Studies on Detection of Avian Pathogens and Control of Avian Diseases by Enhancement of the Biosecurity of the Poultry Industry

（家禽病原体の検出と家禽産業のバイオセキュリティ強化による家禽疾病の制御方法に関する研究）

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DISSERTATION CONTENTS

Background

Poultry industry is constantly threatened by a wide range of pathogens, such as viruses, bacteria, fungi, protozoa and parasites. Such infectious microorganisms, which cause outbreaks of diseases at the poultry farms, still constitute a major problem for the poultry industry across the world. Different modes of pathogen transmission and their long term survivability make farmers unable to prevent disease outbreaks, and always facing them with lots of challenges. Despite applications of various preventative and controlling measures, such as vaccination, disinfection, all-in and all-out policy, installation of barriers around the farms to prevent contact of wild birds with poultry, plus good management systems in some developed countries, outbreaks of certain infectious diseases such as avian influenza (AI), Newcastle disease (ND), salmonellosis, colibacillosis and many more are happening and remain as main problem for poultry industry. Infectious disease outbreaks such as AI, ND, salmonellosis and colibacillosis annually cause millions of dollars loss for only the poultry industry, while outbreaks of such diseases are also threatening public health, as there are several avian diseases (zoonoses) that are transmissible to human through their direct contacts or via contaminated poultry products, across the world. Thus, these situations mean that further investigation is required to find-out better ways for diseases prevention and control at poultry farms. The present thesis assumes that the enhancement of biosecurity at farms can be the only and effective way to overcome the infectious disease outbreaks and to manage their prevention and control strategies. Searching for materials with capacity to inactivate various kinds of pathogens, alongside with evaluating their safety to farm animals and their consumers, are very necessary to choose and apply such materials for enhancement of biosecurity at the poultry industry.

Aims and outlines

The aims behind the present studies were to search for biosecurity enhancement materials that were obtained from different sources, and to evaluate their efficacies for inactivating various kinds of pathogens, under different conditions and in different ways, to confirm their safety towards farm animals, and finally to find suggested applications for such materials at the poultry farms, in order to enhance their biosecurity. And at the same time, another aim is to find sensitive detection systems for pathogens, which enable to monitor the biosecurity.

Three materials, namely slightly acidic hypochlorous acid water (SAHW) at pH 6.0, bioceramic (BCX) at pH 13, and food additive grade of calcium hydroxide ($FdCa(OH)_2$) at pH 13, were evaluated for their ability to inactivate avian pathogens. SAHW, a novel chlorine based solution was evaluated in the liquid form and in the spraying form, namely, through its direct exposure with pathogens in liquid, and via direct or indirect exposure through its spraying onto pathogens on the surfaces or in the air. Avian pathogens such as low pathogenic avian influenza virus (LPAIV) subtype H7N1, Newcastle disease virus (NDV) strain B1, *Escherichia coli* (*E. coli*) and *Salmonella* Infantis (*S.* Infantis), were used in order to find out SAHW's efficacy to inactivate pathogens in the mentioned ways and to prevent horizontal transmission via air and surfaces of contaminated objects by its application as an ideal disinfectant at poultry farms, thus demonstrating a perfect spraying system, in order to enhance biosecurity at the poultry production.

BCX at pH 13 derived from chicken feces and $FdCa(OH)_2$ originated from natural limestone were the other two materials, the efficacies of which were evaluated on bacteria (*E. coli* and *S.* Infantis) in feces, in order to prevent infection transmission via litter or contaminated poultry bedding materials to other animals, farms and the environment at large, and finally to enhance biosecurity at livestock facilities.

Abbreviations

AAF	Amnio-allantoic Fluids
AI	Avian Influenza
AIV	Avian Influenza Virus
ANOVA	Analysis of Variance
APEC	Avian Pathogenic *E. coli*
APMV	Avian Paramyxovirus
BCX	Bioceramic
C	Celsius
$Ca(OH)_2$	Calcium Hydroxide
CaO	Calcium Oxide
CFU	Colony Forming Unit
CK	Chicken Kidney
CPE	Cytopathic Effect
DHL	Deoxycholate Hydrogen Sulfide Lactose
DIVA	Differentiation of Infected from Vaccinated Animals
DNA	Deoxyribonucleic Acid
Dpc	Days post Challenge
Dpe	Days Post Exposure
dpi	Days Post Inoculation
dW_2	Double Distilled Water
E. coli	*Escherichia coli*
EMEM	Eagle's Minimum Essential Medium
ExPEC	Extra-Intestinal Pathogenic *E. coli*

FAO	Food and Agriculture Organization of the United Nations
FBS	Fetal Bovine Serum
$FdCa(OH)_2$	Food Additive Grade of Calcium Hydroxide
Fig	Figure
FT	Fowl Typhoid
GM	Growth Medium
GPV	Goose Parvovirus
HA	Hemagglutination
HACCP	Hazard Analysis Critical Control Point
HAIVP	Human Avian Influenza Virus Pandemic
HOCl	Hypochlorous Acid
HPAIV	Highly Pathogenic Avian Influenza Virus
IB	Infectious Bronchitis
IBD	Infectious Bursal Disease
IBDV	Infectious Bursal Disease Virus
JSFA	Japan Standard of Food Additive
LB	Luria-Bertani
loNDV	Low Virulence Newcastle Disease Virus
LPAIV	Low Pathogenic Avian Influenza Virus
M	Molar
MAFF	Ministry of Agriculture, Forestry and Fisheries
MDCK	Madin-Darby Canine Kidney
Min	Minute
ml	Milliliter

MM	Maintenance Medium
mM	Millimolar
mm	Millimeter
NA	Neuraminidase
NaOCl	Sodium Hypochlorite
NC	Not Challenged
ND	Newcastle Disease
Nd	Not Detectable
NDV	Newcastle Disease Virus
NDVs	Newcastle Disease Viruses
NMEC	Neonatal Meningitis *E. coli*
Nr	Not Remarkable
OCl^-	Hypochlorite Ion
OIE	Office International Des Epizooties
PBS	Phosphate Buffered Saline
PD	Pullorum Disease
ppm	Part Per Million
RBC	Red Blood Cell
RF	Reduction Factor
RNA	Ribonucleic Acid
RO	Reverse osmosis
S. Infantis	*Salmonella* Infantis
SAHW	Slightly Acidic Hypochlorous Acid Water
SD	Standard Deviation

SE	*Salmonella* Enteritidis
Sec	Second
ta	Converted Titer into Log_{10} of the Recovered Virus or Bacteria from Treated Samples
TCID	Tissue Culture Infectious Dose
tpc	Converted Titer into Log_{10} of the Recovered Virus or Bacterial Titer of Untreated Samples
UPEC	Uropathogenic *E. coli*
VN	Virus Neutralization
vNDV	Virulent Newcastle Disease Virus
w/v	Weight per Volume
w/w	Weight per Weight
WHO	World Health Organization
µl	Microliters
µm	Micrometer

Chapter 1

“General Introduction, Biosecurity, and Biosecurity Materials”

I. 1. General introduction

I. 1. 1. Poultry industry, principal pathogens, and the problems to be coped with

Poultry industry has played a unique role in the feeding of large world populations, by providing cheap and valuable sources of protein such as meat and eggs, along history, and is continuously playing its role at the present, as well as would it in the future. Millions of tons of poultry meats are produced across the globe, annually, as food for millions of world population [128]. In order to meet the increasing demands of world population, poultry industry needs to expand more. In 2014, world poultry meat production was around 109,970 thousand metric tons, while it was projected to reach more than 111,000 thousand metric tons in 2015 [138].

Poultry eggs are another valuable source of protein for the human being, which are produced at a rate of more than millions tons, annually, and both of the poultry products (meat and eggs) consumption by every individual person per year/kg is arising since year 2000 and is predicted to continue up to 2040 and forwards (https://www.wattagnet.com/ext/resources/uploadedFiles/WattAgNet/ Footer /Footer Content/2011_Poultry_Trends.pdf) [209].

Pathogens such as avian influenza virus (AIV), Newcastle disease virus (NDV), *Escherichia coli* (*E. coli*), *Salmonella spp.* and etc. are the infectious agents which principally affect the poultry industry, and cause outbreaks of diseases such as avian influenza (AI), Newcastle disease (ND), colibacillosis, salmonellosis and etc, and these infectious diseases are strongly threatening the poultry industry and public health, as there are several avian diseases with zoonotic potential, which are transmissible to humans via direct contacts with the infected poultry, or through consumption of contaminated poultry products [85, 104]. Significantly, increases in the global demands for poultry products, clearly pose a great challenge to be coped with in the control and prevention of infectious disease outbreaks, by enhancement of biosecurity at the poultry industry.

I. 1. 2. The general impacts of related pathogens on the poultry industry

Impacts of pathogens infecting poultry are vast, as they cause millions of dollars losses and constitute major problems for the poultry industry. Normally, infectious disease impacts in the poultry industry depend on the kinds of infectious agents and their transmissibility, farm location, and the countries strategies for disease control. For example, outbreaks of highly pathogenic avian influenza (HPAI) and virulent ND, which result in up to 100 % morbidity and mortality in the affected farms, are of very high impacts, and normally result in depopulation of the affected, and at times neighboring farms, in order to control their ongoing spread and for their eradication.

Among several infectious diseases of the poultry, the author selected four most common viral and bacterial infections, namely AI, ND, colibacillosis, and salmonellosis. Their causative agents have the potential to transmit to and infect the human and were taken understudy for evaluation of the selected candidate disinfectants for enhancement of biosecurity. Below, each of selected viral and bacterial infections of the poultry would be discussed, individually.

I. 1. 3. Avian influenza (AI)

AI is a disease with the ability of causing extremely high morbidity and mortality within poultry. It was described first in 1878 in Italy, by that time known as fowl plague [147]. Finally, an influenza A virus was identified as the causative agent of that fowl plague in 1955 [147, 165].

AIVs belong to the family of *Orthomyxoviridae,* the genus influenza virus type A. Due to its two different surface glycoproteins haemagglutinin (H or HA) and neuraminidase (N or NA) type A influenza virus has been divided into different antigenic subtypes, including eighteen for H (H1 to H18) and eleven for N (N1 to N11); theoretically, with the possibility of 198 different HA-NA combinations [8, 54, 63, 130, 192].

AIVs have been detected in 12 of the 50 Orders of birds at large, including 100 species of wild birds [36, 179, 205]. Among birds, AIVs are the most in

number, variety and prevalence in waterfowl, Order Anseriformes [74], which is compatible with the fact that AIVs are basically waterborne.

For its pathogenicity in infected birds, AIVs have been divided into two different groups: One comprises low pathogenic AIVs (LPAIV), which include all the avian subtypes (1~16 H and 1~9 N) and usually cause low pathogenic AI (LPAI) in birds, both domestic and wild. The second group includes only two subtypes, H5 and H7, which by definition may cause highly pathogenic AI (HPAI) in chickens, and in a variety of other domestic and wild birds, at large. In addition, there are a few subtypes, namely H17, H18, N10, and N11, which were detected in bats only [127, 192, 217]. All in all, this means that, viruses with H5 and H7 are sometimes LP and sometimes HP. In the poultry populations, foremost among chickens and turkeys, principally subtypes H7 and H5 can cause HPAI. According to the Office International Des Epizooties (OIE), from the end of 2003 to April 10th 2016, outbreaks of HPAI (H5N1) have been reported from 54 countries across the world, and among them, Vietnam stands in the top with 2,744 outbreaks [215]. Since the first report of a HPAI outbreak in 1959 [146], it still remains as high-priority concern of the poultry industry.

AIVs are highly contagious and easily spread through droplet of upper respiratory system of an infected bird, in addition to fecal-oral transmission, aerial transmission happens through direct contact with the infected poultry or via indirect contacts with their secretions. In nature, it is basically a waterborne virus. HPAI viruses are usually transmitted to the susceptible hosts via exposure to infected birds, feces, or respiratory secretions, and the transmission usually happens as a result of contaminated fomites movements, including people, equipments, vehicles and air [199].

Phylogenetic analysis of HPAI and LPAI viruses suggested that the outbreaks may occur as a result of a single introduction of LPAIV that subsequently undergoes mutations to the virulent form, occurring in a single lineage which would acquire ability of adaptation chiefly in the sense of pantropism of the virus towards

a new host species [16]. As outbreaks of HPAI bring about up to 100% morbidity and mortality in gallinaceous poultry, and occasionally in wild birds, this disease is hence included in the OIE-listed diseases, infections and infestations in force in 2016 [214].

Control and eradication of HPAI outbreaks are cost-effective [73]. For example, outbreaks of HPAI in the United States of America (USA) from December 2014 to June 2015 resulted in euthanizing of more than 48 million birds, which generated doubling of egg price [200]. In developed countries such as the USA, the authorities implement control measures that include surveillance and diagnosis, quarantine and movement control, epidemiological investigations, appraisal and compensation, depopulation of affected and endangered poultry, carcass disposal and finally cleaning and disinfection. In addition, some developing countries like Indonesia, China, Egypt, Vietnam, and Korea are implementing active immunization of flocks as part of their control strategies, but vaccination could not stop outbreaks.

Japan experienced HPAI (H5N1) outbreaks for the first time in 1925 [123, 133]. Since then, there were several outbreaks of HPAI caused by H5N1 and H5N8 strains in different parts of Japan between December 2003 to January 2015 [123, 162, 163, 170, 201]. In all cases, series of control measures against HPAI were strictly and rapidly introduced. These measures were based on a stamping-out policy without vaccination, and movement control, cleaning, destruction, burial, and disinfection of affected premises [82]. HPAI outbreaks not only cause huge economical impact, but the outbreaks also raise concern for the public health. According to the World Health Organization (WHO), reported cumulative number of confirmed human cases of influenza type A reach to 854 cases by HPAIV subtype H5N1, reported from 16 countries, and Egypt stands on the top according to the number of cases (from 2003- 30 July 2016). Among them, 450 cases ended with death of the infected persons, with Indonesia on top according the mortality of infected persons [215]. In addition, a total of 10 laboratory-confirmed cases of

human infection with HPAIV subtype H5N6, including 6 human deaths have been reported from China since 2013 [212]. Although other H5 influenza viruses such as H5N2, H5N8, and H5N9, have the potential to infect humans, no human fatal cases have been reported by those subtypes, yet. Further, a total of 722 laboratory-confirmed cases of human infection with another avian influenza A viruses subtype H7N9, including 286 human deaths also have been reported [212]. AIV infections, especially in the current epidemiological situation, represent a risk, in terms of both the direct infection of humans from the avian hosts, and the consequences of genetic re-assortment between a mammalian IV and AIV, which could be the base for generation of a new pandemic virus [145, 149, 198, 208]. That is an additional reason for a critical need to be focused on the AI prevention and control by biosecurity enhancement of the poultry industry.

I. 1. 4. Newcastle disease (ND)

ND is highly contagious, with high morbidity and mortality rates and therefore an economically important viral disease of poultry. ND affects many species of birds and results in significant economic losses and trade restrictions [4, 9, 47, 161, 167].

