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Studies on the control of influenza and classical swine fever

(インフルエンザと豚熱の制御に関する研究)

Enkhbold Bazarragchaa

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Abbreviations

AAALAC International	Association for Assessment and Accreditation of Laboratory Animal Care International
ABSL-3	animal biosafety level 3
AI	avian influenza
AIV	avian influenza virus
APMV	avian paramyxovirus
ASF	African swine fever
BSL-3	biosafety level 3
CI	confidence interval
COVID-19	coronavirus disease-2019
CPE	cytopathic effect
CSF	classical swine fever
CSFV	classical swine fever virus
Ct	cycle threshold
dpc	days of post-challenge
dpi	days post-infection
dsDNA	double-stranded deoxyribonucleic acid
EID_{50}	50% egg-infective dose
ELISA	enzyme-linked immunosorbent assay
FCS	fetal calf serum
FMD	food and mouth disease
GM	geometric mean
H3N2v	variant H3N2
HA	hemagglutinin
HPAI	high pathogenicity avian influenza
HPAIV	high pathogenicity avian influenza virus
hpi	hour of post-inoculation
IAV	influenza A virus
IBDV	infectious bursal disease virus
IBV	infectious bronchitis virus
IC	immunochromatographic
ILTV	infectious laryngotracheitis virus

IPX	immunoperoxidase
kb	kilobase
LAMP	loop-mediated isothermal amplification
LPAIV	low pathogenicity avian influenza virus
MAFF	Ministry of Agriculture, Forestry, and Fisheries
MDCK	Madin-Darby canine kidney
MEM	minimal essential medium
min	minute
NA	neuraminidase
NASBA	nucleic acid sequence based amplification
NDV	Newcastle disease virus
NEAR	nicking enzyme amplification reaction
NP	nucleoprotein
NT	neutralizing antibody
OIE	World Organization for Animal Health
PA	polymerase acid
PB2	polymerase basic 2
PBS	phosphate buffered saline
PCR	polymerase chain reaction
RNA	ribonucleic acid
RT-cPCR	conventional reverse transcription-polymerase chain reaction
RT-qPCR	real-time reverse transcription-polymerase chain reaction
S/P	sample-to-positive
SNT	serum neutralization test
TAD	transboundary animal disease
$TCID_{50}$	50% tissue culture infective dose
TD	transboundary disease
USA	United States of America
UTR	untranslated region
VTM	viral transport medium
WV	whole-virus

Notes

This thesis contains two articles published as follows.

1. **Bazarragchaa E.,** Okamatsu M., Ulaankhuu A., Twabela A.T., Matsuno K., Kida H., Sakoda Y. Evaluation of a rapid isothermal nucleic acid amplification kit, Alere™ i Influenza A&B, for the detection of avian influenza viruses. ***J. Virol. Methods*. 2019, 265, 121−125. doi: 10.1016/j.jviromet.2019.01.004**
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2. **Bazarragchaa E.,** Isoda N., Kim T., Tetsuo M., Ito S., Matsuno K., Sakoda Y. Efficacy of oral vaccine against Classical Swine Fever in wild boar and estimation of the disease dynamics in the quantitative approach. ***Viruses*. 2021, 13, 319. doi: 10.3390/v13020319**
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Preface

In the world, the safe and sustainable production of livestock and livestock products are economically important regarding food provision, consumption, and nutrient management. The constant increase in the human population raises the demand for meat and related products [1]. Behind the global increase in both human and livestock animals, there is the occurrence of infectious diseases that cause new adverse conditions for naïve populations to affect different hosts and spread easily via close interaction [2]. Some diseases have been recognized to infect humans or animals with rapid spread among populations without any border barrier. These diseases are categorized as transboundary diseases (TDs), whose spread is generally enhanced by human activities and animal movements, and much attention is needed to grasp their preventive and control measures.

Regarding human health, the emerging diseases, such as pandemic influenza, coronavirus disease 2019 (COVID-19), and Ebola are typical illnesses and threaten humans, resulting in a severe situation with high transmission rate [3]. For animals, foot-and-mouth disease (FMD), avian influenza (AI), classical swine fever (CSF), and Africa swine fever (ASF) are also typically highly contagious diseases that have caused enormous economic loss in the livestock. Due to their widespread, TDs cause huge damages, especially health damage in public health and economic damage in animal health [2,4]. To control the TDs promptly and efficiently, the development and reinforcement of some key activities including early and accurate diagnosis, effective prevention measures based on vaccine development and its scheme, filed epidemiology in the respect of biosecurity measures and disease transmission dynamics, and good risk communication are generally required as a common agreement in international security [2]. However, there is still necessary to strengthen these measures, due to that the diseases in each country are cross-border infectious diseases and the implementation of control measures are not launched effectively.

