhancement on livestock farms is one of the strategies to reduce the occurrence of pathogens in farm. The microbial reduction at the farm level reshapes antimicrobial consumption and reduces the cost of livestock vaccination. Our previous studies demonstrated that bovine rotavirus A, bovine torovirus, bovine enterovirus and bovine coronavirus can be employed as viral pathogen indicators to monitor the biosecurity levels of bovine farms (Hasan et al, 2022; Takahashi et al, 2020).

Mammalian orthoreoviruses (MRVs) - non-enveloped double-stranded RNA viruses which belong to the Reoviridae family –, and *Sapelovirus A* (SV-A) formerly known as porcine sapelovirus (PSV) – non-enveloped single-stranded RNA virus which belongs to the *Sapelovirus* genus and *Picornaviridae* family -, are ubiquitous pathogens on pig farms. These infections among pigs have been reported worldwide, including Japan (Fukase et al, 2022; Harima et al, 2020a; Qin et al, 2017; Sunaga et al, 2019). The serotypes of MRVs were classified into 4 types (MRV1 to 4, respectively) based on the S1 segment (Dermody et al, 2013), and MRV1 to 3 serotypes were isolated from pigs (Harima et al, 2020b; Kwon et al, 2012; Lelli et al, 2016; Luo et al, 2020; Ye et al, 2020). MRVs infect other mammalian species (Decaro et al, 2005; Lelli et al, 2013; Yang et al, 2015), including humans (Rosa et al, 2019; Yamamoto et al, 2020). PSV, on the other hand, has a single serotype and only infects pigs. The genetic diversity of PSV has been investigated based on the VP1 gene (Bai et al, 2018; Son et al, 2014). Although both MRVs and PSV often cause asymptomatic pigs, several studies have shown that these isolates can cause diarrhea and other in experimentally infected piglets and pigs (Chen et al, 2022; Thimmasandra Narayanappa et al, 2015; Wang et al, 2021). These results indicate that these viruses can cause serious problems in the swine industry.

Fecal-oral transmission is regarded as one of the common infection routes of MRVs and PSV among pigs (Lan et al, 2011; Wang et al, 2021). Although plans to develop effective vaccines toward pathogens, including these ubiquitous viruses, remain a high priority, a long term period of the time is required to secure and supply sufficient vaccines. Due to the inadequate supply of vaccines, proper

daily disinfection is necessary in order to optimize the reduction of these viruses in pig farms.

In Chapter 3, MRVs and PSVs were used as pathogen indicators of the biosecurity level of pig farms. Additionally, the virucidal activities of disinfectants were evaluated toward the isolated MRV by suspension and carrier tests in the presence of organic materials, to ensure that the disinfections would be practical.

3.2. Materials and methods

3.2.1. Information on pig farms and sample collections from pigs

Based on the owner's agreement toward sampling in pig farms, two different specific-pathogen-free (SPF) farms (A and B) that are located in the Kanto district of Japan were selected. Farm A has 190 sows from farrowing to finishing (Fig. 3.1); farm B has 5,500 grow-to-finish fattening pigs on a regular basis, with approximately 400 10-week-old piglets brought in each week (Fig. 3.2). According to information from

farm B, the post-all-out disinfectant solution has changed in effect in Sep 2019. Previously, only QAC was used for piggery disinfection and foot baths; starting Sep 2019, Mix-500 which is a more effective disinfectant than QAC-500, even in severe conditions such as low temperature (Kabir et al, 2021) - has been utilized. The sow farm introducing piglets to farm B has also adopted disinfection with Mix-500 since Sep 2019.

A total of 199 fecal specimens (95 individual and 104 pooled feces) were obtained from apparently healthy pigs in farms A and B. At every sampling, fresh five feces from pigs were randomly collected from the floor of each barn in both farms. In farm A, through 11 samplings from Feb 2020 to Aug 2022, a total of 380 fecal specimens (320 from growing pigs and 60 from sows) were collected from pigs ranging in growing stages from 2-weeks-old piglets to sows. In farm B, through 11 samplings from Feb 2020 to Jan 2022, a total of 220 fecal specimens were collected from pigs ranging in age from 10 to 23 weeks. The collected five feces from growing pigs (300 of 320 from farm A, and 220

from farm B) of the same barn were pooled into one bag and regarded as one composite sample, based on the basic data (their age and sampling day), resulting in 60 composite samples and 80 individual samples for farm A and 44 specimens for farm B, all intended to be used in the laboratory testing.

Then, additional samplings were conducted in farm B to further evaluate the introduction risk of MRVs. In Jun 2022, five fecal specimens on the truck which transported 10-week-old piglets from a supplier SPF farm, and five fecal specimens from those piglets at three days post introduction were collected, respectively. Finally, in Sep 2022, five feces from the 23-week-old pigs, which were introduced as the forementioned piglets in Jun 2022 were also collected. They were all used as independent specimens in the laboratory testing.

To make a 10% (w/v) homogenate, all fecal specimens were suspended in PBS. The homogenates were clarified by centrifugation at 1,220 x g for 15 minutes at 4℃, and the supernatants were aliquoted and preserved at -80℃ until use.

3.2.2. RT-PCR testing

The viral RNA genome was extracted from the supernatants with the use of a QlAamp Viral RNA Mini Kit (QIAGEN, Hilden, Germany), in accordance with the manufacturer's instructions. The RNA was amplified by using reverse transcription polymerase chain reaction (RT-PCR) for the detection of L1 gene of MRVs with virus-specific primers as shown in Table 3.1 (Zhang et al, 2021). Just before RT-PCR assay, 2 μL of extracted RNA was mixed with 1 μL of dimethyl sulfoxide (DMSO) (Wako Pure Chemical Industries, Co, Ltd, Japan) and 4.5 μL of dW_2, then heated at 98℃ for 5 min. The RT-PCR was carried out with the use of a PrimeScript One Step RT-PCR Kit ver.2 (Takara Bio Inc. Kusatsu, Japan). In brief, 7.5μL of DMSO-treated viral RNA was mixed with 12.5 μL of 2 x 1-Step Buffer, 3.8 μL of RNase-Free dH_2O, 0.2 μL of the primer mixture (final concentration of 0.4 μM each), 1 μL of PrimeScript 1 step Enzyme Mix, in a total volume of 25 μL. The RT-PCR assay was performed under the following conditions: reverse transcription at 50℃ for 30 min and denaturation

step at 94℃ for 2 min, followed by 35 cycles at 94℃ for 30 sec, 53℃ for 30 sec, 72℃ for 60 sec, and a final incubation at 72℃ for 7 min.

To distinguish among the three serotypes of MRVs (MRV1 to 3), RT-PCR with three primer sets for the S1 gene of MRVs shown in Table 3.1 was performed. Primers were designed using National Center for Biotechnology Information (NCBI) primer basic local alignment search tool (BLAST) (https://www.ncbi.nlm.nih.gov/tools/primer-blast/index.cgi?GROUP_TARGET=on) for amplification of S1 gene of MRVs according to the multiple gene sequences of MRVs in the database. The utilized GenBank accession numbers for MRV1 primer design were as follows: KT947000, KX263313, KR296778, KT444528, LC613225, MN788300. For MRV2, MN233092, LC705298, KX384852, MG457114, LC482244 were used. For MRV3, LC705308, LC705318, LC705328, KT224510, KY419126, MZ541851, LC579757, JX486063, JF829220, HQ642775, DQ911244, KT444558. The specificity of each primer was confirmed using BLAST (https://blast.ncbi.nlm.nih.gov/Blast.cgi). DMSO treatment and RT-PCR assay were performed with the use of the products as described above, except for the new primer sets. The RT-PCR stages were the following: reverse transcription at 50℃ for 30 min and denaturation step at 94℃ for 2 min, followed by 35 cycles at 94℃ for 30 sec, 57℃ for 30 sec, and 72℃ for 60 sec, and final incubation at 72℃ for 7 min.

The RNA was also tested using RT-PCR amplifying a 270 bp fragment in the conserved 5′ untranslated region (UTR) of PSV with a pair of primers as shown in Table 3.1 (Chelli et al, 2020). In brief, 2.0 µL of RNA was mixed with 12.5 µL of 2 x 1-Step Buffer, 9.3 µL of RNase-Free dH_2O, 0.2 µL of the primer mixture (final concentration of 0.4 µM each), 1 µL of PrimeScript 1 step Enzyme Mix, in a total volume of 25µL. The RT-PCR assay was performed under the following conditions: reverse transcription at 50℃ for 30 min and denaturation step at 94℃ for 2 min, followed by 35 cycles at 94℃ for 30 sec, 53℃ for 30 sec, 72℃ for 40 sec, and a final incubation at 72℃ for 7 min.