Historically, the first outbreak of ND occurred in chickens in Java, Indonesia and in Newcastle upon Tyne region in England in 1926 [9]. ND takes its name from an outbreak of the disease that occurred in a poultry farm near Newcastle-on-Tyne of the England [51], and its name was chosen to differentiate the disease from HPAI [7]. In many countries, ND remains as one of the major problems and due to its devastating effects on the poultry industry, it is also included in the OIE-listed diseases, infections and infestations in force in 2016 [214]. There are 13 serotypes of avian paramyxovirus (APMV-1 ~ 13) capable of infecting avian species [11, 29, 93, 94, 129, 161]. The avian paramyxovirus serotype 1 (APMV-1), synonymous with NDV, belongs to the genus *Avulavirus* in the subfamily of *Paramyxovirinae* and the family *Paramyxoviridae* [108], and encompasses a diverse group of enveloped single-stranded, negative sense,

nonsegmented RNA viruses, that contain six genes and encodes seven proteins [44]. The virulent form of virus that exhibits an intracerebral index (ICPI) of ≥ 0.7, is the cause of ND which must be reported to OIE [143, 216].

NDV is also highly pathogenic to turkeys, and the clinical signs are similar to those in chickens but are less severe [10, 25]. Ducks, geese, pigeons and doves, upland game birds, cormorants, and pet birds (Psittacine) are other hosts which are infected by NDV [134, 175]. Overall, APMV-1 is known to infect more than 250 bird species affiliated with 27 orders, however, other avian species are also likely to be susceptible [5, 39, 92, 98, 161].

Epizootics of ND continue to occur on a regular basis in Central and South America, Africa, and Asia, while sporadic epizootics occurs in Europe [9]. Where chickens are raised commercially, either in developing or developed countries, ND outbreaks have occurred in many locations, causing massive economic damage through control efforts and trade loss. For instance during the last major outbreak in California, in the USA, it cost of 160 million US dollar to control the outbreaks [34].

The primary route of NDV transmission is either by ingestion of contaminated objects or inhalation of droplets containing the pathogen, as infected birds are normally shedding the virus through their feces and respiratory discharge [9, 113, 167]. When the virus reaches the mucous membranes of susceptible birds, the virus is likely to infect the upper respiratory tract. Thus, newly infected birds allow for the potential to expose more birds and the virus easily spreads through the flocks [9, 161].

In the USA, the first case of a virulent ND outbreak was reported in 1950 [72]. In 1971, a major outbreak occurred in commercial flocks in southern California after arrival of a shipment carrying infected parrots from Latin America and was eradicated back in 1974 [202]. The most recent outbreak in the USA occurred in 2003 - 2004, affected poultry industry in several states: Arizona, California, Nevada, and Texas. The outbreak resulted in depopulation of 3.4 million

birds, California alone spent more than 160 million US dollar to control the outbreak, and the USA has been free of ND outbreaks since 2004 [34, 35].

In Japan, the first ND outbreaks were recorded in 1930 [131], and large outbreaks continued until a live vaccine (Hitchner B1/47 strain) was approved for application to stop virulent NDV (vNDV) in 1967 [205]. Since then, fewer outbreaks occurred in Japan in small flocks or in some commercial poultry farms that were not immunized against NDV or were vaccinated improperly [123, 196].

The first human infection with NDV, which resulted from a laboratory accident, was reported in 1942 [32]. Human ND cases are most often associated with direct contact with either birds or concentrated virus. It produces a subclinical and at times local and systemic response in humans, which is usually limited to a transient conjunctivitis not affecting the cornea.

I. 1. 5. Colibacillosis

Avian colibacillosis is a bacterial infection of the poultry, caused by a group of pathogenic avian *E. coli* strains. *E. coli* is considered as one of the principle pathogens, causing morbidity and mortality of the poultry, associated with huge economic loses [140]. Although colibacillosis is known for over a century, it still remains as one of the major endemic diseases affecting the poultry industry, worldwide. Currently, *E. coli* strains causing systemic disease (avian colibacillosis), in poultry are termed as avian pathogenic *E. coli* (APEC), which is presently considered an outstanding pathogen for poultry industry, due to severe economic losses [53, 117].

E. coli isolates from colibacillosis cases are often being subjected to biotyping and serotyping. In most countries, *E. coli* O1, O2, and O78 serogroups represent the major isolates from infected birds [42, 43, 57, 176], while other serogroups such as O6, O8, O21, O46, O88, O106, O111, and O143 are also involved in chicken infections [102]. Among them, serogroups O2 and O78 are particularly associated with colibacillosis outbreaks in poultry and represent 80% of disease cases, worldwide [53].

As *E. coli* mainly colonizes the gastrointestinal tract of infected animals, clinical or sub-clinical infected animals normally shed *E. coli* through their feces into the environment [90, 139]. *E. coli* can survive for long time in water, lettuce, soil, feces, manure, and porous and non-porous surfaces; such ability increases its chance for transmission to another hosts, flocks, and farms [14, 89, 107].

Among the many transmissible infection of poultry to human, *E. coli* is also a potential food-safety pathogen, and colonization by antibiotic resistant *E. coli* from the intestinal tract of chickens and turkeys, have been shown in human volunteers [204]. Furthermore, studies reported involvement of several mammals and birds species as reservoirs for human extra-intestinal pathogenic *E. coli* (ExPEC) serogroups [21, 58, 174], and that includes the Uropathogenic *E. coli* (UPEC), and neonatal meningitis *E. coli* (NMEC), with APEC sub-pathotypes [58].

I. 1. 6. Salmonellosis

Avian salmonellosis such as fowl typhoid (FT) and pullorum disease (PD) are considered to be other major bacterial diseases of the poultry industry, worldwide. Salmonellosis is a bacterial infection of poultry caused by *Salmonella* spp. by its association with various diseases conditions, as a primary or secondary source of infections [171, 203]. *Salmonella* serotypes have been isolated from poultry, human, cattle, pigs, insects, rodents, and wild birds which are living inside or around the hen houses [12, 13, 126].

Salmonellosis is important in the poultry industry, because of causing clinical and subclinical disease of chickens, and food-borne diseases of humans. In the *Enterobacteriaceae* family; the genus *Salmonella* is a Gram negative and facultative intracellular pathogen that causes localized and systemic infections, with chronic asymptomatic carrier state [171, 172]. Based on the internationally recognized standard method of *Salmonella enterica* serotyping in the USA and the Kauffman-White scheme, according the O (somatic) and H (flagellar) surface proteins, the species now includes > 2,500 different serotypes [28].

According to pathogenic characteristics, *Salmonella* is divided roughly into two groups; the one that includes the majority of recognized serovars, and those which produce gastroenteritis in human by virtue of their ability to colonize the alimentary tract of poultry and contaminate carcasses during processing, thereby easily entering the food chain and transmitting to humans [2, 194].

FT caused by *Salmonella* Gallinarum is an acute or chronic septicaemic disease that usually affects adult birds, although birds of all ages may be infected. PD caused by *Salmonella* Pullorum is an acute systemic infection of young birds. This pathogen was identified in the beginning of the 19th century and was associated with endemic intermittent mortality or with outbreaks characterized by mortality of 100% and reduction in the productivity [100, 153-155] with huge economic loses [150, 171]. Although FT and PD have been eliminated from many countries by serological detection and selective slaughtering of the infected birds, they still cause outbreaks in some commercial farms [18, 150].

Salmonellosis is an important food-borne pathogen, worldwide. Majowicz, *et al.* [120] estimated that approx. 93.8 million human cases of gastroenteritis and 155,000 deaths occur due to *Salmonella* infections, annually, across the world. Also, salmonellosis is a common infection of humans in the USA, with an estimation of 1.4 million cases occurring annually [207], which result in 15,000 hospitalizations and 400 deaths every year. However, among the *Salmonella* spp, *Salmonella Enteritidis* (SE) is the main cause of human infections in the USA, and mainly its 4 serotypes are responsible for almost one-half of all human isolates [26]. At the beginning of 1970s, the incident of salmonellosis dramatically increased in the USA, and by 1994, it was the most commonly reported agent with an incident of >10 laboratory–confirmed infection per 100,000 population [26]. Contaminated eggs, eggshell, and contaminated chicken meat are identified as the major vehicle of SE infection to the human.

I. 2. Farm's biosecurity

"Biosecurity" stands for all measures which are taken on hands in order to keep infectious diseases away from farms, and includes all preventive and control measures for coping with infectious agents. Biosecurity forms the first line of defense against infectious agents. Quick and simple measures built into daily practice will help farmers to protect their farms, their bank balance, and their industry from the costly consequence of disease outbreaks. The objectives of a good biosecurity practice at farms include prevention of the introduction of infectious agents into the farms, prevention of disease agents spread from one infected area or flock to another, and minimizing the incidence of diseases and their public health concerns.

Biosecurity measures are normally divided into two parts, namely offensive and defensive. To establish a good biosecurity practice, it is important to analyze and identify the risk, to find out the critical points in terms of pathogen introduction and spread, and finally to implement good control measures according to the level of risk. According to the FAO animal production and health manual, cleaning and disinfection of all surfaces (cages, walls, and poultry feeding and water areas) regularly should be performed to prevent bird flu, which clearly highlights the role of biosecurity in prevention of diseases [61].

I. 3. Biosecurity materials

I. 3. 1. Slightly acidic hypochlorous acid water (SAHW): SAHW is a novel chlorine-based solution with slightly acidic pH at 6, produced through electrolysis process from saturated brine by OSG Co., Ltd. (Osaka, Japan). In the condition of pH at 6 the ratio of hypochlorous acid (HOCl) molecule is 97.18 % and hypochlorite ion (OCl-) is 2.82 % at 25 °C [211].

Normally, chlorine-based solutions include different chlorine species, such as chlorine gas (Cl_2), hypochlorous acid (HOCl), hypochlorite ion (OCl^-) and sodium hypochlorite (NaOCl), while their concentration and presence in the solution are mainly related to the pH value of the solutions. Among them, HOCl is the most active ingredient, with the highest oxidative potential, because the

uncharged “HOCl” molecule, but not “OCl^-” ion, can pass cell membranes effectively [211]. It has a high capacity for killing pathogens by irreversibly denaturing the critical components of cells, such as nucleic acids (DNA/RNA), mitochondria, enzymes and surface proteins [211]. Chlorine-based solutions at the pH 4-5, include HOCl about >99 %, and in the mentioned pH, they are very strong and broad spectrum disinfectants [211]. But solutions in such low pH as 4-5, seem to be corrosive for the metallic objects, and harmful for the farm animals.

The novel product “SAHW” is a slightly acidic solution (pH 6), and contains high concentration of free available chlorine in the form of HOCl. Its specific disinfection capacity towards different pathogens, its safety to farm animals, and its non-corrosivity for the metallic objects are to be confirmed.

I. 3. 2. Bioceramic (BCX): BCX powder is another novel product from NMG Environmental Development Co., Ltd. (Tokyo, Japan), which is produced from chicken feces through sintering process. Within the 24 hr sintering process at 800 °C, chicken feces were heated under alkaline conditions, so that all carbon and nitrogen were removed from the feces, and the final product, so called BCX was harvested (Fig. 1. 1).

Fig. 1. 1 Processing plant of producing bioceramic by NMG Environmental Co., Ltd.

BCX at pH 13 contains calcium oxide (CaO) as its active ingredient, but its capacity to inactivate pathogens also depends on its pH value. Increase in the BCX pH, enhances its disinfection capacity. BCX acts as a trapping agent that absorbs the pathogens and causes their inactivation, but the real disinfection mechanism is not clear, yet. BCX disinfection property to inactivate viruses, already have been evaluated by Takehara *et al.* [185], as they demonstrated its efficacy to inactivate AIV for the first time. Then, Thammakarn *et al.* [188] reported its capacity to prevent fecal-oral transmission of infectious bursal disease virus (IBDV), alongside with its safety to chicks.

I. 3. 3. Food additive grade of calcium hydroxide ($FdCa(OH)_2$): $FdCa(OH)_2$ is a white dry alkaline powder, which is made from natural calcium carbonates derived from limestone through calcinations process by Fine Co., Ltd. (Tokyo, Japan), and contains more than 97% calcium hydroxide ($Ca(OH)_2$) as its main composition (Table 1. 1). Historically, $Ca(OH)_2$ has been widely used as cost-effective biosecurity material to control certain pathogens. Besides that, slaked lime is listed as an effective disinfectant in many national regulations and guidelines. It is recommended as in situ disinfectant on a regular basis and in case of epidemic outbreaks of diseases such as avian influenza, Aujeszky's diseases and African swine fever [56, 62, 103]. Slaked lime has been used as a trapping agent in Japan around the livestock farms, in order to enhance livestock farms biosecurity.

The focuses of the present studies were based in the below points:

1. To help farmers to enhance their farm's biosecurity by means of introducing ideal biosecurity materials - foremost disinfectants - to them, which they can be safely applicable at the presence of farm animals, and can be useable as both offensive and defensive measures at the poultry industry.
2. To evaluate SAHW disinfection capacity towards different viruses and bacteria, alongside with its safety to farm animals and objects present at farms, in order to confirm SAHW being a good biosecurity material for the poultry industry.

3. To evaluate BCX for its further efficacy to inactivate bacteria in feces in the powder form, alongside with its bactericidal durability in litter, in order to prove its capacity as a broad spectrum disinfectant, and finally to suggest its application as an environment-friendly biosecurity enhancement material at the poultry farms.
4. To evaluate FdCa$(OH)_2$ efficacy against bacteria with a perfect method, in order to find out the exact concentration and required time for its bactericidal efficacy, alongside with its durability in litter, so as to help farmers to upgrade their strategy for enhancing their farms biosecurity.

Table 1. 1 $FdCa(OH)_2$ compositions analyzed by Fine Co., Ltd. (Tokyo, Japan) and its comparison to Japan standards of food additive (JSFA).

Compositions	$FdCa(OH)_2$ (%)	JSFA (%)	Acceptable/ denial
$Ca(OH)_2$	97.06	> 95.0000	Acceptable[a)]
Hydrochloric acid (HCl) in soluble matter	0.010	< 0.0500	Acceptable
Carbonate (CO_3)	Nd[b)]	Nr[c)]	Acceptable
Diarsenic trioxide (As_2O_3)	0.008	< 0.0004	Acceptable
Led (Pb)	ND	< 0.0040	Acceptable
Barium (Ba)	ND	< 0.0300	Acceptable
Magnesium (Mg) and others	0.560	< 6.0000	Acceptable

a) Acceptable according to the Japan standard for food additive (JSFA).

b) Not detectable.

c) Not remarkable.