Poultry and pig industries grow and satisfy the food demand worldwide, though poultry and pig species are susceptible to various devastating pathogens that frequently invade the hosts [5,6]. AI and CSF are among the major diseases that affect poultry and pig population, respectively. Influenza is one of the severe diseases that threaten both animals and humans. It is caused by the influenza A virus (IAV) belonging to the family *Orthomyxoviridae* [4]. Chicken is one of the most susceptible avian species for AI virus (AIV) infection and its pathogenicity is classified into high and low according to the characteristics of AIVs in chickens. Based on the antigenic characteristics of surface

glycoproteins, hemagglutinin (HA) and neuraminidase (NA), regarding the disease, there are extremely variable subtype viruses already known (H1–H16 for HA and N1–N9 for NA) [7]. AIVs are derived from wild waterfowls as natural reservoirs of these viruses. Moreover, pigs play a role as a "mixing vessel" by expressing both human- and avian-type receptors to influenza viruses in respiratory epithelial cell and it allows to increase zoonotic potential, resulting in human infection with H1N1 and H3N2 subtype viruses recently circulating among pig and human population [8].

CSF is a multisystemic viral disease of pigs caused by the CSF virus (CSFV), which belongs to the *Flaviviridae* family and is closely related to other members of the genus, including bovine viral diarrhea virus genotypes 1 and 2, and the border disease virus [9]. CSFV is genetically classified into three genotypes (1, 2, and 3) and variable sub-genotypes (1.1–1.7, 2.1–2.3, and 3.1–3.4) [5. Among them, CSFV genotype 1.1, 2.1, 2.2, and 2.3 were circulated in Europe and Asia with different degrees of virulence [10,11]. Swine species including domestic pigs and wild boar are susceptible reservoir hosts for CSFV. Through direct and indirect contact, there are high risk on the disease transmission and maintenance of CSFV infection among host populations, especially in wild boar population due to virulence of CSFV. In addition, wild birds and wild boars play an essential role in disease transmission of these causative pathogens. According to these situations, it is important to establish effective control and prevention strategies to reduce burdens of not only influenza and CSF, but also for other TDs and the outcomes allow to set up better actions on livestock production and zoonotic risk.

To maintains better conditions for both animal and human health regarding sustainability of livestock production, effective control measures following with biosecurity enhancement in farms, reinforcement of the surveillance system of the diseases using effective methods adapted in each setting and establishment of preventive methods to reduce the risk of viral infections in susceptible animals should be implemented. Moreover, in the case of outbreaks of transboundary animal disease (TAD), including AI and CSF, the stamping-out policy is recommended as a standard control measure that can reduce the further spread of infectious pathogens and prevent the invasion to other hosts or locations [4,12]. However, due to the circulation of various strains including non-pathogenic AIV and CSFV, silent infection with an asymptomatic disease in susceptible animals was reported [13-15], which led to the challenging point to implement early warning and quick response against the diseases. Thus, in addition to control measures, it is essential to improve the strategy for the diagnosis of TDs in terms of early detection of causative pathogens. Overall,

such comprehensive effort is expected to be best practiced in animal and human health, and it should have a positive impact on the decrease of public health risk in terms of zoonotic spillover.

For these reasons, as representative TDs, the present study aims to evaluate the rapid diagnostic tool for AI in the field, to assess the preventive measures for the potential emergence of swine-origin influenza in the human population and to evaluate the effectiveness of vaccination for CSF in wild boar using epidemiological and virological approach. The output of this study is provided in three different chapters. In chapter I, the evaluation of the applicability of the rapid diagnostic kit was conducted for AI in poultry, and the sensitivity and specificity of this kit was evaluated for AIVs. In chapter II, vaccine development strategy for humans was established on non-human H3N2 virus infection, and a study model for further vaccine preparation was discussed. In chapter III, the efficacy of the oral vaccine against CSFV in wild boar was evaluated using the virological and epidemiological approaches. Additionally, the disease dynamics of existing CSFV infection forms were analyzed.