These RT-PCR products were analyzed by 1.5% agarose gel electrophoresis.

3.2.3. Isolation of MRVs and PSV

Virus isolations of MRVs and PSV were performed by cell-culture assays using the supernatants of fecal specimens, respectively. For MRVs isolation, the supernatants that contained both L1 and S1 genes-positive RNAs of MRVs were inoculated onto monolayers of MDCK cells in MM (Thammakarn et al, 2015), supplemented with 2 μg/mL of trypsin.

For PSV isolation, the 47 supernatants that were positive for PSV were randomly selected from all positive samples of both farms, and then inoculated onto monolayers of African green monkey kidney (Vero) cells in MM, porcine kidney (PK-15) cells, and baby hamster kidney (BHK-21) cells, in Dulbeco's Modified Eagle Medium (DMEM, Nissui Pharmaceutical Co. Ltd, Tokyo, Japan) (Jahangir et al, 2010), respectively.

These inoculated cells were cultured at 37℃ in 5 % CO_2 and observed daily to monitor for CPE. The infected cell cultures were frozen and thawed three times, then centrifuged at 1,220 x g for 15 min at 4℃, and the culture supernatants were aliquoted and preserved at -80 ℃. Two more serial passages were carried out using the supernatants from the previous passage into fresh monolayers. The respective supernatants from the third passages were tested to verify the prevalence of MRVs or PSV by RT-PCR.

3.2.4. Purification of MRVs and sequence for phylogenic analysis

MRV isolates were subjected to three consecutive plaque purifications by plaque assay, as described previously (Jahangir et al, 2010). The plaque from the third plaque cloning was tested to verify that the virus was a plaque-purified strain of MRVs, based on RT-PCR with the use of S1 gene primer pairs as described above.

The PCR products with primer sets for partial S1 genes were purified with a QlAquick Gel Extraction Kit (Qiagen Inc, Tokyo, Japan) according to the manufacture`s instruction. Both strands of the purified PCR products were sequenced by Fasmac Corp. (Atsugi, Japan) to verify the positive

results and the sequence data were deposited to DDBJ. The accession numbers of the sequences of part of the S1 gene were LC730310 to LC730312. The sequence similarities between the plaque-purified strains in this study and the reference strains in GenBank were investigated with the use of BLASTN searches. Phylogenetic tree based on S1 genes of MRVs was computed by using the neighbor-joining method in MEGA-X software with a bootstrapping set of 1,000 replicates.

3.2.5. Disinfection test toward MRV1

3.2.5.1. Test virus, tested solutions, and blocking solution

The virucidal activities of disinfectants toward MRV1 in liquid and on surfaces were examined through suspension and carrier tests, as shown in Chapter 1. Porcine MRV1, a plaque-purified strain in this study, designated MRV1-GmmTN1 was propagated in MDCK cells; the cell culture was frozen and thawed three times, then centrifuged at 1,220 x g for 15 min. The supernatants supplemented with 10 μg/mL of trypsin were incubated at 37℃ for 30 min, aliquoted and then stored at -80℃ until use. The virus titer of the stock was estimated to be around 10^9 PFU/mL by plaque assay with MDCK cells.

As disinfecting agents, QAC-500, 0.17% $FdCa(OH)_2$ solution (Fine Co, Ltd, Tokyo, Japan), Mix-500（QAC-500 in 0.17% $FdCa(OH)_2$) and 100 ppm of NaClO solution (NaClO-100) and NaClO-1000 were used in this study. All the tested solutions were each prepared just before the disinfectants were tested and used. As a water control, dW_2 was used instead of disinfectants in each test. As a neutralizing agent to terminate the disinfectant`s action, a blocking solution was prepared, as previously described in Chapter 1.

3.2.5.2. Suspension tests for the evaluation of virucidal activities of disinfectants toward MRV1 in liquid

Suspension tests were conducted at room temperature (RT: 25 ± 2°C) as described in Chapter 1. In brief, 400 μL of 0.17% $FdCa(OH)_2$, Mix-500, NaClO-100 and NaClO-1000, were mixed with 100 μL of MRV1-GmmTN1 containing 5% FBS in a microtube. In case of QAC treatment, 400 μL of QAC-

500 was mixed with 100 µL of the virus with and without 5% FBS, respectively. All the resulting solutions were then subjected to vortex mixing. Following the given exposure periods, the disinfectant`s action was immediately stopped by adding 500 µL of the blocking solution into the microtube. In parallel, other reactions were performed to evaluate the neutralization effect of the blocking solution. In brief, 400 µL of each tested solution was mixed with 500 µL of the blocking solution in a microtube, and then 100 µL of MRV1-GmmTN1 with and without 5% FBS was added into the mentioned mixture. These reactions were regarded as 0 sec treatment and contact time.

3.2.5.3. Carrier tests for the evaluation of virucidal activities of disinfectants toward MRV1 on surfaces

Carrier tests were conducted using abiotic carriers with the dropping and wiping techniques, as described in Chapter 1. Prior to each assay, plastic carrier coupons (around 5.0 cm x 5.0 cm) in Petri dishes were sterilized with microwave irradiation in a microwave oven for 5 min, and then placed in a class II biosafety cabinet. An amount of 100 µL of MRV1-GmmTN1 containing 5% FBS was inoculated onto the surface of each coupon and subsequently spread by a sterile glass spreader onto the coupons. Each coupon was air dried for 60 min inside the safety cabinet at RT. In the carrier test with the dropping technique, 500 µL of each tested solution was dropped on each contaminated coupon and incubated for 1, 5 and 30 min. After inoculation, the virucidal activities of the tested solutions were neutralized by soaking each carrier in stomacher bags containing 2 mL of the blocking solution. Afterwards, the remaining infectious virus particles were recovered from the surface via rubbing vigorously by finger over the stomacher bag.

In the carrier test with the wiping technique, the contaminated coupons, as described above, were wiped for 30 sec, while using a rayon-polyester sheet (5 cm x 5 cm) that was folded into four pieces (2.5 cm x 2.5 cm) and soaked in 500 µL of each disinfectant. After wiping action, according to the method described above, the wiped carries were soaked in 2.5 mL of the blocking solution, and then remaining virus particles on the carriers were recovered. Each of the wiping sheets were transferred

into stomacher bag containing 2 mL of the blocking solution, and then the remaining virus particles were recovered from each sheet with the use of a BagMixer.

3.2.5.4. Infectivity assay and data analysis

Each disinfectants test was repeated three times, separately. For the infectivity assay, the recovered infectious virus particles were quantified by plaque assay with MDCK cells. Plaques were counted at 6 dpi and the virus titer was calculated as PFU.

The results were reported as $\log_{10}$ PFU/mL with mean ± SE in triplicates. The log reduction of virus titer was presented as the difference between the treated titer of the virus with disinfectants and that of the controls: 0 sec treatment in the suspension test and dW_2 treatment in the carrier tests. More than 3 $\log_{10}$ reductions were regarded as evidence of effective virucidal activities of disinfectants (OECD, 2013; Sattar et al, 1989, US EPA, 2018)).

3.3. Results

3.3.1. Prevalence of MRVs and PSVs in pigs

As shown in Fig. 3.3, the specific amplicons of RT-PCR products for L1 and S1 genes of MRVs were detected from pig fecal samples (samples I to III were collected from farm B in Feb, Sep, Oct 2020, respectively, and sample IV was collected from farm A in Mar 2021). Samples I and III were positive for MRV2 and MRV1, respectively, whereas the concurrent infections of MRV1 and MRV2 were detected in sample II. Sample IV was negative for both L1 and S1 genes of MRVs.

The prevalence of MRVs in 199 fecal samples from farms A and B is presented in Table 3.2. The 140 fecal samples from farm A were negative for MRVs, whereas 12 of 59 fecal samples (20.3%) from farm B were positive for MRVs. Either of the two serotypes of MRVs was detected in the 10 out of 12 positive fecal specimens: MRV1 from one sample collected in Oct 2020 (sample III) and MRV2 from nine samples collected in Feb (sample I etc.), Jun, Sep, Oct 2020, Mar 2021 and Sep 2022. The

remaining 2 samples collected in Feb and Sep 2020 (sample II) contained both MRV1 and MRV2. MRV3 was not detected at all in the fecal specimens we collected.