Chapter 2

“Efficacy of Slightly Acidic Hypochlorous Acid Water to Inactivate Viruses”

II. 1. Introduction

Outbreaks and fast transmission of some avian viral diseases like avian influenza (AI), Newcastle disease (ND), and infectious bronchitis (IB), with their high morbidity and mortality rates, are largely attributed to infection via aerosol [48, 68, 76, 112, 169, 178, 219]. Considerable amounts of airborne pathogens are often present in poultry farms. They reduce the productive capacity of the poultry, and act as a potential threat for the poultry industry, as well as for the farms personnel [30, 66, 69, 221, 223]. Infected birds shed viruses directly to the air by droplets during sneezing or coughing, and indirectly through feces [161, 167, 177], thus contaminating the air and floor of the farms and the objects which are nearby. AIV and NDV remain infective for long time in the environment, both on the surfaces of contaminated objects and in the air [79, 99, 161, 190]. Susceptible hosts contract those viruses directly via inhalation of contaminated air, or indirectly through ingestion of contaminated materials [3, 112]. Among the avian diseases, AI and ND are the most fatal, with large numbers of outbreaks [6, 7, 91, 167]. The stability and transmissibility of aerosolized AIV and NDV has been tested under different conditions [99, 112, 190]. As the virus is inhaled directly into the deeper respiratory system, smaller amounts of the virus are required to infect the chicks [45, 46, 101]. Inactivation of viruses found on the surfaces and in the air of the poultry farms will significantly reduce and or limit the chance for their circulation and outbreaks. Therefore, application of an ideal disinfectant with the capacity to inactivate AIV and NDV in the air and on the surfaces through an aerosol spraying system would be the best way to cope with these plagues.

Demonstration of such spraying system would limit the chance of infection transmission via contaminated air and objects, as well as reduce the chance of AIV and NDV circulation within the farms and flocks. Therefore, in this chapter the author evaluated slightly acidic hypochlorous acid water (SAHW) for its virucidal efficacy against AIV and NDV in the aqueous phase, and in direct and indirect spraying forms, in order to find SAHW's virucidal capacity, and to establish a

spraying system for its application towards the mentioned viruses on the surfaces of objects and in the air of the poultry farms.

II. 2. Materials and methods

II. 2. 1. SAHW: SAHW containing 50 ppm chlorine (SAHW 50 ppm) was prepared by a generator "Well Clean–TE" (OSG Co., Ltd., Osaka, Japan) in our laboratory, with normal tap water on the day of use. SAHW preparations containing 100 and 200 ppm free available chlorine concentration (SAHW 100 ppm and SAHW 200 ppm, respectively) were kindly supplied by OSG Co., Ltd.

II. 2. 2. Sprayers: A nanoscale aerosol sprayer "Nanoscale aerosol sprayer", was kindly provided by Nano-scale Co., Ltd. (Kawasaki, Japan), with the ability of spraying 500 ml/hr aerosol with a particle size less than 20 µm in diameter. In addition, aerosol sprayers in the form of nebulizer (NE-C28 Camp A-I-R), with ability of spraying 12 ml/hr and producing small aerosol particle (size <3 µm in diameter) were purchased from OMRON Corp. (Kyoto, Japan).

II. 2. 3. Spraying boxes: Plastic boxes (regular boxes in which spraying was conducted) measuring W360×D290×H112 mm and W513×D359×H230 mm were purchased from a local market.

II. 2. 4. Viruses: A low pathogenic AIV (LPAIV) A/duck/Aomori/395/04 (H7N1) isolated from wild ducks [86], NDV strain Sato [186] and vaccine strain B1 (shown below) were used during this study. Individual stocks of the AIV subtype H7N1 and NDV strain Sato were propagated in 10-day-old embryonated chicken eggs, individually, and viral infected amnio-allantoic fluid (AAF) was harvested on 3 days post inoculation (dpi) into 50 ml conical centrifuge tube. After harvesting, fluid was centrifuged at 440 × *g* for 15 min to sediment cell particles. Then, the supernatant was collected and aliquoted to the serum tube as 500µl/tube and stored at -80 °C. ND live vaccine (NDV- B1: lyophilized, 5,000 doses, > $10^{9.2}$ EID_{50}/vial) was purchased from Nisseiken Co., Ltd. (Tokyo, Japan). On the day of use, the ND vaccine was reconstituted in 50 ml of double distilled water (dW_2). After its

titration on primary chicken kidney (CK) cells (shown below in II. 2. 5.), the vaccine titer was 7.25 $\log_{10}$ $TCID_{50}$/ml.

II. 2. 5. Cell culture: Madin-Darby canine kidney (MDCK) cells were cultured in 96-well tissue culture plates. Eagle's minimum essential medium (EMEM; Nissui pharmaceutical Co., Ltd., Tokyo, Japan), supplemented with 5 % fetal bovine serum (FBS), 0.3 mg/ml L-glutamin, 1.4 mg/ml $NaHCO_3$, and antibiotic-fungicide cocktail (100 IU/ml penicillin, 0.1 mg/ml streptomycin and 0.5 µg/ml Amphotericin B) were employed to grow the cells. Cell monolayer containing 1 µg/ml trypsin (final concentration) (trypsin, from bovine pancreas 10,000 BAEE units/mg protein, Sigma, St. Louis, MO, USA), was subjected to AIV, while the MDCK cells with no trypsin were used for NDV titrations and their titer was determined as 50 % tissue culture infective dose ($TCID_{50}$)/ml according to the Behrens and Kärber's method [124]. CK cells were prepared from kidneys of 1 to 7 day-old chicks as described previously [95, 197]. Briefly, chicks were dissected and their kidneys were removed using aseptic technique, and then kidneys were washed with phosphate buffered saline (PBS: 0.14 M NaCl, 2 mM KCl, 3 mM Na_2HPO_4, 1.5 m KH_2PO_4, pH 7.4). After stirring with a magnetic bar for 5 min, tissues were treated sequentially for three to four times trypsinization for 5 min with trypsin EDTA (0.05 % trypsin, 0.05 mM EDTA, in PBS). The resulted cell suspension was centrifuged at 440 ×*g* for 5 min. Then the cell pellet was re-suspended in growth medium described above and cultured in plates at 0.3 % cell concentration. The cell suspension was seeded at 4 ml onto tissue culture dishes of 60 mm in diameter and 100 µl per well, over 96 micro-plates.

II. 2. 6. Determination of spraying time for inactivation of AIV: Reverse osmosis (RO) water was sprayed by Nanoscale aerosol sprayer directly on to the 3×3 cm double fold rayon sheets from 30 cm distance (sprayer nasal to dish) or indirectly inside a spraying box for different spraying times, then the amount of RO water present on the rayon sheets was observed for its humidity, and its weight was checked by balance for determination of water amount. Besides that, within 10 sec

direct spraying onto the rayon sheets, around 280 µl RO water was present in the sheets. But, for indirect spraying form into the box, for 10 sec spraying time, box was found full of RO water particles. Therefore, 10 sec spraying time was selected as desired duration of spraying time for SAHW evaluation.

II. 2. 7. Sampling procedure and virus isolation: Oropharyngeal swabs were collected using a rayon cotton bulb swab from Eiken Chemical Co., Ltd. (Tochigi, Japan), from 2-5 days post-exposure (dpe) of chicks to ND live vaccine via aerosol spraying, from all chicks. The swabs were put in vials containing transport medium (3.7 % brain heart infusion broth, 1,000 IU/ml penicillin, 1 mg/ml streptomycin, and 5 µg/ml Amphotericin B) [144], vortexed and kept for 1 hr at room temperature (25 ± 2 $^{\circ}$C), then stored at -30 $^{\circ}$C up to the day of inoculation. Swab samples were titrated on a monolayer of CK cells in 96 well micro-plates. Serial ten-fold dilution was prepared per swab sample in maintenance medium (MM) described below and inoculated to CK cells seeded in 96 well-plates of 100 µl/ well and 4 wells per dilution. Cytopathic effects (CPE) were observed for daily in the inoculated plate, and hemagglutination (HA) test was performed at 5 dpi to confirm the result. MM was prepared from EMEM supplemented with 100 IU/ml penicillin, 100 µg/ml streptomycin, 0.5 µg/ml Amphotericin B, and 4 mM L-glutamine.

II. 2. 8. Virus neutralization (VN) test: VN test was performed in CK cells to calculate chicken's maternal antibody titer by plaque-reduction method, with a constant amount of virus and varying serum dilution as previously described [158]. Briefly, sera samples were collected from commercial chicks, without any vaccination, hereafter designated "conventional chicks" before virus spraying (at 3 day-old), diluted in a serial four-fold dilution in PBS and mixed with the equal volume of NDV strain Sato [186]. The neutralizing antibody titer was calculated at 50 % plaque reduction point by Behrens-Kärber's method [124].

II. 2. 9. Animal: Animal work was performed in strict accordance with animal care guidelines of Tokyo University of Agriculture and Technology (Tokyo, Japan) with permit numbers 25-37 and 26-45. Day-old conventional chicks were purchased

from Kanto Co., Ltd. (Gunma, Japan), labeled and settled in rat cages (CLEA-0108-3, Clea Japan, Inc., Tokyo, Japan) inside the isolator (CL-5443, Clea Japan, Inc.), and kept up to becoming three-day-old, then used for the experiments.

II. 2. 10. Calculation of reduction factor (RF): The RF was determined using the following equation.

$$RF = tpc - ta$$

Where *tpc* is the titer of untreated sample in $\log_{10}$ units, while *ta* is the titer of recovered virus from treated samples. Inactivation was acceptable when RF was found greater than or equal to 3 [114, 185, 189].

II. 2. 11. AIV inactivation

II. 2. 11. 1. Assay in liquid:

A. Two hundred twenty five microliters of SAHW 50 ppm or SAHW 100 ppm was mixed with 50 µl of AIV (titer about 7.7 $\log_{10}$ $TCID_{50}$/ml) in a reaction tube and kept for 5 sec exposure time. Then 225 µl of FBS was added on SAHW-virus mixture to stop SAHW's activity in order to know the exact required time for AIV inactivation. Then serial ten-fold dilution was performed and inoculated on MDCK cells as described above for titration of the remaining virus. To confirm whether adding of 225 µl of FBS can stop activity of the same volume of SAHW, first, they were mixed in the same volume in a reaction tube, and then 50 µl of AIV (titer about 7.7 $\log_{10}$ $TCID_{50}$/ml) was inoculated into their mixture. As there was no contact between the virus and SAHW before adding FBS, it was marked as 0-sec contact.

B. SAHW was sprayed by Nanoscale aerosol sprayer and harvested back using a petri dish from 1 or 30 cm distance (sprayer nasal to dish), respectively and challenged with AIV, applying the same design and the same exposure times as in A, in order to know whether still it retains its virucidal efficacy. For the positive control, 50 µl of AIV (titer about 7.7 $\log_{10}$ $TCID_{50}$/ml) was inoculated into 450 µl MM in a micro tube as a positive control. All the experiments were carried out in triplicate (including II. 2. 11. 2., and II. 2. 11. 3.).

II. 2. 11. 2. Assay through direct spraying: Hundred microliters AIV (titer about 7.7 $\log_{10}$ $TCID_{50}$/ml) was inoculated on to the 3×3 cm double fold rayon sheet, placed on to a 5×5 cm glass plate inside of a 90 mm diameter petri dish without lid, then transferred into the spraying box. Different concentrations of SAHW were subsequently sprayed by Nanoscale aerosol sprayer directly to the AIV spiked on rayon sheets from 30 cm distance (spray nasal to dishes) (Fig. 2. 1), for a certain spraying time (10 sec). RO water was sprayed for the positive control and the procedure was run at the same condition as treatments. After spraying, the samples (rayon sheet and glass together) were transferred directly or after a few minutes contact time into stomacher bags (size 100×150×0.09 mm, capacity 80 ml; Organo Co., Ltd., Tokyo, Japan), containing 900 µl MM to stop the SAHW activity, and to harvest the remaining virus using Bagmixer® 100 "MiniMix®" CC® (Practical Japan Inc., Chiba, Japan). Serial 10-fold dilution/sample was prepared in MM and inoculated to the MDCK cells for remained virus titration according to the procedure described above.

II. 2. 11. 3. Assay through indirect spraying form: Hundred microliters of AIV (titer about 7.7 $\log_{10}$ $TCID_{50}$/ml) was inoculated on the rayon sheets placed on the glass plate as in II. 2. 11. 2., but the samples were transferred and kept in petri dish with the lid closed during SAHW spraying inside the spray box (Fig. 2. 2), for certain spraying time (10 sec). After stopping SAHW spray, the lid of petri dish was removed and the lid of the spray box closed, immediately, and kept for 10 min contact time to let the sprayed SAHW particles react with the virus. RO water was sprayed for the positive control and same procedure was applied for them, as in II. 2. 11. 2.

II. 2. 12. NDV inactivation

II. 2. 12. 1. Direct exposure to NDV strain Sato: To confirm the virucidal efficacy of SAHW against NDV strain Sato, evaluation was conducted according the same design as explained in II. 2. 11. 1. Then, the samples were titrated on

MDCK cells in a 96 micro-plate to calculate the remaining virus titer, as previously described.

II. 2. 12. 2. Aerosol delivery of NDV strain B1 and the effects of maternal immunity: The experiment was conducted to find out chickens' maternal immunity titer, with the required dose of vaccine to infect chicks to the maximum (100 %). Three-day-old conventional chicks were numbered, and 0.5 ml blood was collected per chicks prior of their exposure to the vaccine spray, in order to analyze the maternal antibody titer. Then, the chicks were divided into four groups of 5 birds and placed in the spraying boxes, separately. Different doses of NDV B1 (10, 25, 50, and 100 doses/2 ml) were prepared from the vaccine solution (100 doses/ml) with PBS, and the 2 ml of volume was sprayed completely into each box within 9 min by nebulizer. The lids of the boxes were kept closed for 5 min after spraying to let chicks inhale the virus. Then, the chicks were transferred to their cages in a single isolator. Chicks were observed for clinical signs, and oropharyngeal swab samples were collected from 1 to 6 dpe per chicks into viral transport medium to isolate the virus, followed by chick dissection at 6 dpe. Virus neutralization assay was performed to find their maternal antibody titer. This experiment was repeated for two times, except for the group exposed to 100 doses.

II. 2. 12. 3. Aerosol disinfection capacity of SAHW in the air: This experiment was designed to evaluate virucidal capacity of SAHW towards NDV in the air. Fifteen 3-day-old conventional chicks were numbered and divided into three groups of five birds. The vaccine dose of NDV determined in experiment II. 2. 12. 2. as such that causes 100% infection towards conventional chicks has been sprayed on each group in the spraying box, separately (Fig. 2. 3). Control group was sprayed with RO water and treatment groups were sprayed with SAHW 50 ppm or SAHW 100 ppm. SAHW or RO water was sprayed for 13 min; from 2 min before to 2 min after the NDV spraying, which was conducted, thus, for 9 min (Fig. 2. 4). Chicks were kept in the box with lid closed for 5 min to let them inhale the virus, if remained, then transferred to their cages in the isolator. Clinical signs were

observed for, and sampling was performed from 2 to 5 dpe, as in II. 2. 12. 2. Chicks were dissected at 5 dpe after sampling. This experiment was repeated for three times, except for 50 ppm treatment (repeated twice).