Chapter I

Evaluation of a rapid isothermal nucleic acid amplification kit, Alere™ i Influenza A&B, for the detection of avian influenza viruses

Introduction

Influenza virus belongs to the family of *Orthomyxoviridae* and it characterized as segmented virus consists of negative-strand ribonucleic acid (RNA) genomes. Influenza A and B viruses commonly infect humans and animals. The genome of influenza viruses involves eight segments. Among them, hemagglutinin (HA) and neuraminidase (NA) proteins are glycoproteins that existed on the surface of influenza viruses and responsible for viral infectivity. Moreover, the genes on the polymerase basic 2 (PB2) and polymerase acid (PA) proteins are highly conserved in avian influenza viruses (AIVs) of all H16 subtype viruses [4]. Avian species including chickens, are susceptible to infections of low pathogenicity avian influenza virus (LPAIV) and high pathogenicity avian influenza virus (HPAIV) [2,7]. Initial action should provide for chickens and quick detection requires the control of avian influenza (AI). The AI is a disease that can transmit to bird species caused by influenza A virus (IAV). The viruses are naturally circulating among wild birds and poultry especially chickens that are high susceptible to AIV infection. In the laboratory level, the detection of AIV is performed by standard methods such as virus isolation, viral gene detection and viral antigen detection [4,16]. Virus isolation using embryonated chicken eggs remains a sensitive method that can provide a presence of the virus. However, virus isolation is time-consuming and requires a highly equipped laboratory to identify influenza viruses.

Polymerase chain reaction (PCR)-based methods, such as conventional reverse transcription-polymerase chain reaction (RT-cPCR), real-time RT-PCR (RT-qPCR) and gene sequencing allow rapid and sensitive gene detection as well as characterization of influenza viruses within a short period. Therefore, PCR-based methods are widely used for both surveillance and diagnostic purposes of IAV [16,17]; however, these assays require sample processing such as RNA isolation before the reaction, molecular diagnostic laboratory, and temperature-sensitive reagents [18]. Antigen capture immunochromatographic (IC) test can be used for the early detection of infection of AIV in suspected cases on site [19,20]; however, the analytical sensitivity of IC test is much lower than virus isolation and RT-qPCR [16,21].

In recent years, several companies have developed simpler and more rapid diagnostic kits for the diagnosis of seasonal influenza in hospitals. One of the rapid molecular diagnostic assays that has recently been developed is Alere™ i Influenza A&B that could provide the qualitative detection and characterization of influenza A and influenza B viruses via

amplification of nucleic acid under isothermal condition [22,23]. The assay is designed based on the nicking enzyme amplification reaction (NEAR) and targets the PB2 genes of influenza A virus and PA genes of influenza B virus. To identify specific amplification, fluorescently labeled molecular beacons are bind to the amplicons. In brief, after swab samples suspended into heated lysis buffer, viral RNA releases from the virus particles. The recognition regions of template bind to the target region of viral RNA and are extended by the polymerase enzyme then next identical template binds to the same region to make more copies of complementary regions. The different recognition regions bind to these complementary regions and extended, resulting in the generation of double-stranded DNA (dsDNA) with nicking sites that allow to cut by nicking enzyme. Next, the amplification using the gene-specific primers initiates from this dsDNA which has more cuts on one strand of the duplex by the nicking enzyme, resulting in rapid synthesis of amplificated product. Finally, the result is judged based on the absorption of the fluorescent signals from the amplicons then classify whether influenza A or B virus [24]. The rapid kit has been deployed in hospitals to diagnose human influenza in the United States of America (USA) [25]. The test allows to perform the individual test without pre-treatment of specimens and the test result of a sample is available within 15 minutes (min) from sample collection [21,26].

To better control management, the implementation of rapid and accurate diagnostic kit is essential for quick responses against potential infection at the farm level and for both human and animal health in terms of one health concept. With these reasons, this study aims to evaluate the applicability of the Alere™ i Influenza A&B kit for the diagnosis of AI in chickens by determining the sensitivity and specificity of this kit.

Materials and Methods

Viruses

For the determination of the sensitivity of Alere™ i Influenza A&B kit, 20 AIVs were tested. These strains included sixteen LPAIVs [16 HA subtypes (H1–H16) and 9 NA subtypes (N1−N9)], three H5 HPAIVs, and one strain of H7N9 HPAIV (Table 1) [27-30]. For the virus propagation, each of the AIV strains was diluted with serum-free minimal essential medium (MEM) and inoculated into 10-day-old specific antibody-negative embryonated chicken eggs which were derived from conventional white-leghorn chicken. The allantoic fluids containing viruses were stored at −80°C until further use. In addition, 14 other avian viruses, 11 avian paramyxoviruses (APMV), the infectious bronchitis virus (IBV), infectious laryngotracheitis virus (ILTV), and infectious bursal disease virus (IBDV), were selected from the virus library and used to evaluate the specificity of the kit.