Figure 3.4 shows the change over time in the number of MRV detections among tested specimens from farm B. The pooled fecal specimen from each group ranging in age (10 to 23-weeks-old piglets) was collected, and the sampling was conducted 11 times from Feb 2020 to Jan 2022. Fifteen individual feces were collected at 3 different stages (before and after the introduction, and shipment) from same groups of piglets from Jun to Sep 2022. The positive ratio peaked by 100% in Feb 2020 and decreased by 50% in Jun 2020. In Sep 2020 and Oct 2020, the ratio pointed at 25% and 50%, respectively. In Jan 2021, it dropped to 0% and no positive samples were detected from May 2021 to Jun 2022. Although the 10 weeks old piglets introduced in Jun 2022 were negative for MRVs, two out of the five (23-week-old, which were ready for shipment) specimens from the pigs were positive for MRV.

As shown in Fig. 3.5, PSV was also detected from the fecal supernatants. Of the total of 199 specimens obtained from two farms, 138 (69.3%) were positive for PSV (Table 3.2). The fecal specimens from piglets were positive for PSV in two farms investigated, with a prevalence of 90% (72/80) from farm A and 94.9% (56/59) from farm B. Among the fecal specimens from sows in farm A, PSV was detected in 16.7% (10/60).

3.3.2. Isolation of MRVs and PSVs

After the second or third passages, CPE was detected in the inoculated MDCK cells with the supernatants of all 12 MRVs RT-PCR-positive fecal specimens (Fig. 3.6). At 2 to 3 dpi, inoculated MDCK cells had become rounded and detached from cell culture plates. Samples showing CPE were verified to be MRVs positive by means of RT-PCR, using the primer set for L1 gene.

Of the cell lines (Vero, PK-15 and BHK-21) that were inoculated with the feces from pigs, unfortunately, PSV could not be isolated.

3.3.3. Phylogenic analysis of MRVsS1 genes

Viral cultures from samples I to III (containing either of MRV1 and MRV2, and both MRV1 and MRV2) were subjected to plaque purification. By means of RT-PCR using the primer sets for S1 genes, only MRV2 was detected from sample II containing both MRV1 and MRV2, which verified that a single serotype was purified from the sample after third plaque purification. The 3 samples led to three plaque-purified strains of MRVs, namely: one MRV1, designated MRV1-GmmTN1 and two MRV2, designated MRV2-GmmTN9 and MRV2-GmmTN13. To genetically characterize those isolates, the partial sequence of S1 gene segment was obtained from each of them. The phylogenic analysis of the 3 strains and reference strains are shown in Fig. 3.7. Sequence analysis showed that MRV1-GmmTN1 was closely related to MRV1/Bat/Korea/B19-02/2019 (Lo et al, 2022), a variant isolated from a bat in South Korea, with 94.31% nucleotide identity; two MRV2 strains (MRV2-GmmTN9, MRV2-GmmTN13) were closely related to those of MRV2/Pig/USA/66848/2005 that was isolated from pigs in the United States (no reference) (92.20 and 92.00% identity, respectively).

3.3.4. Virucidal activities results for MRV 1 in liquid

Table 3.3 shows the results of the virucidal activities of disinfectants toward MRV1-GmmTN1 for different exposure periods at RT in the aqueous phase. A 0.17% $FdCa(OH)_2$ solution, Mix500 and NaClO-1000 could inactivate the virus containing 5% FBS below the detection limit in 5 sec. It took 30 sec for QAC-500 to efficiently inactivate the virus (≥ 3 $\log_{10}$ reductions). NaClO-100 was unable to efficiently inactivate the virus within 30 min (0.68 $\log_{10}$ reduction). Since the viral titer for 0 sec was similar to the titer treated with dW_2, it was verified that the blocking solution terminated the disinfectant`s action and thus accurately controlled the disinfection time.

3.3.5. Virucidal activities results for MRV1 on surfaces

Table 3.4 shows the results of the virucidal activities of disinfectants toward MRV1-GmmTN1 containing 5% FBS that were experimentally dried on plastic surfaces in the carrier tests. In the carrier test with the dropping technique, 0.17% FdCa(OH)$_2$ and Mix-500 solutions required 1 min to efficiently inactivate the virus. The virus was efficiently inactivated with QAC-500 after 30 min of action time; NaClO-1000 inactivated the virus below the detection limit after 30 min. The recovery ratio of the virus from the carrier treated with dW$_2$ was around 30%, as compared to the titer of the viral stock.

With Mix-500 or NaClO-1000 wiping for 30 sec, no virus was detected thereafter on the wiped carriers. Moreover, Mix-500 and NaClO-1000 could efficiently inactivate the virus in the wiping sheets within 30 sec, compared with the titer in the wiping sheet with dW$_2$. However, under the same conditions, wiping with QAC-500 and 0.17% FdCa(OH)$_2$ solutions did not efficiently inactivate the virus on surfaces (0.16 and 2.11 log$_{10}$ reductions, compared to the titer on the wiped carriers with dW$_2$, respectively). Besides, QAC-500 and 0.17% FdCa(OH)$_2$ were also incapable of efficiently inactivating the virus in the wiping sheet (0.98 and 2.41 log$_{10}$ reductions as compared to the titer with dW$_2$ treatment, respectively). The recovery ratio of the virus from the wiping sheet with dW$_2$ was around 16 % of the titer of the viral stock.

3.4. Discussion

In the present study, twelve porcine MRVs were detected and ten MRVs were isolated from 59 fecal specimens in one SPF pig farm (farm B) in Japan. No MRVs were detected from 140 specimens in another SPF farm (farm A). Additionally, although 138 of 199 specimens in farm A and B were positive for PSV, PSV could not be isolated in this study. In Japan, Japan SPF Swine association defines SPF pigs as free of the following five pathogens: Aujeszky's disease, atrophic rhinitis, swine mycoplasma disease, swine dysentery and toxoplasmosis (Japan SPF Swine Association, 2022).

MRVs and PSV are not included for SPF conditions and are not monitored. Additionally, the virucidal activities of disinfectants toward MRV1-GmmTN1 in the presence of organic materials were evaluated through suspension and carrier tests to appraise the efficacy of application for daily cleaning in pig farms. Most of the piglet fecal samples collected from both farms were positive for PSV during the study period. On the other hand, the number of MRVs detections in farm B declined after introduction of enhanced biosecurity, which suggests that daily effective disinfection contributes, potentially significant benefits to the swine industry.

Both MRV1 and MRV2 have been previously isolated from pigs in Japan (Fukutomi et al, 1996; Hirahara et al, 1988). The latest report showed that five MRVs (two of MRV2 and three of MRV3) were isolated from 230 porcine feces in Japan (Fukase et al, 2022). In the present study, MRVs were not detected in farm A, whereas MRV1 and MRV2 were isolated from farm B, which suggests that multiple serotypes were present in that pig farm. Phylogenetic analysis based on the partial S1 gene showed that the MRV1 isolate was most closely related to the MRV1 isolated from a bat in South Korea (Lo et al, 2022) (94.31 % identity). This result suggests that the MRV1 isolate may have been derived from the viruses recently circulating in South Korean bats populations. Besides, two MRV2 isolates shared 92.20 and 92.00 % nucleotide identities with porcine MRV2 from the United States. The finding indicates that porcine MRV2 in Japan and the United States shares a common origin. Reassortant MRV strains were generated when two or more lineages of MRVs infected the same host (Dermody et al, 2013). The exchange of RNA segments results in genetic diversity, viral evolution, cross-species-transmission of MRVs (Kitamura et al, 2021; Qin et al, 2017; Wang et al, 2015). This indicates that novel MRVs with high pathogenicity have the potential to cause a devastating impact on the swine industry and human health, particularly when concurrent antigenic changes take place. Hence, the enhancements of biosecurity in pig farms are increasingly required to control MRVs infection among pigs.