II. 2. 12. 4. Influence of SAHW on the normal chick growth: This experiment was designed to understand whether SAHW affects the chick's normal growth or causes any abnormal condition. Fifteen 3-day-old conventional chicks were divided into three groups of five birds, one group for the control and two for the treatments. For treatment 1, chicks were weighted and placed in the spray box/group, then SAHW 100 ppm was sprayed for 6 days, daily for 13 min, twice: once in the morning and once in the afternoon; whereas for treatment 2, SAHW 100 ppm spraying was conducted once a day, every morning, for a total of 6 days. Control group received RO water spray for 6 days, twice a day, as in treatment 1. Chicks were observed for any abnormal condition during the experiment. After 6 days of SAHW spraying, chicks were weighted and their body weight was statistically analyzed using Prism v.6.05 (Trail) software (GraphPad Software Inc. La Jolla, CA, USA), and values are expressed as mean ± standard deviation (SD). One-way ANOVA followed by Bonferroni's multiple comparisons test was used to analyze and compare body weights between the control and treatments. This experiment was done for one time.

II. 3. Results

II. 3. 1. Results obtained from AIV inactivation experiments

When AIV was used without dilution, AIV titer was decreased more than 3 $\log_{10}$ $TCID_{50}$/ml with SAHW 200 ppm, but not with SAHW 100 ppm or SAHW 50 ppm. Therefore, AIV was diluted ten times with PBS (pH 7.4), just before used in the experiments. As ten times diluted virus was used, the virus detection limit was ≥ 2.5 $\log_{10}$ $TCID_{50}$/ml. At the 0-sec point, whereby 50 % FBS was mixed with SAHW before inoculation of AIV, the titer of virus did not decrease at all (RF = 0.00). This means that virucidal efficacy of SAHW was stopped at any time by adding 50 % FBS to the reaction tube.

II. 3. 1. 1. Inactivation in liquid: Table 2. 1 summarizes inactivation of AIV in liquid form. Original SAHW 50 ppm that was not sprayed, and its harvested solution after spraying from 1 cm distance reduced titer of AIV to lower than detectable level (≤ 2.5 $\log_{10}$ $TCID_{50}$/ml) (RF ≥ 5.2) within 5 sec of contact time; however, its harvested solution after spraying from 30 cm distance did not reduce titer of AIV to the acceptable level (≥ 3 $\log_{10}$ $TCID_{50}$/ml) (RF = 1 ± 0.68). It could not further reduce the titer of virus even with longer incubation times (3 min). In comparison to SAHW 50 ppm sprayed, SAHW 100 ppm and SAHW 200 ppm harvested from the same distance (30 cm), could reduce the titer of AIV down to lower than detectable level (RF ≥ 5.3).

II. 3. 1. 2. Inactivation by sprayed SAHW: Table 2. 2 summarizes the results of AIV inactivation through direct spraying form. Within 10 sec spraying time, SAHW 50 ppm reduced the titer of AIV from 5.5 $\log_{10}$ $TCID_{50}$/ml to 4.17 $\log_{10}$ $TCID_{50}$/ml (RF = 1.33) directly after spraying, which is lower than acceptable level. But while it was kept for 3 min contact time after stopping SAHW spraying, it reduced the titer of AIV down to lower than detectable level (≤ 2.5 $\log_{10}$ $TCID_{50}$/ml) (RF ≥ 3.2). In addition to that, SAHW 100 ppm and SAHW 200 ppm were able to reduce the titer of AIV to lower than detectable level (≤ 2.5 $\log_{10}$ $TCID_{50}$/ml) (RF ≥ 3.1), immediately after their spraying.

II. 3. 1. 3. Inactivation through indirect spraying: Table 2. 3 show the results of SAHW efficacy via indirect spraying. By 10 sec spraying of SAHW inside the spraying box, and 10 min contact time, SAHW 50 ppm reduced the titer of AIV from 5.5 $\log_{10}$ $TCID_{50}$/ml to 4.5 $\log_{10}$ $TCID_{50}$/m*l* (RF = 1.00), and SAHW 100 ppm brought about a reduction from 5.96 $\log_{10}$ $TCID_{50}$/m*l* to 4.02 $\log_{10}$ $TCID_{50}$/m*l* (RF = 1.94) which is under the acceptable level, while SAHW 200 ppm reduced AIV titer to lower than detectable level (RF $\geq$3.28).

When AIV titer 6.7 $\log_{10}$ $TCID_{50}$/ml was inoculated on the rayon sheets, the recovery ratio through stomacher was around 5.7 $\log_{10}$ $TCID_{50}$/ml, which is ten times lower than the inoculated virus titer.

II. 3. 2. Results obtained from NDV inactivation experiments

II. 3. 2. 1. Inactivation of NDV strain Sato: In the aqueous phase experiment, the vNDV strain Sato was inactivated down to the detection limit ($\leq$ 2.5 $\log_{10}$ $TCID_{50}$/ml) within 5 sec (data not shown).

II. 3. 2. 2. Evidencing 100% of chicken's infectedness and their maternal immunity titer: Most of the vaccine-sprayed chicks started virus shedding at 2 dpe and continued up to 5 dpe, while some chicks started shedding from 1 dpe and some continued up to 6 dpe (Table 2. 4). In the first group which received 10 doses of the vaccine, chicks number 2 and 4 were infected while other chicks were not, and chick number 5 was found infected at 6 dpe, probably due to contact with the infected birds in the same cage. However, in groups 2-4, which received higher doses of the vaccine virus (25-100 doses), all chicks were infected and the result from VN test showed that all of them had high titer of maternal immunity (Table 2. 4).

II. 3. 2. 3. Evidencing the ability of SAHW for inactivation of NDV in the air: In the control group receiving the vaccine virus at 25 doses sprayed with RO water, all chicks showed sneezing as a clinical sign since 3 dpe, and the virus was recovered from their oropharyngeal swab samples from 2 dpe (Table 2. 5). In the SAHW 50 ppm treatment group, all chicks showed sneezing and the virus was recovered from their oropharyngeal swab samples too, while in the SAHW 100 ppm treatment group receiving ND vaccine, no clinical sign was observed, and no virus was isolated from their oropharyngeal swab samples (Table 2. 5), except for one chick which was found to be infected on 5 dpe. There was no significant difference in their gained weight within 5 days of the experiment (data not shown).

II. 3. 3. Monitoring effects of SAHW on the normal growth of chicks: The chicks that were sprayed with SAHW 100 ppm for 13 min once or twice per day, gained body weight not significantly in difference from the control group, which was sprayed with RO water twice a day for 13 min for a total of 6 days (Table 2. 6). There was no abnormal condition observed in any group of chicks.

II. 4. Discussion

AI and ND still persist as important animal and public health concerns, worldwide. In spite of applying strict management systems by some developed countries, AIV and NDV are still present, frequently infecting poultry industry in a disastrous manner, which means that, taking only defensive measures cannot prevent their outbreaks. Hence, there are needs for farmers to handle some offensive measure, such as application of disinfectants to remove pathogens. Selection and application of appropriate disinfectants at farm is the key point enabling farmers to reach the mentioned goal.

SAHW original solution could inactivate both challenged viruses, an ordinary AIV (H7N1) and a virulent NDV (Sato) in liquid, and its harvested solution performed fast and strong inactivation of the mentioned AIV, except for SAHW 50 ppm harvested from 30 cm distance (Table 2. 1), which was probably due to its free chlorine loss during the sprayed SAHW travel along distance [222]. This finding evidenced that the inactivation activity of SAHW is faster than other chlorine containing solutions [157, 187]. In the direct spraying form SAHW 100 ppm and SAHW 200 ppm could inactivate AIV > 99.9% directly after spraying, while SAHW 50 ppm concentration required at least 3 min contact time (Table 2. 2). The indirect spraying form required higher concentration of SAHW in comparison to the direct spraying form (Table 2. 3) and longer exposure times, but still it is faster than the findings in previous report [70]. Furthermore, aerosol spraying of SAHW 100 ppm could inactivate NDV in the air within sec, as it could prevent chicks infection with sprayed vaccine strain in 100 % (Table 2. 5), which confirms previous findings (Tables 2. 2 and 2. 3). SAHW safety to chicks is another important point, which makes it a perfectly ideal disinfectant, as even SAHW 100 ppm concentration spraying to chicks twice a day, every time for 13 min, did not affect their growth performance (Table 2. 6).

Short time direct spraying of SAHW is applicable for disinfection of clothes of farm's personal or visitors, just before they enter the farms or at the time of their

movement from one flock to another. SAHW efficacy is directly related to its concentration, sprayer distance from the application area and its exposure time. Indirect spraying of SAHW inside the box and its efficacy on AIV confirmed its ability to inactivate virus in the air, while spraying it on NDV in the air through aerosol sprayer confirmed its applicability as a good aerosol disinfectant. Spraying of SAHW in a lower concentration, from an appropriate distance for longer spraying times at the presence of poultry inside the farms, will reduce the bioaerosol contaminants in the air, including AIV and NDV. The ability of a sprayer to release smaller particles may help sprayed SAHW's particles to be better dispersed in the air, hover for a long time, and form wider contact with pathogens, and thus efficiently inactivate them.

II. 5. Conclusion

Installation and application of a perfect spraying system at the entrance of and inside the poultry farms from an appropriate distance, with an ideal disinfectant such as SAHW in a proper concentration, would potentially reduce the chance of AIV and NDV transmission via air and surfaces of objects. As the farm condition is totally different from the laboratory condition, further investigation is required to evaluate its efficacy in farm conditions.

Table 2. 1 AIV Inactivation in liquid, by original or sprayed SAHW within 5 sec of exposure time.

SAHW[a)] (ppm)	Distance from sprayer to sample (cm)	Log_{10} $TCID_{50}$/ml		
		Control	Treatment	RF[b)]
50	*	7.7[c)] ± 0.55	≤ 2.5 ± 0.00	≥ 5.2 ± 0.00
	1	7.8 ± 0.76	≤ 2.5 ± 0.00	≥ 5.3 ± 0.00
	30	7.6 ± 0.68	6.6 ± 0.68	1.0 ± 0.68
100	30	7.8 ± 0.55	≤ 2.5 ± 0.00	≥ 5.3 ± 0.00
200	30	8.0 ± 0.00	≤ 2.5 ± 0.00	≥ 5.5 ± 0.00

Original SAHW or its harvested solution after spraying from 1 or 30 cm distance, were directly exposed to the AIV in a reaction tube, and then inoculated to the MDCK cells to titrate the remaining virus.

a) Slightly acidic hypochlorous acid water.

b) Reduction factor = $\log_{10}$ (titer of control/ml) – $\log_{10}$ (titer of treated samples/ml).

c) Data represent mean ± standard deviation of three individual reactions.

* = Original solutions.

Table 2. 2 Inactivation of AIV on the rayon sheets through 10 sec direct spraying of SAHW.

SAHW[a] (ppm)	CT[b] (min)	Log_{10} $TCID_{50}$/ml		RF[c]
		Control	Treatment	
50	0	5.50[d] ± 0.00	4.17 ± 0.75	1.33 ± 0.57
	3	5.70 ± 0.44	≤ 2.5 ± 0.00	≥ 3.2 ± 0.00
100	0	5.50 ± 0.00	≤ 2.5 ± 0.00	≥ 3.0 ± 0.00
200	0	5.62 ± 0.15	≤ 2.5 ± 0.00	≥ 3.1 ± 0.00

SAHW was sprayed directly by Nanoscale sprayer on the virus on rayon sheets for 10 sec spraying time, and then the samples were immediately, or after 3 min exposure, transferred to the stomacher bags containing 900 µl MM to harvest the remaining virus.

a) Slightly acidic hypochlorous acid water.

b) Contact time.

c) Reduction factor = log_{10} (titer of control/ml) – log_{10} (titer of treated samples/ml).

d) Data represent mean ± standard deviation of three different experiments.

Table 2. 3 AIV inactivation through 10 sec indirect spraying of SAHW, and 10 min contact time.

SAHW[a] (ppm)	Log_{10} $TCID_{50}$/ml		RF[b]
	Control	Treatment	
50	5.50[c] ± 0.29	4.50 ± 0.20	1.00 ± 0.20
100	5.96 ± 0.46	4.02 ± 0.60	1.94 ± 0.60
200	5.78 ± 0.26	≤ 2.50 ± 0.00	≥ 3.28 ± 0.00

SAHW was sprayed by Nanoscale sprayer inside box, where the samples were kept inside petri dishes with the lid closed for 10 sec spraying time, then, the lids of petri dishes were removed and the lid of the box kept closed for 10 min to let the sprayed SAHW particles react with the virus on the rayon sheets and inactivate them.

a) Slightly acidic hypochlorous acid water.

b) Reduction factor = $\log_{10}$ (titer of control/ml) – $\log_{10}$ (titer of treated samples/ml).

c) Data represent mean ± standard deviation of three different experiments.

Table 2. 4 Determination of ND vaccine strain dose for attaining 100% infectivity in chicks.

Groups	Vaccine doses	Chick No	Days post-exposure						VN titer
			1	2	3	4	5	6	
1	10	1	-	-	-	-	-	-	956.7
		2	-	-	+	+	+	+	347.7
		3	-	-	-	-	-	-	1054.0
		4	-	+	+	+	-	-	249.3
		5	-	-	-	-	-	+	>1280.0
2	25	6	-	+	+	+	+	+	766.3
		7	-	+	+	+	+	+	1025.0
		8	-	+	+	+	+	+	>1280.0
		9	-	+	+	+	+	-	>1280.0
		10	+	+	+	+	+	+	290.4
3	50	11	-	+	+	+	+	-	844.4
		12	+	+	+	+	+	+	357.5
		13	-	+	+	+	+	-	1068.0
		14	-	+	+	+	-	-	>1280.0
		15	-	-	+	+	+	+	1178.0
4	100	16	-	+	+	+	+	+	>605.4
		17	+	+	+	+	+	+	1280
		18	-	+	+	+	+	-	320.0
		19	-	+	+	+	+	-	>1211
		20	+	+	+	+	+	+	>1178

The preliminary experiment was conducted to find the required dose of NDV to be sufficient for 100 % infection per group of chicks having maternal immunity upon

the day of vaccination (3 days-old). Different doses of NDV-B1 (10, 25, 50, or 100 doses per box) were sprayed by nebulizer. VN titers were shown at 3 day-old.

+ = virus was isolated from an oropharyngeal swab.

- = virus was not isolated from an oropharyngeal swab.

VN = virus neutralization.

Table 2. 5 The virucidal efficacy of SAHW towards NDV in the air.

Groups	Experiment No	Days post exposure			
		2	3	4	5
0 ppm	1	5/5[a)]	5/5	5/5	5/5
	2	4/5	5/5	5/5	5/5
	3	4/5	5/5	5/5	4/5
50 ppm	1	2/5	5/5	5/5	4/5
	2	3/5	5/5	5/5	5/5
	3	NC[b)]	NC	NC	NC
100 ppm	1	0/5	0/5	0/5	1/5
	2	0/5	0/5	0/5	0/5
	3	0/5	0/5	0/5	0/5

The vaccine virus at 25 doses was sprayed with RO water by nebulizers or with SAHW 50 ppm or SAHW 100 ppm towards chicks inside the spraying boxes. The experiments were repeated 3 times.

a) Number of infected chicks/challenged chicks. When the virus was recovered from an oropharyngeal swab samples, the chick was considered to be infected.

b) Not challenged.

Table 2. 6 SAHW's (100 ppm) effects on chick growth within the study period.