Virus titration

Embryonated chicken eggs were used for virus titration. According to the method recommended by World Organization for Animal Health (OIE) [4], 10-fold dilutions of the viruses in phosphate buffered saline (PBS) (pH 7.2) were inoculated into the allantoic cavity of 10-day-old embryonated chicken eggs. After the incubation at 35°C for 48 h, harvested allantoic fluids were subjected to the hemagglutination (HA) test and virus titration was expressed by 50% egg-infective dose (EID_{50}) and calculated using the Reed and Muench method [31].

Madin-Darby canine kidney (MDCK) cells maintained in MEM (Nissui Pharmaceutical, Tokyo, Japan), supplemented with 0.3 mg/ml L-glutamine (Nacalai Tesque, Kyoto, Japan), 100 U/ml penicillin G (Meiji Seika Pharma, Tokyo, Japan), 0.1 mg/ml streptomycin (Meiji Seika Pharm), 8 mg/ml gentamicin (Takara Pharmaceutical, Saitama, Japan), and 10% fetal calf serum (FCS; Sigma-Aldrich, St. Louis, MO, USA) were only used for the determination of the virus infectivity of tissue samples collected from experimentally infected chickens. Ten-fold serial dilutions of viruses with serum-free MEM were inoculated into MDCK cells and incubated at 37°C under 5% CO_2. After the incubation for 72 h, the cytopathic effects (CPE) of the cells were observed and 50% tissue culture infective dose ($TCID_{50}$) was calculated according to the Reed and Muench method [31].

Table 1. The list of avian viruses in the study

Virus	Strain name
AIV	Low pathogenicity avian influenza virus
	A/duck/Tottori/723/1980 (H1N1)
	A/duck/Hokkaido/17/2001 (H2N3)
	A/duck/Mongolia/4/2003 (H3N8)
	A/duck/Czech/1956 (H4N6)
	A/duck/Pennsylvania/10218/1984 (H5N2)
	A/duck/Hong Kong/960/1980 (H6N2)
	A/seal/Massachusetts/1/1980 (H7N7)
	A/turkey/Ontario/6118/1968 (H8N4)
	A/turkey/Wisconsin/1966 (H9N2)
	A/chicken/Germany/N/1949 (H10N7)
	A/duck/England/1/1956 (H11N6)
	A/duck/Alberta/60/1976 (H12N5)
	A/gull/Maryland/704/1977 (H13N6)
	A/mallard/Astrakhan/263/1982 (H14N5)
	A/duck/Australia/341/1983 (H15N8)
	A/duck/Hokkaido/WZ/82/2013 (H16N3)
	High pathogenicity avian influenza virus
	A/duck/Vietnam/HU5-1571/2016 (H5N1)
	A/chicken/Kumamoto/1-7/2014 (H5N8)
	A/chicken/Hokkaido/002/2016 (H5N6)
	A/duck/Japan/AQ-HE29-22/2017 (H7N9)
APMV	NDV/LaSota
	NDV/B1 Hitchner
	APMV-2/Chicken/Yucaipa/1956
	APMV-3/Turkey/Wisconsin/1968
	APMV-4/Duck/Mississippi/320/1975
	APMV-5/Budgerigar/Japan/TI/1975
	APMV-6/Duck/Hong Kong/199/1977
	APMV-7/Duve/Tennessee/4/1975
	APMV-8/Goose/Delaware/1053/1976
	APMV-9/Duck/New York/22/1978
	APMV-10/Penguin/Falkland Island/324/2007
IBV	B-42
ILTV	NS-175
IBDV	Saga

Abbreviation: AIV, avian influenza virus; APMV, avian paramyxovirus; NDV, Newcastle disease virus; IBV, infectious bronchitis virus; ILTV, infectious laryngotracheitis virus; IBDV, infectious bursal disease virus.