The virucidal activities of disinfectants were evaluated toward MRV1-GmmTN1 in the presence of organic materials through suspension and carrier tests. As shown in Tables 3.3 and 3.4, a longer exposure period was required to efficiently inactivate MRV1 on surfaces than in liquid. This trend was also observed in previous studies (Alam et al, 2018b; Hatanaka et al, 2022), indicating that pathogens on surfaces are more resistant to disinfectants than in liquid. Thus, carrier tests need to be conducted to evaluate the efficacy of daily disinfection in pig farms, in addition to suspension tests. Mix-500 and NaClO-1000 showed the stable virucidal activities toward MRV1 in the organic materials through both the suspension and carrier tests. NaClO-1000 is not practical due to its high chlorine concentration and corrosive nature. These results suggest that Mix-500 can be applied as the preferable disinfectant agent for the daily disinfection in pig farms. Wiping with QAC-500 could not efficiently inactivate MRV1 on surfaces within 30 sec, whereas QAC-500 after 30 min of reaction time efficiently inactivated MRV1 on surfaces. These findings were unexpected because QACs have been recognized to be incapable of inactivating non-enveloped viruses such as MRVs (Shirai et al, 1997; Tuladhar et al, 2012). However, some studies also demonstrated that QACs inactivated reoviruses in suspension and carrier tests (Mor et al, 2014; Nemoto et al, 2014). To investigate the mechanism of QACs action toward MRVs, further study is needed.

Detection by means of RT-PCR allowed a precise MRV and PSV determinations in pig farms. As shown in Table 3.2, MRVs were detected (20.3%, 12/59) from farm B, whereas MRVs were not detected from a total of 140 pig feces collected from farm A during the study period. By contrast, PSV was detected in both farms on a regular basis during the study period (69.3%, 138/199). These results suggest that PSV infections among pigs have been widespread in Japan. In a previous study, Chelli et al. showed that 98.4% (63/64) and 14.3% (4/28) of pooled fecal samples from young growers (aged between 1–3 months old) and sows (animals older than 1 year) of three different farms in Northern Italy were positive for PSV, respectively (Chelli et al, 2020). Of the samples in the present study, 90 % (72/80) and 94.9 % (56/59) of piglet feces from farm A and B were positive for PSV, respectively,

whereas only 16.7 % (10/60) of feces from sows in farm A tested positive for PSV. Besides, one feces from a sow that was housed in the same barn with PSV positive suckling piglet (2-week-old) was positive for PSV, whereas other 4 sows were negative as well as their suckling piglets showed negative (data not shown). The high viral loads of PSVs in suckling piglets are fairly similar to previous studies in China and Hungary (Boros et al, 2020; Ibrahim et al, 2022). These findings suggest that sows could become initial sources of PSV infection of piglets. Most of feces from piglets were positive for PSV, which might be associated with a decrease of maternal antibodies from colostrum in sows as they grow. Fecal-oral transmission is regarded as one of main infection routes of MRVs and PSV (Lan et al, 2011; Wang et al, 2021). However, previous study showed that PSV was detected not only from pig fecal samples but also from aerosol samples in pig farms (Ramesh et al, 2021), which indicates that PSV infects pigs through aerosol transmission, in addition to fecal-oral transmission. These data indicates that piglets were under the risk of continuous exposure to PSV. In fact, its infections were rapidly spread among them. Therefore, further enhancement of biosecurity is required to control PSV infections among pigs, such as aerosol disinfection.

On the other hand, it is considered that farm A had higher biosecurity levels than farm B from the difference of positive ratio of MRVs. Farm A has sows from farrowing to finishing; farm B has grow-to-finish fattening pigs on a regular basis. It is considered that introduction of pigs from a positive farm may be an important risk, in case of farm B. The porcine feces were positive for MRVs at all ages (from 10 to 23 weeks old) in Feb 2020 (Fig.3.4). Although two of the five feces collected in Sep 2022 (23-week-old, which was ready for shipment) were positive for MRVs, MRVs in farm B have been decreasing since Jun 2020. These findings indicate that farm B increased the biosecurity levels. It is considered that one of the reasons for this is the fact that both farm B and the supplier farm changed the daily cleaning disinfectant from QAC to Mix-500 since Sep 2019. The results showed that Mix-500 efficiently inactivated MRV1 on surfaces for shorter periods than QAC-500 treatment. Besides, previous studies confirmed that Mix-500 is a more effective disinfectant than QAC-500, even in

severe conditions such as low temperature (Kabir et al, 2021). Therefore, it is likely that the enhancement of biosecurity by using Mix-500 for daily cleaning prevented or decreased MRVs infections among pigs.

In conclusion, this study has highlighted the MRVs and PSV infections in pigs and the susceptibility of MRVs to disinfectants on surfaces. Moreover, the number of MRV detections declined after the enhancement of biosecurity in pig farms, probably due to more effective disinfection that prevented MRVs infection among pigs. It is hoped that this research contributes to potential benefits to the swine industry from a biosecurity perspective and reduces the occurrence of pathogens.

3.5. Tables and figures

Table 3.1. Primers used in this study.

Virus specificity	Target	Sequence (5`-3`)	Amplicon size	Reference
MRVs[a]	L1	TCCATCGTAAATGATGAGTCTG GAAATCAGTTCTAACATCCTCTG	410	Zhang et al.
MRV1	S1	TGCTATCGAACCACGAGTTG TCAGCCATTATTGAATTAGTTGTCA	822	This study
MRV2	S1	GGACTCGAGGAAGTCAAGAAA CTGACCACTGACCAGTACG	916	This study
MRV3	S1	CTAACTATAGGTTCAGGCAGAGC GGTCTGATCCTCACGTGAAAC	447	This study
PSV[b]	5'UTR[c]	CGTGCTCCTTTGGTGATTC GAAAGAGTAGTAGTAGATTCC	270	Chelli et al.

[a] Mammalian orthoreoviruses. [b] Porcine sapelovirus. [c] Conserved 5' untranslated region of PSV.

Table 3.2. Number of mammalian orthoreoviruses (MRVs) and porcine sapelovirus (PSV) positive pigs in relation to the age in farms.

Farm	Life stage	Week-old	Sampling	Positive/total samples MRVs	PSV
A	Piglets	2	Individual	0/5	1/5
		3	Pooled	0/10	10/10
		4	Individual	0/5	5/5
		6	Pooled	0/10	10/10
		8		0/10	10/10
		10		0/10	10/10
			Individual	0/5	5/5
		13	Pooled	0/10	10/10
		16		0/10	9/10
		21	Individual	0/5	2/5
	Sows			0/60	10/60
		Subtotal (%)		0/140 (0%)	82/140 (58.6%)
B	Piglets	10	Individual	0/10	10/10
			Pooled	4/11	11/11
		14		3/11	11/11
		18		1/11	11/11
		23		2/11	11/11
			Individual	2/5	2/5
		Subtotal (%)		12/59 (20.3%)	56/59 (94.9%)
		Total (%)		12/199 (6.0%)	138/199 (69.3%)

A total of 199 fecal specimens (95 individual and 104 pooled feces) were obtained from apparently healthy pigs in two different specific-pathogen-free (SPF) farms (A and B) that are located in the Kanto district of Japan.

Table 3.3. Virucidal activities of the tested solutions toward mammalian orthoreovirus type 1 (MRV1) strain GmmTN1 in liquid.

Tested solutions	FBS (%)	Viral titer at different incubation times				
		0 sec	5 sec	30 sec	1 min	30 min
QAC-500	0	8.97 ± 0.10	8.30 ± 0.10	5.95 ± 0.05*	3.90 ± 0.16*	< 2.70 ± 0.00
	5	8.78 ± 0.03	8.62 ± 0.03	5.92 ± 0.05	4.98 ± 0.08*	< 2.70 ± 0.00
$FdCa(OH)_2$		8.76 ± 0.02	< 1.70 ± 0.00	< 1.70 ± 0.00	< 1.70 ± 0.00	NT
Mix-500		8.95 ± 0.03	< 2.70 ± 0.00	< 2.70 ± 0.00	< 2.70 ± 0.00	NT
NaClO-100		8.79 ± 0.03	8.74 ± 0.02	8.70 ± 0.05	8.70 ± 0.02	8.31 ± 0.02
NaClO-1,000		8.84 ± 0.01	< 1.70 ±0.00	< 1.70 ± 0.00	< 1.70 ± 0.00	NT
dW_2		NT[a]	NT	NT	8.89 ± 0.02	8.99 ± 0.08

Virucidal activities of disinfectants toward MRV1-GmmTN1 containing 5% fetal bovine serum (FBS) in the aqueous phase were evaluated. Quaternary ammonium compound (QAC) diluted 500 times with redistilled water (QAC-500), 0.17% food additive glade calcium hydroxide ($FdCa(OH)_2$) solution, QAC diluted with 0.17% $FdCa(OH)_2$ solution (Mix-500), sodium hypochlorite at 100 or 1,000 parts per million of total chlorine (NaClO-100 or NaClO-1000, respectively) were used as the tested solutions. The viral titers are shown as mean ± SE in triplicate. Viral titer is measured as $\log_{10}$ PFU/mL. Single asterisk indicates effective viral reduction (≥ 3 $\log_{10}$ reductions). Viral titer < 1.70 and < 2.70 $\log_{10}$ PFU/mL show the detection limits in the suspension tests.