Groups	Chick's body weight (g)		Gained weight (g)	
	at 3 day old	at 9 day old	per 6 day	Per 1 day
RO water twice	44.9±2.46	91.5±3.38	46.6±1.87	7.77±0.31
SAHW once	46.7±1.52	94.6±2.48	47.8±1.39	7.98±0.24
SAHW twice	44.9±1.08	91.7±2.06	46.7±1.44	7.79±0.23

Control group was sprayed with RO water twice a day, while treated groups were sprayed with SAHW 100 ppm once or twice a day (every time for 13 min), for a total of 6 days. Data represent chicks body weight (mean ± SD), and statistically there was no significant difference (*p-value* > 0.05).

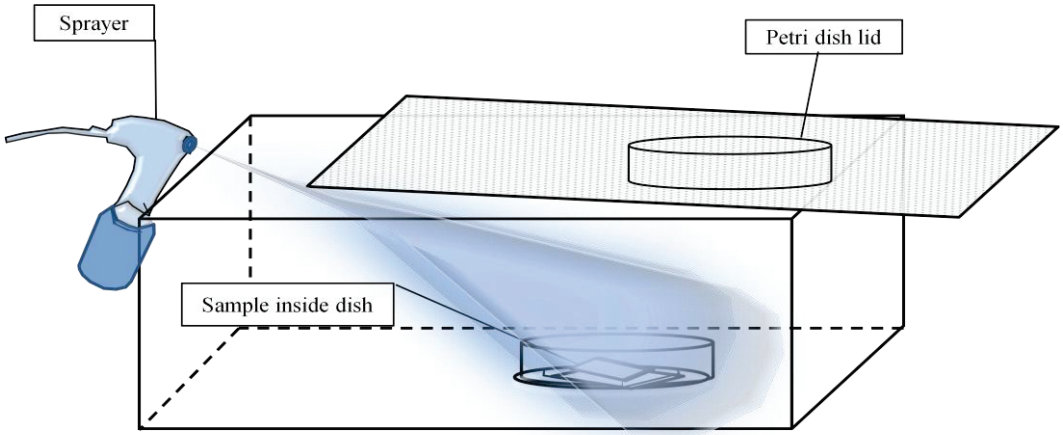

Fig. 2. 1 Direct spraying of SAHW by Nanoscale sprayer. SAHW was sprayed directly on AIV present on the rayon sheet for 10 sec spraying time, then the samples were directly transferred to stomacher bags containing MM to stop SAHW activity and to harvest the remaining virus, or it was kept for certain period of time to let sprayed SAHW to inactivate the virus.

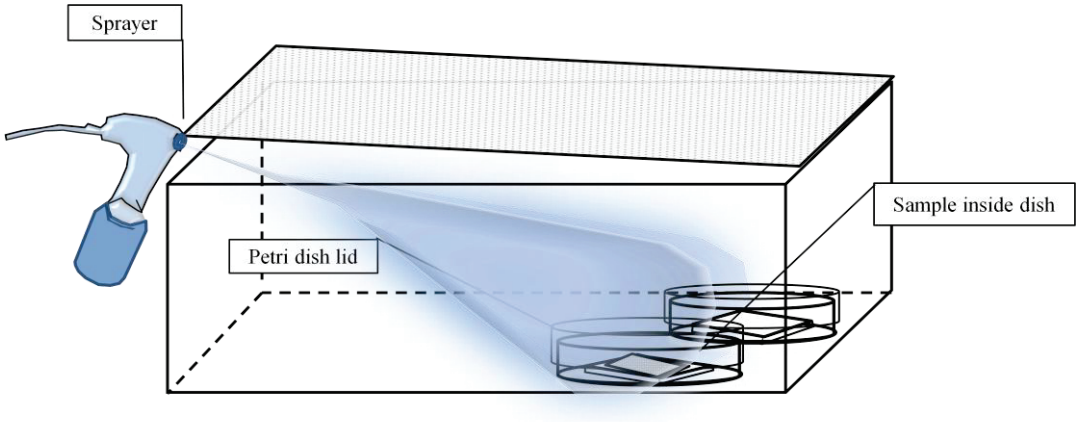

Fig. 2. 2 Indirect spraying of SAHW by Nanoscale sprayer. Petri dishes' lids were kept closed while SAHW was sprayed for 10 sec inside the box. Then, the petri dishes lids were removed and the lid of the spraying box was kept closed for 10 min in order to let the sprayed SAHW particles react with AIV present on the rayon sheets.

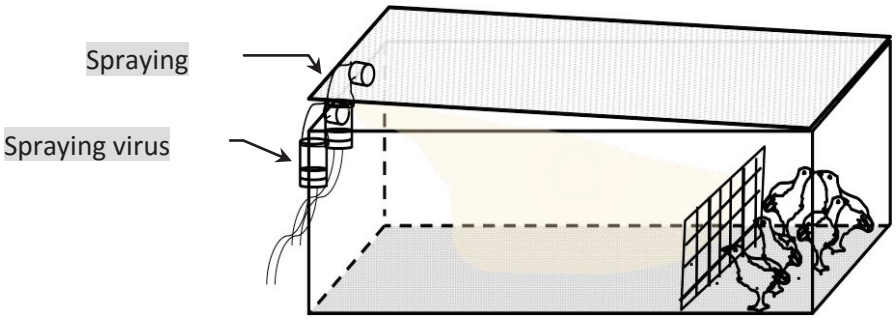

Fig. 2. 3 Spraying by nebulizers of ND vaccine virus and SAHW. Chicks were kept in one side of the box with a wire net, while ND vaccine and SAHW/RO were sprayed from the other side.

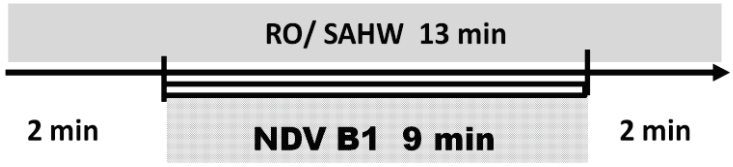

Fig. 2. 4 A scheme of the spraying duration of ND vaccine virus and SAHW/RO. SAHW or RO water were sprayed for 13 min (2 min before and 2 min after ND spraying). Within 9 min, 25 doses of ND vaccine were sprayed.

Chapter 3

“Efficacy of Slightly Acidic Hypochlorous Acid Water on Bacteria”

III. 1. Introduction

Bacterial contamination is a significant and recurring problem affecting the poultry industry worldwide. This problem varies from severe acute infections with sudden and high mortality, to mild infections of a chronic nature with low morbidity and mortality rates, but always with injurious impacts on meat and egg production, egg hatchability, and public health [80, 81, 97, 117, 152, 195, 218]. A wide variety of bacteria are present in the air, and on surfaces of the equipment and facilities of farms and hatcheries [97, 119, 180]. In addition, after eggs are laid in inadequate environments, eggshells become highly contaminated with various kinds of bacteria [97, 125, 136]. Bacteria found on the eggshell can be easily distributed from farms to hatcheries. Given that hatcheries play critical roles in collecting hatching eggs from breeder farms and selling newly hatched chicks to commercial farms [65], hatcheries are a potential source of various infectious disease contaminations across farms. Furthermore, these contaminations cause significant economic losses for the poultry industry [97, 152, 182].

Among the bacterial infections affecting the poultry industry, *Salmonella* spp. and *Escherichia coli* are the most common; they are widely distributed in nature, and have been isolated from large numbers of animal species and humans [27, 64, 77, 88, 106, 156, 164, 166, 193]. *E. coli* is a common pathogen of commercial poultry farms, causing colibacillosis worldwide [1, 17, 23, 50, 152, 180]. Salmonellosis is another important bacterial disease of the poultry industry, causing heavy economic losses through chick mortality and reduced meat and egg production [55, 117, 195]. Infected chicks shed these enteric bacteria through their feces and products (meat and eggs), and contaminate environments and nearby objects including air, food, water, manure, bedding materials, and soil, and the bacteria survive for up to several months [27, 59, 69, 105, 106, 117, 164]. The long-term survival of these bacteria in the environment increases their chance of transmission to sensitive hosts via the ingestion of such contaminated products, food, and water, or through their contact with such inanimate objects [27, 59, 77,

164]. Furthermore, farm personnel also play a role in terms of mechanical transmission of bacteria within and among the farms through their contact with contaminated hands, clothes, and boots [19, 75, 78, 160].

Bacterial survival in food, water, soil, and porous and non-porous surfaces plays a critical role in the transmission of bacterial infections within and between the farms and flocks [77, 88, 115]. Among the bacterial diseases transmissible from poultry to human, salmonellosis is the primary concern for public health among food born infections [84, 151, 191], although the risk of colibacillosis cannot be ignored [55, 60, 75, 117, 164, 210]. Thus, their inactivation on the surfaces of objects through application of materials with strong and broad-spectrum disinfectant capacity is very important to prevent infection and colonization in poultry farms, as well as their transmission to humans. To reach these goals, enhancement of biosecurity within the poultry industry is essential. In Chapter 2, SAHW exhibited excellent virucidal efficacy toward AIV on the surfaces and NDV in the air, and was completely safe for chicks. In the present chapter, sprayed slightly acidic hypochlorous acid water (SAHW) was evaluated for its bactericidal capacity on *Salmonella* Infantis and *E. coli* on rayon sheets and glass plates, as models for porous and non-porous surfaces, respectively, to confirm its applicability to biosecurity enhancement of poultry production.

III. 2. Materials and methods

III. 2. 1. SAHW: SAHW containing different concentration of free chlorine (50 ppm, 100 ppm: SAHW 50 ppm, SAHW 100 ppm, respectively) at pH 6 was provided by the same sources mentioned in II. 2. 1.

III. 2. 2. Aerosol sprayer and spraying box: the same nebulizer was used for SAHW spraying with the same spraying box as described in II. 2. 2., and II. 2. 3.

III. 2. 3. Inocula preparation: *E. coli* strain NBRC106373 was purchased from the National Institute of Technology and Evaluation Biological Resource Center (NBRC) (Chiba, Japan), and *S.* Infantis was kindly provided by Prof. Hiroshi Fujikawa (Laboratory Public Health, Department of Veterinary Medicine, Tokyo

University of Agriculture and Technology, Tokyo, Japan). Both bacterial species were stored in 10 % skim milk at -80 °C until they were used. For the experiments, both bacteria were sub-cultured by plating on Luria-Bertani (LB) agar (containing 1 % Bacto tryptone, 0.5 % Bacto yeast extract, 1 % sodium chloride and 1.5 % Bacto agar, pH 7.4), followed by overnight incubation at 37 °C. Colonies were then picked from the overnight culture and cultivated in LB medium (containing 1 % Bacto tryptone, 0.5 % Bacto yeast extract and 1 % sodium chloride, pH 7.4) as previously described [121]. After incubation at 37 °C for 4 or 6 hrs with shaking at 150 rpm, then log phase of bacterial culture were centrifuged at 1,750 × g for 20 min at 4 °C to remove organic materials. Cell pallets were re-suspended twice in PBS and then adjusted to a bacterial concentration of about 2×10^8 colony forming units (CFU)/ml. Both *E. coli* and *S.* Infantis were enumerated by surface plating on deoxycholate hydrogen sulfide-lactose (DHL) agar after serial tenfold dilution in PBS, followed by overnight incubation at 37 °C. The number of colonies was then determined and converted into $\log_{10}$ CFU/ml.

III. 2. 4. Computation of reduction factor (RF): RF was calculated using the equation below, after conversion of sample titer into $\log_{10}$ CFU/ml:

$$RF = tpc - ta$$

In the above equation, *tpc* is bacterial titer from an untreated sample in $\log_{10}$ CFU/ml, whereas *ta* is the titer of recovered bacteria from treated samples. The inactivation rate was acceptable when the RF was greater than or equal to 3 [114, 185, 189].

III. 2. 5. Experimental design

III. 2. 5. 1. Direct exposure: Fifty microliters of *E. coli* (titer around 8.35 Log_{10} CFU/ml) and *S.* Infantis (titer around 8.76 Log_{10} CFU/ml) were individually inoculated into 225 µl of SAHW 50 ppm or SAHW 100 ppm, in a reaction tubes and mixed, using a vortex mixer. After different exposure times, 225 µl of FBS was added to stop SAHW activity, and to determine the time required for bacterial inactivation. To confirm whether 225 µl of FBS is able to completely stop SAHW

activity, a reaction mixture was prepared with the same volumes of SAHW and FBS, and then 50 µl of *E. coli* or *S.* Infantis was inoculated into the mixture. Given that there was no contact between these bacteria and SAHW before adding FBS, this exposure time was marked as the zero-second contact time. For the positive control, 50 µl of *E. coli* (titer around 8.35 Log_{10} CFU/ml) and *S.* Infantis (titer around 8.76 Log_{10} CFU/ml) were individually inoculated in 450 µl of PBS and indicated as treatments. This experiment was performed in triplicate.

III. 2. 5. 2. Indirect exposure:

A. Dry condition. One hundred microliters of *E. coli* (titer around 8.5 Log_{10} CFU/ml) and *S.* Infantis (titer around 8.4 Log_{10} CFU/ml) were individually inoculated onto a 5 × 5 cm glass plate placed inside a 90-mm diameter petri dish without a lid and incubated for 30 min to facilitate evaporation of PBS and attachment of bacteria on the surface of the glass plate. Then, the petri dishes with lids were transferred to a spraying box and SAHW 50 ppm or SAHW 100 ppm was sprayed for 3, 5, or 7 min inside the box, using a nebulizer from one side, and the samples were kept covered on the opposite side of the box (Fig. 3. 1). RO water was sprayed for the positive control under the same conditions as for SAHW treatments. After stopping aerosol spraying of SAHW or RO water, the lids of the petri dishes were removed and the lid of the box was kept closed for 5 min. The samples were then removed and placed inside a stomacher bag (size 100 × 150 × 0.09 mm, capacity 80 ml; Organo Corp., Tokyo, Japan) containing 900 µl of 20 % (v/v) FBS in PBS, to stop the activity of SAHW and to manually harvest the remaining viable bacteria. The supernatant was then transferred into a microfuge-tube for titration of the remaining viable bacteria.

B. Wet condition. In this test, samples were prepared and bacteria were inoculated in the same way as for the dry condition on the glass plate, except that samples were directly transferred to the spraying box after inoculation and exposed to the sprayed SAHW.

C. Inactivation on the rayon sheets. In this experiment, bacteria were inoculated on to the 3 × 3 cm double-fold rayon sheets, which were placed on the 5 × 5 cm glass plate surface inside a 90-mm diameter petri dish, and then transferred to the spraying box and sprayed with SAHW, according to the wet and dry conditions described above. The rest of the procedure was performed identical to that in A and B. All experiments were conducted in triplicate.