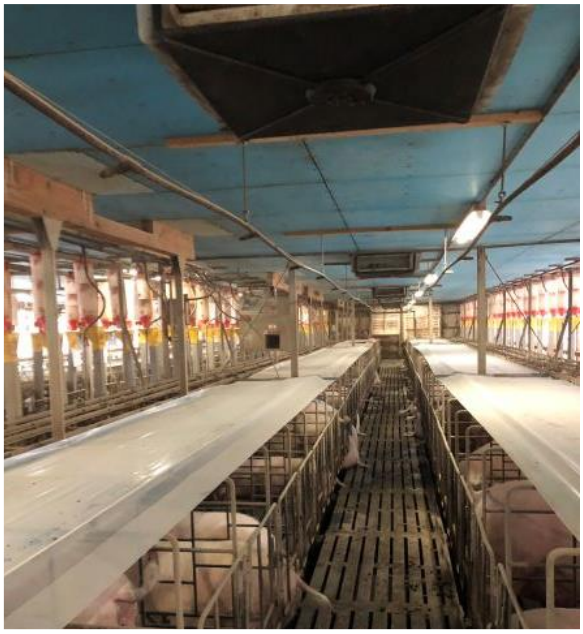
[a] Not tested.

Table 3.4. Virucidal activities of the tested solutions toward mammalian orthoreovirus type 1 (MRV1) strain GmmTN1 on surfaces.

Tested solutions	FBS (%)	Viral titer on the carrier or in the sheet				
		Dropping-carrier			Wiped-carrier	Wiping-sheet
		1 min	5 min	30 min	30 sec	30 sec
QAC-500	5	8.34 ± 0.07	6.88 ± 0.12	3.92 ± 0.22*	6.32 ± 0.02	7.24 ± 0.02
$FdCa(OH)_2$		4.40 ± 0.09*	3.99 ± 0.11*	3.78 ± 0.29*	4.37 ± 0.13	5.81 ± 0.09
Mix-500		4.64 ± 0.31*	3.66 ± 0.11*	3.29 ± 0.09*	< 2.09 ± 0.00	4.89 ± 0.26*
NaClO-1000		6.17 ± 0.08	3.76 ± 0.15*	< 2.09 ± 0.00	< 2.09 ± 0.00	4.19 ± 0.49*
dW_2		8.80 ± 0.04	8.57 ± 0.02	8.50 ± 0.05	6.48 ± 0.11	8.22 ± 0.07

Virucidal activities of disinfectants toward MRV1-GmmTN1 with 5% fetal bovine serum (FBS) on surfaces were evaluated with dropping technique and wiping technique. Quaternary ammonium compound (QAC) diluted 500 times with water (QAC-500), 0.17% food additive glade calcium hydroxide ($FdCa(OH)_2$) solution, QAC diluted with 0.17% $FdCa(OH)_2$ solution (Mix-500) and sodium hypochlorite at 1,000 parts per million of total chlorine (NaClO-1000) were used as the tested solutions. Figures are shown as mean ± SE in triplicate. Viral titer is measured as $\log_{10}$ PFU/mL. Single asterisk indicates effective viral reduction (≥ 3 $\log_{10}$ reductions). Viral titer < 2.09 $\log_{10}$ PFU/mL shows the detection limit in the carrier tests.

Breeding sows.



Sows and suckling piglets (2-week-old).

Weaning piglets (4-week-old).

Fattering piglets (about 21-week-old).

Fig. 3.1. Pigs at farm A in the Kanto district of Japan. Farm A has 190 sows from farrowing to finishing.

Pig shipment (23-week-old). All in / all out management (disinfecting).

Early fattering piglets (10-week-old). A piglet sorter by weight (about 14-week-old).

Fig. 3.2. System at farm B in the Kanto district of Japan.

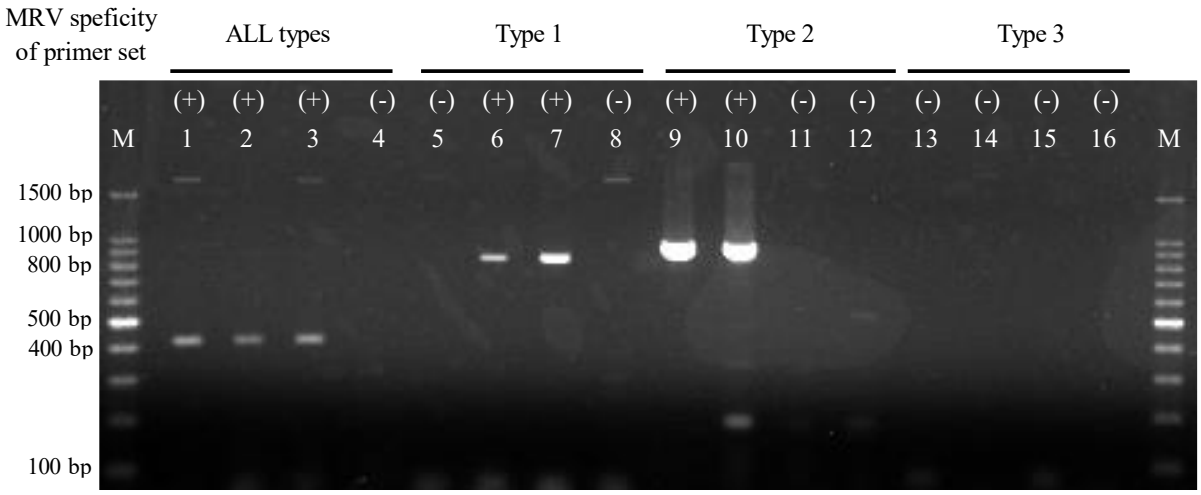


Fig. 3.3. Reverse transcription polymerase chain reaction (RT-PCR) specific for the L1 and S1 genes of mammalian orthoreoviruses (MRVs) in fecal supernatants with use of the primer-pairs (N=4).

Four fecal supernatants (sample I to III collected from farm B in Feb, Sep, Oct 2020, respectively, and the sample IV collected from farm A in Mar 2021 and sample IV from farm A) were used. Ethidium bromide stained 1.5% agarose gel shows the amplicons of 410 base pair (bp) for all types, 822 bp for type1, 916 bp for type2, 447 bp for type3. Lane 1, 5, 9, 13: fecal sample I, Lane 2, 6, 10, 14: fecal sample II, Lane 3, 7, 11, 15: fecal sample III, Lane 4, 8, 12, 16: fecal sample IV and Lane M: Marker (100 bp).

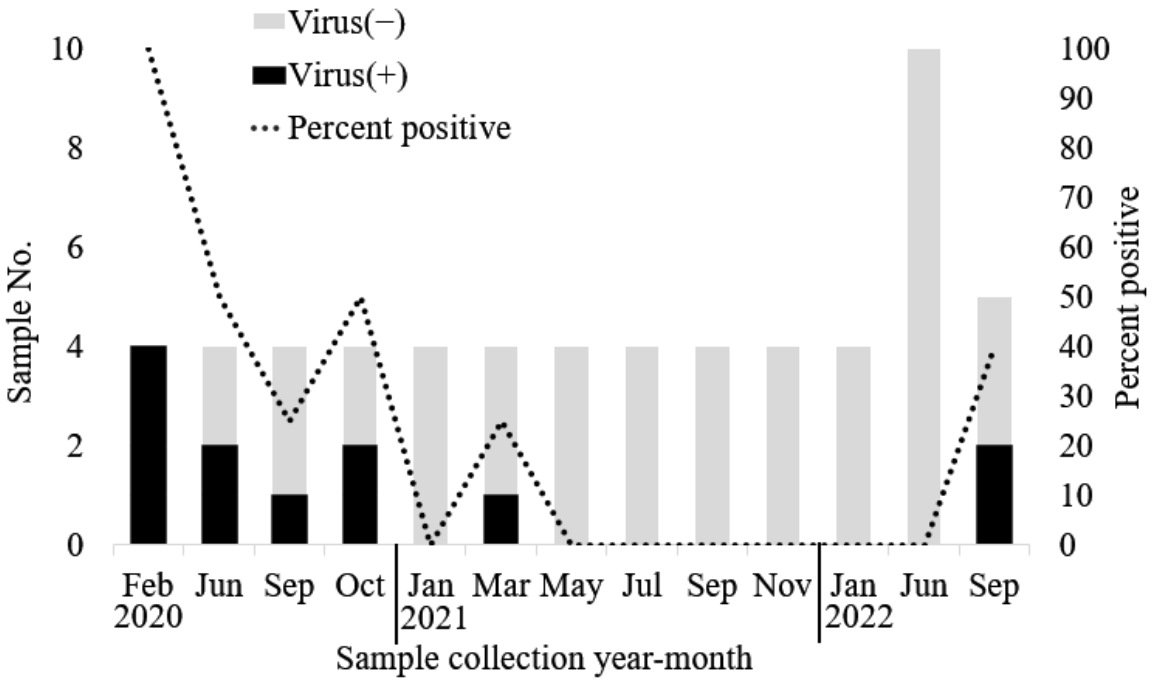


Fig. 3.4. Fecal sample collection in farm B and mammalian orthoreoviruses (MRVs) detection.

The pooled fecal specimen from each group ranging in age (10 to 23-weeks-old piglets) was collected, and the sampling was conducted 11 times from Feb 2020 to Jan 2022. Fifteen individual feces were collected at 3 different stages (before and after the introduction, and the shipment) from same groups of piglets from Jun to Sep 2022. The feces were positive for MRVs at all ages in Feb 2020 (percent positive: 100%), whereas MRVs have been decreasing since Jun 2020 (50%).

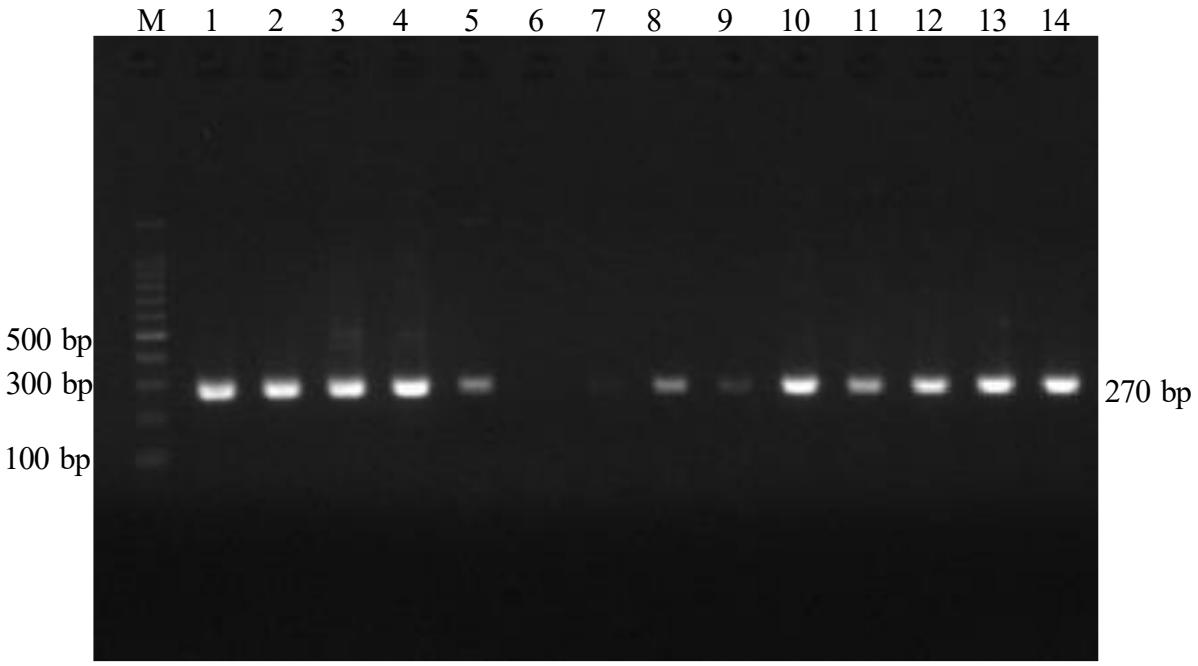


Fig. 3.5. Reverse transcription polymerase chain reaction (RT-PCR) specific for the 5'UTR gene of porcine sapelovirus (PSV) in fecal supernatants with use of the primer-pair (N=14).

Electrophoretic analysis of PSV using 5'UTR gene primer-pair. Fourteen fecal supernatants were used. Ethidium bromide stained 1.5% agarose gel shows the amplicons of 270 bp for PSV. Lane 1–5, 7-14: positive samples, Lane 6: negative sample and Lane M: Marker (100 bp).

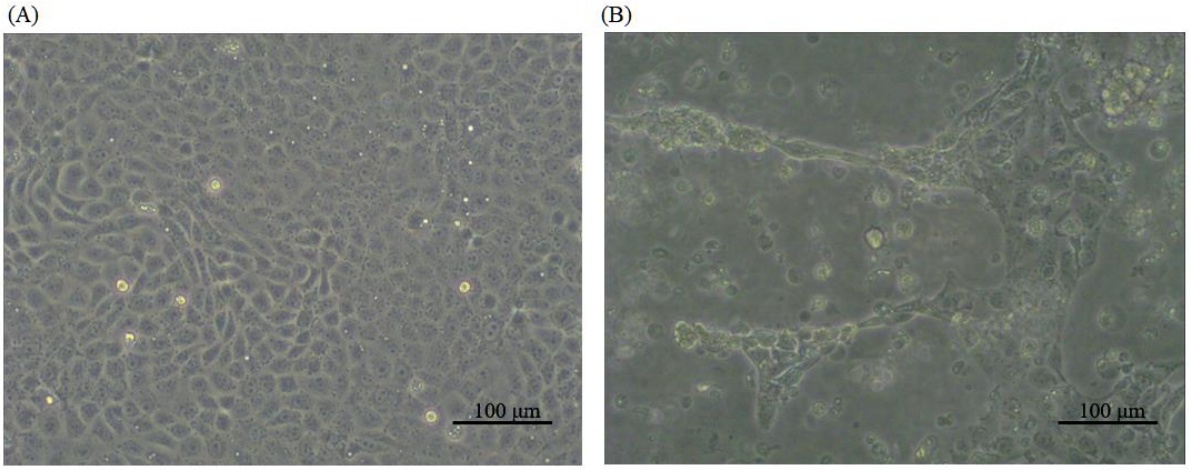


Fig. 3.6. Microscopic images of Madin-Darby Canine Kidney (MDCK) cells.

Fecal specimens from pigs with MRVs infection were inoculated to MDCK cells. Panel A shows the uninfected cells. Virus induced cellular changes in MRV-infected cells were observed at 72 h post-infection (Panel B). Scale bars represent 100 µm.