III. 2. 5. 3. Direct exposure of non-stainless metals to SAHW: This exposure was performed to examine the corrosivity of SAHW towards metallic objects. Fifty milliliters of SAHW 50 ppm or SAHW 100 ppm was added to 90-mm diameter petri dishes, and then non-stainless steel screws with or without a flat washer representing models for the flat and rough surfaces of metallic objects were placed inside them and incubated at room temperature (25 ± 2 °C) under a desk in the laboratory (dark area). At the same time, sodium hypochlorite (NaOCl) solutions containing free chlorine at 100 ppm and 400 ppm (NaOCl 100 ppm and NaOCl 400 ppm), pH 7.97 and 9.64, respectively, were evaluated to compare their corrosivity. RO water was used as the negative control. These liquids neither changed nor refilled until the end of the experiment. The experiment was performed in duplicate, except for 50 ppm SAHW and 100 ppm NaOCl.

III. 3. Results

III. 3. 1. Inactivation efficacy in liquid (direct exposure): Table 3. 1 summarizes the capacity of SAHW to inactivate bacteria in liquid. Both SAHW 50 ppm and SAHW 100 ppm inactivated *E. coli* (RF ≥ 5.75), as well as *S.* Infantis (RF ≥ 6.16) to below the detectable level (≤ 2.6 $\log_{10}$ CFU/ml) within 5 sec of exposure time. In the 0-sec samples treated with SAHW 50 ppm, no reduction was observed in both bacteria titers (RF < 0.39). This experiment demonstrated that the activity of SAHW was negated by adding an equal volume of FBS.

III. 3. 2. Inactivation by sprayed SAHW: Table 3. 2 presents the results of spraying SAHW to inactivate bacteria on the glass plate or rayon sheet surfaces after the indicated spraying times and 5 min of exposure. In the dry condition, the

sprayed SAHW 50 ppm required 7 min spraying and 5 min contact time to reduce *E. coli* and *S.* Infantis titer to below the detectable level (≤ 2.6 $\log_{10}$ CFU/ml) (RF ≥ 4.34). In addition, in the wet condition, SAHW 50 ppm could not inactivate bacteria even through 7 min spraying and 5 min exposure.

In the dry condition, after 5 min of exposure, SAHW 100 ppm reduced the titer of *E. coli* to below the detection level (≤ 2.6 $\log_{10}$ CFU/ml) (RF ≥ 4.44) at 7 min spraying, as well as the titer of *S.* Infantis (RF = 4.07) at 5 min of spraying, but it was not tested for 7 min of spraying time.

In the wet condition, sprayed SAHW 100 ppm could not reduce bacterial titer to the acceptable level (RF = 2.05) even at 7 min of spraying.

On the rayon sheets (Table 3. 2), sprayed SAHW 50 ppm could not reduce both challenged bacteria titers to the acceptable limit (RF < 1.16) at 7 min of spraying, but SAHW 100 ppm reduced the titer of *E. coli* and *S.* Infantis to the acceptable level (RF > 3), at 5 min spraying, and to below detection limit (≤ 2.6 $\log_{10}$ CFU/ml) at 7 min of spraying time.

III. 3. 3. Corrosivity of SAHW towards metallic objects: Fig. 3. 2 illustrates the results of corrosivity tests of SAHW as well as NaOCl towards metals. Within 83 days of exposure, SAHW 50 ppm did not corrode the non-stainless steel, as there was no change observed in their color, in comparison to NaOCl 100 ppm, which clearly changed the normal color of metallic objects into oxidized iron pigments. Such changes were slightly observed in the RO water-exposed metals, which were used as negative control. Furthermore, within 65 days of non-stainless metal exposure to SAHW 100 ppm, no corrosion was observed in comparison to NaOCl 400 ppm, or RO water.

III. 4. Discussion

Bacterial contamination is always a significant concern for poultry producers, not only in terms of morbidity and mortality of the chicks, but also as the main cause of poor hatchability and chick performance of the hatcheries, and as a potential risk to public health. Understanding the mechanisms underlying effective

bacterial inactivation under different conditions, and selection of an appropriate disinfectant with a capacity for fast and strong disinfection efficacy against a broad range of pathogens in the air, on surfaces and in liquids, is vital for designing proper infection control strategies, enhancement of biosecurity, and prevention of zoonotic infections. To minimize contamination of hatcheries, disinfection of eggs and hatcheries is necessary. There are several methods for disinfection of eggs, such as wiping, spraying, dipping into disinfectant, and most importantly, fumigation of the hatching eggs, which can be performed during incubation (during or right after transfer to the hatchery), but most commonly prior to incubation. The most common disinfectant used as fumigant is formaldehyde, which is an excellent anti-microbial agent, but in comparison to SAHW requires higher concentration and longer exposure time [109, 110, 220]. Furthermore, it is highly toxic and causes serious damage to the embryos if fumigation is not properly carried out [15, 33, 173]. Such damage mostly occurs on the outermost organic layer and cuticle, which constitute an important barrier to microbial invasion; hence, such damage may cause serious problems during incubation [15].

In addition of SAHW's excellent capacity against AIV and NDV, as described in Chapter 2, the present study demonstrated its high performance for inactivation of bacteria in liquid and on porous and non-porous surfaces. Aerosol spraying (indirect exposure) of SAHW caused great reduction of the bacterial titer on the surfaces of glass plates and rayon sheets, and is equivalent to the fumigation method. It is therefore a good candidate for disinfection of eggshells by spraying, and most importantly, fumigation of the egg incubators and hatcheries by aerosol spraying. Along with its excellent capacity for inactivating pathogens, SAHW is also harmless for metallic objects, even less corrosive than RO water, and safe for chicks; hence, it can be applied without hesitation at farms and hatcheries.

III. 5. Conclusion

This study section demonstrated SAHW's fast and strong capacity against bacteria in liquid and on surfaces, and confirmed its non-corrosivity towards

metallic objects. Confirmation of its non-corrosivity towards metallic objects and of its safety for chickens increases its applicability to poultry production. From the results obtained with *E. coli* and *S*. Infantis, its effectiveness towards other bacterial pathogens may well be inferred. Given that study was conducted at the laboratory level, further investigation may be required to practically evaluate its remarkable properties and capacity to inactivate pathogens in poultry production units.

Table 3. 1 SAHW bactericidal effects after direct exposure.

SAHW	Bacteria	Control [b] (Log $_{10}$ CFU/ml)	RF[a]	
			0 [c] sec	5 sec
50 ppm	*E. coli*	8.35 ± 0.36 [d]	0.00 ± 0.00	≥ 5.75 ± 0.44
	S. Infantis	8.76 ± 0.08	0.00 ± 0.00	≥ 6.16 ± 0.08
100 ppm	*E. coli*	8.35 ± 0.44	0.39 ± 0.19	≥ 5.74 ± 0.44
	S. Infantis	8.76 ± 0.08	0.37 ± 0.33	≥ 6.16 ± 0.08

SAHW was directly brought into contact with the bacteria in a reaction tube, and after stopping SAHW's activity, and the remaining bacterial titer was calculated by inoculation on DHL agar.

a) Reduction factor = $\log_{10}$ (titer of control/ml) – $\log_{10}$ (titer of treated samples/ml).

b) Titer of bacteria in the control (log $_{10}$ CFU/ml).

c) Contact time.

d) Data represent means ± standard deviation of three individual experiments.

Table 3. 2 SAHW bactericidal effects on bacteria on glass plates or rayon sheets within 5 min of exposure time.

SAHW	Bacteria	Conditions	RF[a)]		
			3[b)] min	5 min	7 min
50 ppm	*E. coli*	Wet	Not tested	Not tested	0.00 ± 0.00
		Dry	2.48 ± 1.43[c)]	4.03 ± 0.35	4.34 ± 0.15
		On rayon	Not tested	1.14 ± 0.51[c)]	1.16 ± 0.85
	S. Infantis	Wet	Not tested	Not tested	0.00 ± 0.00
		Dry	4.15 ± 0.69	3.88 ± 0.57	4.54 ± 0.15
		On rayon	Not tested	0.40 ± 0.02	0.50 ± 0.08
100 ppm	*E. coli*	Wet	0.73 ± 0.92	1.08 ± 1.25	0.53 ± 0.18
		Dry	3.76 ± 0.89	4.44 ± 0.59	4.44 ± 0.00
		On rayon	1.50 ± 0.41	3.15 ± 0.90	5.89 ± 0.52
	S. Infantis	Wet	1.66 ± 1.58	1.72 ± 0.50	2.05 ± 1.12
		Dry	2.67 ± 1.14	4.07 ± 1.05	Not tested
		On rayon	2.05 ± 1.97	4.45 ± 1.44	5.70 ± 0.00

Various SAHW concentrations were sprayed by nebulizer on the bacteria present on different types of surfaces inside spraying boxes, and then, the remaining bacteria were harvested inside of stomacher bags containing 20 % FBS in PBS.

a) Reduction factor = $\log_{10}$ (titer of control/ml) – $\log_{10}$ (titer of treated samples/ml).

b) Spraying time.

c) Data represent means ± standard deviation of three individual experiments.

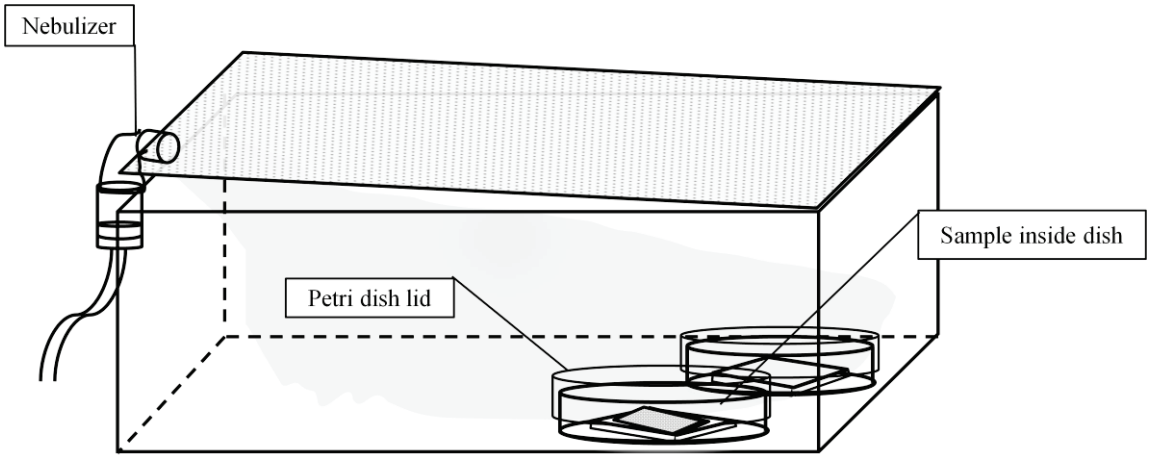

Fig. 3. 1 Indirect spraying of SAHW on bacteria.

SAHW was sprayed using a nebulizer, from the side opposite to that of the petri dishes, into the box for 3, 5, and 7 min, after which the petri dish lids were removed and the bacteria present on the glass plate or rayon sheets were exposed to SAHW for 5 min.

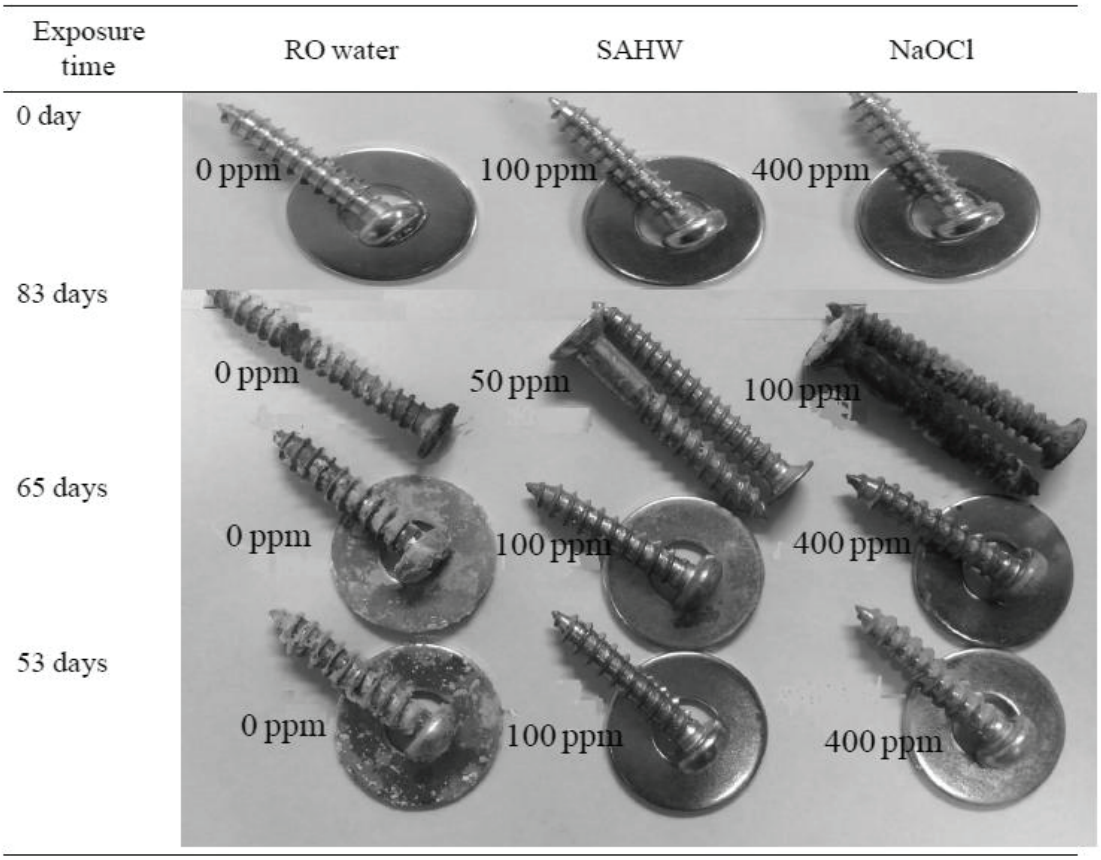

Fig. 3. 2 SAHW effects on the non-stainless metallic objects (steel) and its comparison with NaOCl and RO water.

Different SAHW concentrations were directly brought into contact with metallic objects inside of petri dishes, and kept in a dark place for long exposure times to observe the changes in metals.

Chapter 4

"Efficacy of BCX and FdCa$(OH)_2$ on the Bacteria in Chicken Feces"

IV. 1. Introduction

Disinfectants and their application are an essential part of infection control strategies and of the enhancement of biosecurity at farms, worldwide. Currently, various sources of antimicrobial products are available, and are widely applied for biosecurity enhancement in the livestock industry [52]. Alkaline agents are well known for their strong and long term antimicrobial capacities. Among them, lime's antimicrobial activities have been evaluated by many researchers and most frequently are applied at farms for the control and prevention of biological hazards [20, 22, 118, 137, 189]. For evaluating lime in the powder form in litter or bedding materials, these researchers used distilled water or PBS to recover the spiked microorganisms from treated samples. These recovery steps with addition of water or PBS raise the pH value of the aqueous phase, and the treated microorganisms would be inactivated during their recovery in the aqueous phase but not at the real, earlier time of their exposure to lime in solid [20, 22, 137, 142, 181, 189, 213]. Such misleading of the results may cause outbreaks of infectious diseases in the poultry industry, despite application of lime as disinfectants, and consequently bring about huge economic loses, with animal and public health's concerns.