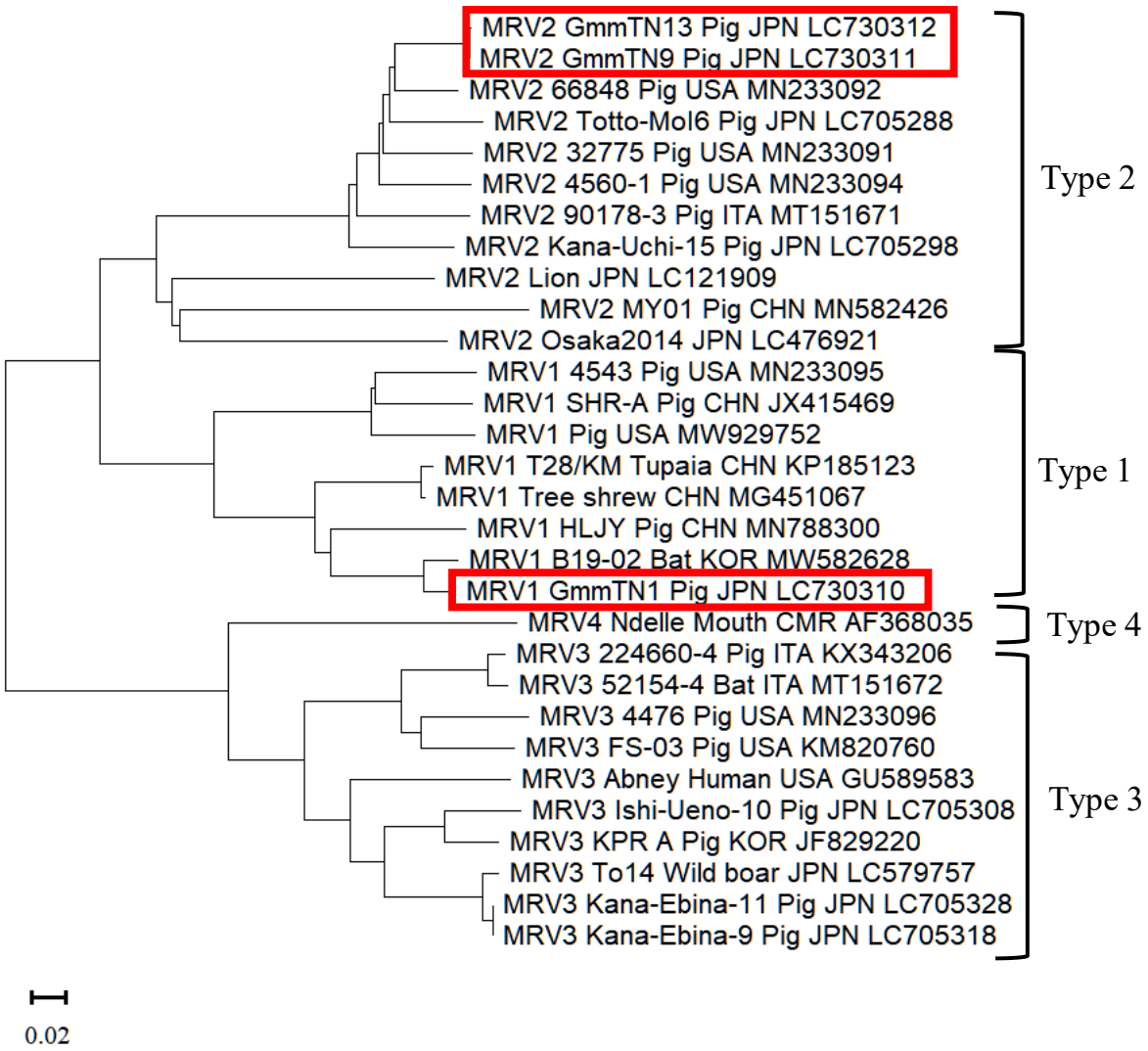


Fig. 3.7. Phylogenic tree of mammalian orthoreoviruses (MRVs) based on the nucleotide sequence data of S1 gene.

The Phylogenetic tree was computed by using the neighbor-joining method in MEGA-X software with a bootstrapping set of 1,000 replicates.

General conclusion

In this thesis, studies on epidemiology of viral pathogen indicators and biosecurity enhancement on pig farms were described. In the first study, convenient and accurate methods evaluating virucidal activities of disinfectants toward viruses on surfaces were developed and established. Moreover, the comparison of the results of suspension and carrier tests revealed that higher concentration of disinfectants or longer exposure periods to disinfectants than in the liquid were required to efficiently inactivate the virus on surfaces. The results conclude that both suspension and carrier tests should be introduced to evaluate disinfectants for the field usage, and that this evaluation system is important and useful to make the finest selection of the disinfectants against viruses on surfaces.

To evaluate the virucidal activity of disinfectant toward viruses on the field usage, experimental disinfection tests of SAHW, including the established methods mentioned in the first study, were performed. The results showed that the usage of wet wipes with SAHW could remove viruses from plastic carries. Besides, a small volume of sprayed SAHW was effective against the viruses on the rayon sheets for daily cleaning in the application area. Moreover, SAHW had a rapid virucidal activity toward aerosolized coronavirus. These findings suggest that the field usage of SAHW for daily cleaning is useful to prevent viral infections.

Finally, in order to improve the level of biosecurity on pig farms, the surveillance of ubiquitous pathogens on pig farms as viral pathogen indicators and evaluated its susceptivity to disinfectants were conducted. Although most of the pigs were positive for PSV at the start of the surveillance, the number of MRV detections declined after the enhancement of biosecurity in pig farms. The results were apparently due to the improved disinfection method which prevented MRVs infection among pigs. Further studies need to be conducted to investigate the relevance of disinfection to the prevention of PSV.

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Abstract

To date, many preventive measures have been established and utilized to minimize the economic losses due to serious infectious diseases in the domestic livestock industry worldwide. Although these preventive measures have been utilized in the livestock industry, outbreaks of viral infectious diseases in livestock have caused significant economic losses worldwide, including Japan. Therefore, advanced preventive measures need to be established in addition to the conventional countermeasures.

On-farm disinfection is considered as one of the most effective actions to control infections on livestock farms. However, published evaluation methods of virucidal activities of disinfectants toward viruses on surfaces are inaccurate and not convenient, especially regarding the wiping technique. Moreover, evaluation methods of efficacy of disinfectants toward microorganisms in the air have not been published. Therefore, effective disinfection methods against viruses on surfaces and in the air need to be established and utilized to enhance the biosecurity at the farm level. Besides, for the improvement and assessment of the biosecurity levels in livestock farms, accurate information about pathogens on the farms needs to be shown. Since the reductions of pathogen indicators on the farms mean that biosecurity has been enhanced, data on ubiquitous pathogens surveillance is necessary to achieve the enhanced biosecurity on farms. Therefore, the main objective of this study is to enhance biosecurity in pig farms using effective disinfection strategies.

In Chapter 1, the efficacy of various disinfectants against infectious bronchitis virus (IBV) in aqueous phase and on plastic surfaces was tested. For the viruses on the surfaces, dropping and wiping decontamination techniques were established and performed. The disinfectants evaluated were 0.17% food additive glade calcium hydroxide ($FdCa(OH)_2$) solution, sodium hypochlorite at 500 or 1,000 parts per million (ppm) of total chlorine (NaClO-500 or NaClO-1000, respectively), NaClO-500 supplemented in 0.17% $FdCa(OH)_2$ (Mixed-500) and quaternary ammonium compound (QAC) diluted 500-fold in water (QAC-500). In the suspension tests, all tested solutions inactivated the IBV to the undetectable level within 30 sec, even with the presence of 5 % fetal bovine serum (FBS). In the

carrier test with the dropping technique, NaClO-1000 and QAC-500 could inactivate the virus with 0.5% FBS to undetectable level within 1 min. $FdCa(OH)_2$ and Mixed-500 efficiently inactivated the virus (≥ 3 $\log_{10}$ reductions). With the wiping technique, all solutions, except NaClO-500, could inactivate the virus on a carrier to undetectable level in the wiping-sheets and wiped-carriers. In this study, the convenient and accurate carrier tests for the evaluating virucidal activities toward viruses were established. Moreover, this study highlighted that longer exposure time or higher concentration of disinfectants compared to the wiping technique was required to inactivate the virus on surfaces with the dropping technique.

In Chapter 2, slightly acidic hypochlorous acid waters (SAHWs) containing different concentrations of free available chlorine (FAC) were evaluated for their virucidal activity toward IBV and a low pathogenic H7N1 avian influenza virus (AIV) in liquid, on surfaces and in the air, with the presence of organic materials. In suspension test, abiotic carrier test, and direct spray test, SAHWs containing different concentrations of FAC - 62, 119, 220, 300, and 540 ppm (SAHW-62, -119, -220, -300, and -540, respectively) were utilized. In aerosol disinfection tests, IBV containing 0.5 % FBS was sprayed and exposed to SAHWs containing of FAC - 100, 200, 300 and 500 ppm (SAHW-100, -200, -300 and -500, respectively) for a few seconds in a closed chamber, before reaching the air sampler. In the suspension test, SAHW-62 could decrease the viral titer of both IBV and AIV by more than 1,000 times (an effective level) within 30 sec. In the carrier test with the dropping technique, IBV on carriers showed high resistance to SAHWs, while AIV on plastic carrier was inactivated to an effective level within 1 min. With the wiping technique, SAHW-62 could inactivate both IBV and AIV on wiped plastic carriers to an effective level within 30 sec. However, SAHW-220 could not inactivate IBV on the wiping rayon sheet to an effective level. In the direct spray test, sprayed SAHW-540 within 20 min, and SAHW-300 within 10 min, could inactivate IBV and AIV on the rayon sheets to undetectable level, respectively. In the aerosol disinfection tests, IBV exposed to SAHW-100 and -200 for a few seconds decreased by 0.21 $\log_{10}$ and 0.80 $\log_{10}$, respectively, compared to the pre-exposed samples to SAHWs as controls. On the

other hand, reductions of 1.16 $\log_{10}$ and 1.67 $\log_{10}$ were achieved following the exposure to SAHW-300 and -500, respectively, within a few seconds. This study indicates that the usage of wipes with SAHWs could eliminate viruses from contaminated carriers, while viruses remained on the wipes. Besides, a small volume of sprayed SAHW is potentially effective toward the viruses on the rayon sheets for daily cleaning in the application area. In addition, SAHWs have rapid in vitro virucidal activity toward aerosolized IBV.

In Chapter 3, the epidemiology of viral pathogen indicators of biosecurity levels in pig farms was investigated. Porcine sapelovirus (PSV) and mammalian orthoreoviruses (MRVs) were used as the pathogen indicators on two pig farms. Although 138 PSVs were detected from a total of 199 fecal specimens of healthy pigs collected from two farms in Japan, no PSV was isolated. On the other hand, 12 MRVs were detected and 10 were isolated from the collected feces in one of two farms. The detection of MRVs has declined after June 2020, probably due to adoption of disinfection with QAC diluted with 0.17% FdCa$(OH)_2$ solution (Mix-500) that restricted MRVs infection among pigs. By sequencing based on the partial S1 gene of MRVs, MRV isolates were classified as MRV1 and MRV2. Additionally, the virucidal activities of disinfectants toward the isolated MRV1 were evaluated by the suspension and carrier tests in the presence of organic materials in similarity to the method used in Chapter 1. The evaluated disinfectants were the following: QAC-500, FdCa$(OH)_2$, Mix-500, sodium hypochlorite at 100 ppm of total chlorine (NaClO-100) and NaClO-1000. In the suspension test, all disinfectants except for NaClO-100 efficiently inactivated MRV1 within 1 min. In the carrier test with the dropping technique, 0.17% FdCa$(OH)_2$, Mix-500 and NaClO-1000 required 5 min to efficiently inactivate MRV1, whereas it took 30 min for QAC-500 to efficiently inactivate the virus. With the wiping technique, Mix-500 and NaClO-1000 could inactivate MRV1 below the detection limit on the carrier and to the effective level on the wiping-sheet, respectively, within 30sec. The results of this study suggest that PSV are widespread in pig farms in Japan. On the other hand, although different serotypes of MRVs are circulating among pigs, the occurrence of MRVs in the farms decreased as a result of the disinfection

using Mix-500.

In this study, convenient and accurate evaluation methods of virucidal activity of disinfectants have been established and utilized. Moreover, the virucidal activity of SAHWs toward viruses has been evidenced by means of tests simulating the practical application of appropriate disinfectants. In addition, although PSV infection among pigs could not be controlled, effective disinfection markedly restricted MRVs infection, which suggests that further development of effective strategy is required to reduce PSV. It is considered that this research contributes potential benefits to the livestock industry from a biosecurity perspective and reduces the occurrence of pathogens.

和文要旨

これまで世界中の畜産業では，家畜の感染症による経済的な損失を抑えるために様々な防疫方法を開発・発展させてきた。しかし近年，従来の防疫方法では対策が困難な感染症が発生しており，バイオセキュリティの強化のためにはさらなる防疫方法の開発と発展が求められている。防疫方法の一つとして化学的障壁，すなわち消毒剤を用いた病原体の不活化が挙げられる。消毒剤評価法に基づいて，病原体に対して有効な消毒剤の選定は行われる。しかし現在，物質表面上のウイルスに対して拭き取りで利用するための評価法は簡易かつ正確なものではない。また，空間消毒で利用するためのガイドラインとして国際的に通用する評価法は確立されていない。そこで本研究では，適切な消毒による農場のバイオセキュリティの強化を目的として以下の実験・調査を行った。

第 1 章では，鶏伝染性気管支炎ウイルス（IBV）に対し液相混和試験と簡易なキャリア試験（滴下法と拭き取り法）を確立・実施し，消毒剤評価法の違いによる消毒資材のウイルス不活化効果を比較した。供試液には有効塩素濃度 1,000 もしくは 500ppm の次亜塩素酸ナトリウム（NaClO-1000，NaClO-500），0.17%食品添加物規格水酸化カルシウム溶液（$FdCa(OH)_2$），および NaClO-500 との混合液（Mixed-500），逆性石けん（500 倍希釈：QAC-500）を用いた。液相混和試験では 5%牛胎児血清（FBS）を含むウイルス液とそれぞれの供試液を反応させた。キャリア試験では 0.5%FBS を含むウイルス液をプラスチック表面に塗布・乾燥させ，供試液を滴下し 1 分間の反応（滴下法），もしくは供試液を染み込ませたレーヨンシートを用いてプラスチック表面を拭き取る動作を 30 秒間行った（拭き取り法）。液相混和試験では，

全ての供試液が IBV を 30 秒以内に検出限界未満まで不活化できた。滴下法では，NaClO-500 はプラスチック表面の IBV を 1 分以内に不活化できなかった。拭き取り法では，NaClO-500 を除く全ての消毒資材は，拭き取ったシート内の IBV が検出限界未満となった。拭き取り後，全ての消毒資材でプラスチック表面の IBV は検出限界未満となった。以上の結果から，液相混和試験とキャリア試験では同じ消毒資材を用いても不活化効果に違いが認められた。よって，二次スクリーニングとしてのキャリア試験は実際の現場を考慮した消毒剤評価法として必須であると考えられた。

第 2 章では，第 1 章で確立したキャリア試験法および噴霧法を用いて，物質表面上の IBV および鳥インフルエンザウイルス（AIV）に対する微酸性次亜塩素酸水（SAHW）の不活化効果を評価した。消毒剤には有効塩素濃度 62 から 540ppm の SAHW（SAHW-62，-119，-220，-300，-540）を用いた。また，空中に飛散した飛沫中の IBV に対する SAHW の不活化効果を調べた。空間消毒では 100 から 500ppm の SAHW（SAHW-100，-200，-300，-500）を用いた。空間消毒における SAHW と IBV の暴露時間は，ウイルスが噴霧されてから箱内を通過しサンプラーに回収されるまでの数秒間である。滴下法では，SAHW-220 はプラスチック表面の IBV を 1 分以内に不活化できなかったが，SAHW-62 は AIV を 99.9%以上不活化した。レーヨンシート上の AIV と IBV に対して，SAHW は 1 分以内では有効に不活化することが出来なかった。拭き取り法では， SAHW-220 による拭き取りでプラスチック表面の AIV と IBV は検出限界未満となったが，拭き取り後のシート内には AIV と IBV が残存した。噴霧法では，シート上に付着した AIV に対して SAHW-300 を 10 分間，IBV に対して SAHW-540 を 20 分間の

噴霧でそれぞれ検出限界未満にまで不活化した。空間消毒法では SAHW-300 および-500 を噴霧した結果，空気中の IBV が 93%と 97%それぞれ減少し，また統計学的に有意となった。以上の結果から，物質表面上および空気中のウイルスに対して SAHW を用いた様々な消毒法は非常に有効であると考えられた。

第 3 章では，養豚場のバイオセキュリティレベルの目安として豚サペロウイルス（PSV）および哺乳類オルソレオウイルス（MRV）を汚染標病原体とし、疫学調査を実施した。２つの養豚場の繁殖豚および肥育豚由来の糞便試料 199 検体を調べた結果，138 検体（69.3%）が PSV 陽性であったが，PSV の分離は出来なかった。また，1 農場の 12 検体のみが MRV 陽性となり 10 株の MRV が分離された。分離株の系統学的解析の結果，血清型は 1 型（MRV1）および 2 型に分類された。第 1 章で確立した方法を用いて，5%FBS を加えた MRV1 分離株の消毒薬抵抗性を評価した。供試液には $FdCa(OH)_2$，QAC-500 およびそれらの混合液（Mix-500），NaClO-100 および NaClO-1000 を用いた。滴下法では，$FdCa(OH)_2$，Mix-500，NaClO-1000 は MRV1 を 5 分以内に不活化した。拭き取り法では，Mix-500 および NaClO-1000 は拭き取ったレーヨンシート内の MRV1 を有効不活化レベルまで，プラスチック表面の MRV1 を検出限界未満まで減少させた。MRV 陽性農場への Mix-500 の導入以降も疫学調査の継続実施を行ったところ，MRV の検出率は減少傾向が認められた。以上の結果から，MRV に対する有効な消毒法の導入によりバイオセキュリティが強化されたと考えられた。また，PSV の蔓延防止のためには，本研究で得られた情報を基礎としてさらなる防疫方法の開発と発展が重要であると考えられた。