Among the bacterial infections, salmonellosis and colibacillosis are very common, and most frequent. They cause high morbidity and mortality, reducing productivity of the chicks and hatchability of the eggs [67, 83, 97, 117, 135]. In addition to that, annually, billions of tons of poultry bedding materials which are highly contaminated with microorganisms are collected from the poultry farms. Usually, farmers are using such poultry manure (wastes) as fertilizer for their farm's land as a chosen method of the litter disposal, worldwide [31, 96]. Several studies have demonstrated the role of poultry litter or their wastes, after their application in the farm's land, in the contamination of surface water and the environment around [24, 41, 118, 132, 206].

Application of antibiotics as food additive is a way to minimize the chance of bacterial colonization and shedding to the farm's environments through infected

chicks, as well as for controlling their infections and outbreaks. But their application has the risk of producing antibiotic resistant bacteria [40, 49, 111], which is a great concern of the animal and public health. Furthermore, antibiotic residual in the animal products is another risk for the public health [37, 38, 40, 122, 148]. In view point of the above, enhancement of biosecurity at farms is very important as an effective way of preventing and controlling of infectious disease outbreaks, limiting zoonotic infection transmission, and reducing environmental contaminations.

To reach the mentioned goals, it is worthwhile to search for new environmental friendly materials and to establish accurate evaluation methods for their bactericidal capacities, with especial attention to their certain required contact time and concentration, along with duration of their activity.

Bioceramic (BCX) powder is an environmental friendly material, and it seems to be a useful tool for enhancement of biosecurity. Its efficacies to inactivate viruses have been already reported [185, 188], but according to our knowledge, currently there is no data available regarding its bactericidal efficacy in feces in the powder form, along with its persistent bactericidal efficacy in litter.

Food additive grade of calcium hydroxide ($FdCa(OH)_2$) powder is another novel product, about which there is currently no data available regarding its efficacy to inactivate pathogens and its durability in litter. Here in the present Chapter, the author tried to evaluate powder forms of BCX and $FdCa(OH)_2$ for their bactericidal efficacies in feces, along with the duration of their persistent bactericidal efficacy in litter, under chicks exposure at experimental level.

IV. 2. Materials and methods

IV. 2. 1. BCX: BCX powder, which is derived from chicken feces prepared through sintering process under alkaline conditions, was kindly provided by NMG Environmental Development Co., Ltd. (Tokyo, Japan).

IV. 2. 2. $FdCa(OH)_2$: $FdCa(OH)_2$ powder is made of natural calcium carbonates, derived from limestone through calcination process, was kindly provide by Fine

Co., Ltd. (Tokyo, Japan).

IV. 2. 3. Feces: Chicken feces were collected from chickens less than three-weeks old in our laboratory and autoclaved at 121 °C for 15 min, then they were heated at 80 °C for 60 min, in order to reduce their humidity, and stored at 4 °C until used in the experiments.

IV. 2. 4. Litter: Sawdust was purchased from the local market and used as mixed with powders.

IV. 2. 5. Bacterial suspension: *E. coli* and *Salmonella* Infantis were received from the same sources as explained in III. 2. 3., along with their culture and enumeration methods.

IV. 2. 6. Rifampicin resistant bacteria: Hundred microliters log phase culture of both *E. coli* and *S.* Infantis were cultured through their surface plating on DHL agar containing different concentrations of rifampicin (from 0 – 100 µg/ml), individually, and followed by overnight incubation at 37 °C. Colonies which were grown in the area containing higher concentration of rifampicin were picked and sub-cultured on the DHL agar containing 100 µg/ ml rifampicin. After overnight culture, around five colonies were picked up and subsequently cultured in the LB medium containing the same concentration of rifampicin. After incubation at 37 °C with shaking at 100 rpm, log phase of bacteria from the solution culture was used for the experiments, while stocked in 10 % skim milk at - 80 °C until used.

IV. 2. 7. Computation of RF: Enumeration and calculations of treatments and control were set in the explained method in III. 2. 4.

IV. 2. 8. Study design

IV. 2. 8. 1. Recovery of bacteria with PBS: The autoclaved feces were measured in weight and added into 50 ml conical centrifuge tubes according to experiments design, in order to make final concentration of 0, 5, 10, 20, and 30 % (w/w), in the total weight of 0.5 g with BCX or $FdCa(OH)_2$ powders. Hundred microliters of *E. coli* (titer around 7.8 Log_{10} CFU/ml) and *S.* Infantis (titer around 8.3 Log_{10} CFU/ml) were inoculated into the measured feces, individually, and vortexed to mix well;

then, BCX or $FdCa(OH)_2$ powders were added on them, individually, followed by mixing by vortex mixer. Soon afterwards, 10 ml PBS was added on the mixture to harvest the spiked bacteria from feces through mixing by vortex for about 1 min. Serial tenfold dilution was prepared immediately per sample, and plated on DHL agar as described above.

IV. 2. 8. 2. Recovery of bacteria with Tris-HCl: These experiments were designed in the same way as described in IV. 2. 8. 1.; but after adding BCX and $FdCa(OH)_2$ powders to the contaminated feces, following proper mixing, samples were incubated for different exposure times (3, 6, and 20 hrs) at room temperature (25 ± 2 °C) in a dark place. Then, 10 ml 1M Tris-HCl (pH 7.2) was added on the mixture to stop BCX and $FdCa(OH)_2$ powders activities and to harvest the remaining bacteria from feces through mixing by vortex for about 1 min. Then, serial tenfold dilution was prepared per sample and plated on DHL agar as described above.

In order to find out whether Tris-HCl is able to stop bactericidal activities of BCX and $FdCa(OH)_2$ powders, 10 ml of Tris-HCl was added on the inoculated feces, after its incubation for the certain time periods with the control, then followed by adding BCX and $FdCa(OH)_2$ powders, and conducting the same procedure with the other samples. As there was no contact between bacteria with BCX and $FdCa(OH)_2$ powders, it was recorded as 0-min contact time.

IV. 2. 8. 3. Persistent bactericidal efficacy test: An experiment was designed to study the durability of BCX and $FdCa(OH)_2$ powders' bactericidal efficacy in litter under animal exposure. Animal work was conducted in strict accordance with the animal care guidelines of Tokyo University of Agriculture and Technology (Tokyo, Japan), with permit numbers of 26-45 and 27-20. Day-old commercial chicks, with no vaccination, here after called "conventional chicks", were purchased from Kanto Co., Ltd. (Gunma, Japan), and divided into group of 6 chicks in rat cages (CLEA-0108-3, Clea Japan, Inc., Tokyo, Japan) containing 50% and 20 % w/w of BCX and $FdCa(OH)_2$ powders in litter in the total amount of 200 g, respectively, and kept

inside the isolator (CL-5443, Clea Japan), while providing normal feed and water. After properly mixing, about 2 g of bedding materials were sampled daily, per cage, and the harvested samples were evaluated for their bactericidal efficacy, using rifampicin resistant *E. coli* and *S.* Infantis as described in IV. 2. 8. 2.; but, as adding of 5 ml Tris-HCl was enough to reduce the pH of samples to 8, five ml of the mentioned solution was used to stop the activity of the disinfectants and to harvest the remaining bacteria. DHL agar containing 100 μg/ml rifampicin was used for bacterial enumeration of the samples.

IV. 3. Results

IV. 3. 1. Inactivation at the time of recovery: Table 4. 1 shows the efficacy of different concentrations of BCX and $FdCa(OH)_2$ powders towards the bacteria during their recovery by PBS. In this experiment, as shown in IV. 2. 8. 1., there was no incubation period after materials with bacteria were mixed in feces. When treated samples were recovered by PBS, BCX powder had no effect on both bacterial species, as even its 30 % concentration in the feces could not inactivate the challenged bacteria (RF ≤ 0.07). In comparison, even 5 % of $FdCa(OH)_2$ powder inactivated down to the detectable level (≤ 3.6 $\log_{10}$ CFU/ml); *E. coli* (RF = 4.59), and *S.* Infantis (RF = 4.53).

IV. 3. 2. Inactivation within the exposure time.

IV. 3. 2. 1. BCX powder bactericidal efficacy: Table 4. 2 represents the efficacies of different concentrations of BCX powder on the bacteria in feces. In the 0-min contact time, whereby there was no direct contact between *E. coli* and *S.* Infantis with BCX powder in the reaction tube, no reduction was observed in the titer (RF = 0.00). Within 20 hr of 10 % BCX powder incubation with the *E. coli* and *S.* Infantis containing feces, the titer of both bacteria was reduced with RF ≤ 1.5, but not to the acceptable level (RF ≥ 3), while 20 % BCX powder reduced the titer of *E. coli* down to the detectable limit (≤ 3.6 $\log_{10}$ CFU/ml) (RF ≥ 4.18), as well as the titer of *S.* Infantis down to the detectable level (RF ≥ 4.73) within 6 hr of exposure time. But, when they were exposed to 30 % BCX powder for 3 hr of incubation times, the

titer of *E. coli* was reduced, yet not to the acceptable level (≥ 3 $\log_{10}$ CFU/ml) (RF = 2.86), while reduction of the titer of *S.* Infantis to the acceptable level (RF = 3.88) did occur.

IV. 3. 2. 2. $FdCa(OH)_2$ powder bactericidal efficacy: Table 4. 3 shows effects of different percentages of $FdCa(OH)_2$ powder on the *E. coli* and *S.* Infantis in feces. In the 0-min contact time, even application of 30% concentrated $FdCa(OH)_2$ powder could not reduce titer of both tested bacteria (RF ≤ 0.23), while within 3 hr of exposure times, its 5 % concentration reduced titer of *E. coli* (RF = 2.81) and *S.* Infantis (RF = 2.88), which is under the acceptable level (RF ≥ 3); however, when incubation time was increased to 6 hr, it was able to inactivate both tested bacteria down to the detectable level (RF ≥ 4.5). Furthermore, $FdCa(OH)_2$ powder in the higher concentration (10 %) required 3 hr exposure time to decrease titer of tested bacteria down to the detectable limit (RF ≥ 4.3).

IV. 3. 3. Durability of BCX and $FdCa(OH)_2$ bactericidal efficacy in the presence of chicks: Table 4. 4 shows persistent bactericidal efficacy of BCX and $FdCa(OH)_2$ in bedding materials. At 6 hr exposure times, samples that were collected from 50% BCX concentration in litter performed excellent bactericidal efficacy against both *E. coli* and *S.* Infantis (RF ≥ 3) up to 3 days in the presence of chicks, whereas afterwards, it lost its bactericidal efficacy and could not reduce the bacterial titer to the acceptable level. Besides, 20 % of $FdCa(OH)_2$ powder retained its bactericidal efficacy for up to 3 days and reduced bacterial titer to the acceptable level (RF = 3.3), yet lost its activity after 3 days. As 5 ml Tris-HCl was used to stop activity of powders, bacterial detection limit was (≤ 3.3 $\log_{10}$ CFU/ml).

IV. 4. Discussion

Pathogen contaminated feces play critical role in the transmission of infectious disease among animals and among farms, as well as in contaminating the environment at large. Inactivation of pathogens present in the feces plays a fundamental role in the prevention of fecal-oral transmission of infections and enhancement of biosecurity at the livestock farms.

The Ministry of Agriculture, Forestry and Fisheries (MAFF) of Japan established a standard for Hazard Analysis Critical Control Point (HACCP) certification system for the livestock farms in 2009, in order to improve security for prevention of biological hazard contamination at the primary production site of the food chain, that is livestock farms [184]. This system derives farmers to enhance their farms biosecurity through application of disinfectants to good level, in order to be recognized as HACCP certified farms by MAFF [87].

BCX powder is a material which is produced from chicken feces by sintering process in Japan. Takehara *et al.* [185] demonstrated efficacies of BCX to inactivate avian influenza virus (AIV) for the first time; then, Thammakarn *et al.* [188] reported BCX capacity to prevent fecal-oral transmission of infectious bursal disease virus (IBDV), alongside with its safety to chicks. They also demonstrated virucidal efficacy of BCX in contaminated feces at incubation of 8 hr using live chicks as indicator [188]. The present study confirmed BCX efficacy to inactivate bacteria in the feces in the powder form (Table 4. 2) although it required higher concentration and longer incubation time than previously reported [185, 188]. Besides that, its 50 % concentration in litter exhibited good bactericidal efficacy for 3 dpe (Table 4. 4) under chicks' exposure. This BCX exposure in litter with chicks was similar to the farm conditions. As BCX is safe for the farm animals, there is no limitation regarding its application in higher concentration when being used as a mixture with bedding materials.

$FdCa(OH)_2$ powder is another novel product, and demonstrated excellent bactericidal efficacies in feces and litter. In comparison to BCX powder, it required lower concentration and shorter exposure times (Table 4. 3). In addition to that, it had good durability of bactericidal activity, after its exposure to chicks in the experimental conditions (Table 4. 4). $FdCa(OH)_2$ powder efficacy was highly related to its pH value. When it was re-suspended in PBS, its pH was found >13, but when re-suspended in 10 ml Tris-HCl, as in the 0-min contact time, its pH decreased to around 8, resulting in the complete reduction of its bactericidal

efficacy in the recovered aqueous phase (Tables 4. 3). As there was no reduction observed in the titer of exposed bacteria, it confirms our accurate evaluation system.

Demonstration of accurate evaluation method for disinfectants evaluation is very important to prevent misunderstanding the results. Data on Table 4. 1 and 4. 3, shows very big gap between the capacities of $FdCa(OH)_2$ to inactivate bacteria, as the result of inaccurate evaluation method. Such misleading will cause outbreaks of diseases despite application of alkaline agents as disinfectants at farms. Accurate evaluation method would minimize such error during disinfectant application at farms. In addition to that, it will help farmers to design a better strategy for diseases prevention and control, and finally would minimize the animal and public health concerns in regards to infectious diseases.

IV. 5. Conclusion

Both BCX and $FdCa(OH)_2$ powders demonstrated excellent efficacies to inactivate bacteria in feces, and approved their possible application for disinfection of poultry bedding materials (litter) inside the farms, along with their application for disinfection of chicken manure, just after harvesting from poultry farms, in order to prevent environmental contamination. Both products are environmental friendly materials with no risk to the animals and humans health, and can be used as biosecurity materials for the enhancement of biosecurity in the poultry productions. This finding helps farmers to properly apply alkaline agents in appropriate concentrations and exposure times in their farms, in order to prevent and control infectious disease outbreaks and to enhance biosecurity. Finally, this finding may help farmers to implement better strategies for infections control in their livestock farms.

Table 4. 1 Inactivation of bacteria with BCX and $FdCa(OH)_2$ in feces during their recovery within min.

Disinfectant	Concentration (%)	RF[a)]	
		E. coli	*S.* Infantis
BCX	10	0.00[b)] ± 0.00	0.00 ± 0.00
	20	0.00 ± 0.00	0.00 ± 0.00
	30	0.07 ± 0.14	0.03 ± 0.17
$FdCa(OH)_2$	5	≥ 4.59 ± 0.00	≥ 4.53 ± 0.00
	10	≥ 4.59 ± 0.00	≥ 4.53 ± 0.00
	20	≥ 4.59 ± 0.00	≥ 4.53 ± 0.00

Chicken feces were inoculated with bacteria in conical centrifuge tubes, then different amounts of BCX or $FdCa(OH)_2$ powder were added on them and mixed properly, and soon thereafter the remaining bacteria were harvested by adding 10 ml PBS.

a) Reduction factor = $\log_{10}$ (titer of control/ml) – $\log_{10}$ (titer of treated samples/ml).

b) Data represent mean ± standard deviation of three individual reactions.

Table 4. 2 Bactericidal capacity of BCX powder towards the bacteria present in the feces.

BCX in feces	Bacteria	RF[a)]			
		0[b)] min	3 hr	6 hr	20 hr
10%	*E. coli*	0.00 ± 0.00	Not tested	0.64 ± 0.12 [c)]	1.50 ± 0.00
	S. Infantis	0.00 ± 0.00	Not tested	1.39 ± 0.56	1.09 ± 0.00
20%	*E. coli*	0.00 ± 0.00	1.01 ± 0.38	≥4.18 ± 0.00	≥5.14 ± 0.00
	S. Infantis	0.00 ± 0.00	1.54 ± 0.41	≥4.73 ± 0.00	≥5.09 ± 0.00
30%	*E. coli*	0.00 ± 0.00	2.86 ± 1.17	Not tested	≥5.14 ± 0.00
	S. Infantis	0.00 ± 0.00	3.88 ± 1.09	Not tested	≥5.09 ± 0.00

Chicken feces were inoculated with bacteria in conical centrifuge tubes, then different amounts of BCX powder were added on them and mixed properly, kept for different exposure times, and then the remaining bacteria were harvested by 10 ml Tris-HCl.

a) Reduction factor = $\log_{10}$ (titer of control/ml) – $\log_{10}$ (titer of treated samples/ml).

b) Incubation time.

c) Data represent mean ± standard deviation of three individual reactions.

Table 4. 3 Bactericidal effects of $FdCa(OH)_2$ on the bacteria present in the feces.

$FdCa(OH)_2$	Bacteria	RF[a)]			
		0[b)] min	3 hr	6 hr	20 hr
5%	*E. coli*	0.00 ± 0.00	2.81 ± 1.31[c)]	≥4.53 ± 0.00	Not tested
	S. Infantis	0.00 ± 0.00	2.88 ± 1.60	≥4.73 ± 0.00	Not tested
10%	*E. coli*	0.00 ± 0.00	≥4.33 ± 0.00	≥4.53 ± 0.00	≥4.40 ± 0.00
	S. Infantis	0.00 ± 0.00	≥4.73 ± 0.00	≥4.73 ± 0.00	≥5.09 ± 0.00
20%	*E. coli*	0.07± 0.33	Not tested	Not tested	≥4.40 ± 0.00
	S. Infantis	0.23 ± 0.17	Not tested	Not tested	≥5.09 ± 0.00

Chicken feces were inoculated with bacteria in conical centrifuge tubes, then different amounts of $FdCa(OH)_2$ powder were added on them and mixed properly, kept for different exposure times, and then the remaining bacteria were harvested by 10 ml Tris-HCl.

a) Reduction factor = $\log_{10}$ (titer of control/ml) – $\log_{10}$ (titer of treated samples/ml).

b) Incubation time.

c) Data represent mean ± standard deviation of three individual reactions.

Table 4. 4 Persistence of bactericidal efficacy of BCX and $FdCa(OH)_2$ powders in litter under the chicks exposure.

Disinfectant	Bacteria	Days post exposure					
		0	1	2	3	4	5
BCX (50 %)	*E. coli*	≥4.05[a)] ± 0.00	≥4.05 ± 0.00	≥4.05 ± 0.00	3.08 ± 0.24	0.63 ± 0.33	Not tested
	S. Infantis	≥4.47 ± 0.00	≥4.47 ± 0.00	3.16 ± 0.01	2.99 ± 1.01	2.31 ± 0.52	Not tested
$FdCa(OH)_2$ (20%)	*E. coli*	≥3.96 ± 0.00	≥3.96 ± 0.00	≥3.96 ± 0.00	≥3.96 ± 0.00	1.24 ± 0.67	0.75 ± 0.00
	S. Infantis	≥4.47 ± 0.00	≥4.47 ± 0.00	4.17 ± 0.42	2.32 ± 0.14	3.03 ± 0.16	1.23 ± 0.33

BCX and $FdCa(OH)_2$ were mixed with litter and spread on the cages, then 6 chicks were kept per cages, and daily around 3 g samples were collected per cages and subjected to rifampicin resistant bacteria, and incubated for 6 hr exposure times in order to know whether still they kept their bactericidal efficacy.

a) Data represent RF[b)] of two experiments as mean ± standard deviation.

b) Reduction factor = $\log_{10}$ (titer of control/ml) – $\log_{10}$ (titer of treated samples/ml).

Chapter 5

“General Discussion and Final Conclusion”

V. 1. General discussions

Poultry houses are a big source of dust that raises during poultry activity, and such dust contains particles that are originating from feed, bedding materials, skin cells, feather debris and feces, which can be highly contaminated and carry microorganisms to far distance [71]. In compare to cattle farms (0.38 mg/ m^3) and pig farms (2.19 mg/ m^3), the amount of inhalable dust in the poultry houses is very high (3.6 mg/m^3), while at the same time, the amounts of total bacterial and fungal counts in the poultry farms reach 5.8 $\log_{10}$ CFU/ m^3 and 4.1 $\log_{10}$ CFU/ m^3, respectively [168, 183]. However, large size particles present in dust sediment on floor or surfaces of the objects found around, while the small size particles remain suspended in the air for longer time. But in general, dust particles, bacteria and viruses can travel by air for long distance [71]. Thus, inactivation of pathogens in the air, on surfaces and in the bedding materials through application of disinfectants is a very important need for enhancement of biosecurity at farms.

SAHW is an excellent virucidal and bactericidal agent that exhibited excellent capacities to inactivate AIV on the surfaces, NDV in the air, and bacteria (*E. coli* and *S.* Infantis) on the surfaces, through its spraying on the mentioned pathogens, or via its direct contact in liquid, within short period of time.

Aqueous phase of original SAHW 50 ppm or its harvested solution after spraying from 1 cm could reduce the titer of an ordinary AIV, NDV, *E. coli,* and *S.* Infantis, up to more than 99.9 % within 5 sec, but its travel along 30 cm distance after spraying brought about the lost its efficacy (Tables 2. 1 and 2. 5); however, SAHW 100 ppm or SAHW 200 ppm, retained their efficacies after travelling along the mentioned distance and inactivated AIV, NDV, *E. coli* and *S.* Infantis (Tables 2. 1, 2. 5 and 3. 2) to > 99.9%, directly after spraying, or after some moments of contact time. In the indirect spraying form, as less amount of sprayed SAHW had the chance to contact with the AIV present on the rayon sheets, it required at least 10 min contact time and SAHW 200 ppm, while SAHW 100 ppm required 7 min spraying time and 5 min contact time to inactivate both *E. coli* and *S.* Infantis. Such method of spraying disinfectant in the air by an aerosol sprayer seems to be effective for inactivation of pathogens in the air and prevention of aerogenic infection in the livestock farms.

Short time direct spraying of SAHW with a higher concentration will be applicable for disinfection of vehicle or clothes of farm's personal or visitors, just before their entering into the farms and/or their movement from one part to another. Table 5. 1 summarizes SAHW applicability with the form of application and the required concentrations. As its efficacy is directly related to free available chlorine concentration, sprayer distance from application area and the exposure times, those parameters are important to be considered during its application. As application of disinfectants at the presences of those kinds of animals whose products are used as foods for public requires high safety for the animals and their products, chlorine-based compound such as SAHW looks to be the best choice for the mentioned purpose. Aerosol spraying of SAHW in the air or its direct spraying on the surfaces potentially reduces the chance for pathogen introductions and outbreaks. But anyhow, SAHW loses its efficacy at the presence of organic materials quite easily, and this point should be considered during its application at farms.

BCX have good antibacterial and antiviral capacity at the presence of organic materials. As previously discussed in Chapter 1, BCX at pH 13 has the ability to inactivate AIV, goose parvovirus (GPV), NDV, IBDV, as well as to inhibit fecal-oral transmission of IBDV [185, 188]. According to the data obtained in Chapter 4, it exhibited excellent efficacy to inactivate bacteria (*E. coli* and *S.* Infantis) in feces, but required higher concentration and longer incubation times (Table 4. 2). Apart from its good antimicrobial activity in feces and litter, it is also completely safe for the farm animals, and in Japan, MAFF has certified its application as food additive for the farm animals [185]. As clinically or subclinically infected animals shed pathogens via their feces as a usual route of infection spread, farms bedding materials are highly contaminated with various kinds of pathogens [116, 141] and play a key role for infection transmission to the susceptible hosts present around. Pathogen inactivation in the bedding material is essential for prevention of their transmission to the susceptible host along with their colonization in litter through application of disinfectants such as BCX and $FdCa(OH)_2$. Safety of BCX to farm animals makes it a valuable candidate for application at farms, as mixed with litter. Its safety for the animals allows farmers to apply it in higher concentration while using it

being mixed with the bedding materials. However, it also can be used in liquid or powder forms in other parts of the poultry industry (Table 5. 1).

$FdCa(OH)_2$ is another valuable product with stronger disinfection capacity in comparison with BCX, in both aqueous and powder forms. Although this material is newly produced and still there is no enough data available regarding its antimicrobial efficacy against different pathogens, It exhibited excellent efficacy against bacteria (*E. coli* and *S.* Infantis) in liquid, feces, and litter, as it required very low concentration and shorter exposure time to inactivate the mentioned pathogens down to detectable level (Table 4. 1 and 4. 3), and it could retain its bactericidal efficacy for longer time (Table 4. 4), appearing to be highly effective against various kinds of pathogens. Although application of $FdCa(OH)_2$ powder in higher concentration (> 20 %) in litter will be harmful for the farm animals, it has diverse and important applicability at the poultry industry, such as disinfection of the poultry waste (manure) after its removal from the floor, since it can potentially reduce the chance of environmental contamination through such contaminated materials. Besides that, it can be applied as solution for disinfection of various objects (Table 5. 1).

Inactivation of bacteria in solid by alkaline agent such as lime, takes long time and requires high concentration, however, alkaline agents can inactivate microorganisms in liquid within short time (min) during their recovery from solid materials such as feces, using normal solution such as PBS (Table 4. 1). In addition, durability of alkaline agents in the farm condition is another important point to be considered. Thus, the mentioned points raise concern regarding the accuracy of previous reports regarding bactericidal efficacy of lime, in the solid form [159, 181].

It is worthwhile to mention that only application of one kind of disinfectants, or one method, cannot enhance the biosecurity at farms or other parts of the poultry industry, and different disinfectants should be applied in different ways, and they should be employed according to their capacities and applicability at the different parts of the poultry industry. Furthermore, it is necessary to handle various biosecurity measures at the same time, in conjunction, while implementing one measure cannot prevent disease outbreaks.

V. 2. Final conclusion

In this dissertation, SAHW, BCX and $FdCa(OH)_2$ were selected as candidate materials that can enhance biosecurity at the poultry industry, and their efficacy was evaluated against the most deadly pathogens of the poultry industry, such as AIV and NDV, and against the most common bacterial infections of poultry, such as *E. coli* and *Salmonella,* which are the most important concerns of the animal and public health spheres. The obtained data in Chapters 2 and 3 confirmed high efficacy of SAHW against the mentioned pathogens.

Further to the fact that SAHW possesses excellent virucidal and bactericidal capacity, it is also very safe to the chickens and not corrosive to metallic objects. The data provided in Chapter 2, indicated that spraying of SAHW 100 ppm to the chicks did not affect their growth performance, and the data from Chapter 3 as well indicated that direct exposure of metallic objects to the SAHW 100 ppm was not corrosive to them, even during long exposure time.

Application of SAHW in adequate concentration, from an appropriate distance by a spraying system with the ability of producing aerosol particles inside the populated poultry farms can potentially reduce the chance of aerogenic infections transmission. Moreover, SAHW spraying on the surfaces of eggs and objects can limit transmission of pathogens within and among flocks, farms, and hatcheries. SAHW can be used in different forms at different critical points of the poultry industry (Table 5. 1). Lower cost, broad spectrum, easy mass applicability, availability, safety to the animals and their products, and non-corrosivity to metallic objects are the most important factors which may encourage farmers to apply this product in their animal farms.

Data of Chapter 4 presented the excellent efficacy of BCX and $FdCa(OH)_2$ against the bacterial pathogens *E. coli* and *S.* Infantis in feces and litter. Although in comparison to SAHW they required longer exposure times and higher concentrations, their efficacy at the presence of organic materials and their safety to chicks, point them out as the most useful biosecurity materials applicable in mixture with litter or any other bedding materials at the floor of farms, in the powder form. Mixing BCX or $FdCa(OH)_2$ powders in litter would be a very useful tool to prevent fecal oral transmission of infection within the poultry, and to prevent their colonization at farms. Although both BCX and $FdCa(OH)_2$ are able to inactivate bacteria in both liquid and powder forms at the presence of organic

materials (feces), in comparison to BCX, $FdCa(OH)_2$ powder was stronger, as it required lower concentration and shorter exposure time in both evaluated forms.

BCX and $FdCa(OH)_2$ powders are good candidate for enhancement of biosecurity at the poultry farms through their application as mixture with litter. However, they can be applicable for disinfection of boots in boots' baths, or for disinfection of transport vehicle's tires as solution at the entrance of the farms. Further, they can be used for disinfection of eggshells in the egg industry. They are also applicable for disinfection of poultry bedding materials and useful for disinfection of poultry wastes and manure just after their removal from the farms, in order to prevent environmental contamination.

Concomitant application of the three evaluated candidates at the critical points of the poultry industry, with respect to their form of applicability, the required concentration, and the needed exposure time, would potentially reduce the chance of disease outbreaks and certainly would play key roles in the enhancement of biosecurity at the poultry industry.

Table 5. 1 Proposed usage of different biosecurity materials with their application areas, in the poultry industry.

Materials	Concentration	Form	Application area
SAHW	≤ 0.5 ppm	Aqueous	Drinking water treatment.
	50 ppm	Spraying	Farms' or industry personnel cloths or gowns.
	100 ppm	Spraying	Air and surfaces of the objects, eggshells and egg incubators.
	200 ppm or more	Aqueous and spraying	Foot and tire baths, disinfection of eggshells in the egg industry, floors, walls and objects surfaces, after appropriate cleaning.
BCX	1 %	Powder	Food additive.
	30 %	Solution	Foot and tires baths.
	20 ~ 50 %	Powder	Litter or bedding materials, poultry manure and wastes.
	100 %	Powder	Foot baths and around the poultry houses for long-term application.
$FdCa(OH)_2$	5 %	Solution	Foot and tires baths, and disinfection of transport cages, eggshells, and various part of farms after cleaning.
	10 %	Powder	Bedding materials (litter).
	20~30 %	Powder	Feces, manure, carcasses (wastes).
	100 %	Powder	On the surfaces around the poultry houses.

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