Evaluation of the specificity and sensitivity of the Alere™ i Influenza A&B kit

A rapid isothermal nucleic acid amplification kit, Alere™ i Influenza A&B (Alere, Inc. Waltham, MA, USA), was evaluated for the rapid diagnosis of AI in chickens. The kit amplifies RNA by NEAR that is usually performed at 55–59˚C [23]. The kit set for single run contained a sample receiver, transfer cartridge, and test base. The tests were performed using the device under isothermal condition, and the sample was tested individually according to the manufacturer's instructions. Briefly, first, the sample receiver containing the elution buffer was attached to the device and heated for 3 min. Thereafter, 200 µl of undiluted or serial diluted sample was directly added into the heated elution buffer. The transfer cartridge was attached to the receiver to take a small portion of the sample mixture and transferred to the test base which contained the reaction mixture for isothermal amplification. Subsequently, the device was subjected to RNA amplification, and the result was presented on its display after 10 min while approximately 15 min was required for testing a single sample. The detection limit was calculated by showing the lowest virus titer detectable by the Alere™ i Influenza A&B assay, and it was expressed as EID_{50} per test (EID_{50}/test). Moreover, the detection of AIVs using the Alere™ i Influenza A&B kit was compared with the rapid diagnostic IC kit for IAV, ESPLINE® A INFLUENZA (FUJIREBIO, Inc. Tokyo, Japan), which was authorized by the Ministry of Agriculture, Forestry and Fisheries (MAFF), Japan for the screening test of AI. The procedure of the IC kit described in the study by Bai *et al.* [32] was followed. Accordingly, 30 µl of the sample suspension was dropped onto the IC kit. After 15 min of incubation at the room temperature, the results were observed. A single blue line in the control line represented a negative test, whereas double blue lines in the control that represented a positive test.

In addition, to evaluate the specificity of the Alere™ i Influenza A&B kit, overall, 224 tracheal and cloacal swabs were collected from total 83 chickens (*Gallus gallus, Julia*), 8 Muscovy ducks (*Cairina moschata*) and 8 domestic ducks (*Anas platyrhynchos domesticus*). In detail, 134 swab samples were collected from apparently healthy chickens, 90 swab samples were collected from experimentally infected chickens, Muscovy ducks, and domestic ducks. All swab samples were suspended in 2 ml of viral transport medium (VTM) (MEM containing 10,000 U/ml of penicillin G, 10 mg/ml of streptomycin, 0.3 mg/ml of gentamicin and 0.5% bovine serum albumin), followed by test using the Alere™ i Influenza A&B assay. The results were statistically analyzed with Cohen Kappa.

Virus detection from swabs and tissue homogenates of chickens experimentally inoculated with H5N6 HPAIVs

Four 6-week-old chickens were obtained from Hokkai Starchick (Hokkaido, Japan). The chickens were intranasally inoculated with 100 µL of the virus suspension containing $10^{6.0}$ EID_{50} of A/chicken/Hokkaido/002/2016 (H5N6) [29]. Each of the chickens was housed in a self-contained isolator unit (Tokiwa Kagaku, Tokyo, Japan) at the animal biosafety level 3 (ABSL-3) facility of the Faculty of Veterinary Medicine, Hokkaido University. All chickens were monitored daily following virus inoculation.

Tracheal and cloacal swabs were collected from the chickens twice per day until death. The swabs were suspended in 2 ml of VTM. The tissue samples of the brain, trachea, lung, kidney, and colon were collected from dead chickens and homogenized using a Multi-Beads Shocker (Yasui Kikai, Osaka, Japan) to make 10% (w/v) of suspension in VTM. All of these samples collected from experimentally infected chickens were stored at −80°C until testing by the virus isolation using MDCK cells, Alere™ i Influenza A&B kit, and IC kit.

Ethics statements

All animal experiments were authorized by the Institutional Animal Care and Use Committee of the Faculty of Veterinary Medicine, Hokkaido University (approval numbers: 13-0108) and all experiments were performed following the guidelines of the committee. The BSL-3 facility of the Faculty of Veterinary Medicine, Hokkaido University is permitted to store and use HPAIVs by the MAFF, Japan in accordance with the Act on Domestic Animal Infectious Diseases Control (Permission number: 19)

Results

Gene detection of AIVs by the Alere i Influenza A&B kit

The sensitivity of the Alere™ i Influenza A&B kit was evaluated using 20 strains of IAVs including 16 different representative strains of LPAIVs [16 HA subtypes (H1-H16) and 9 NA (N1-N9)] and three H5 and one H7 subtypes of HPAIVs (Table 2). Following virus propagation using embryonated chicken eggs, 10-fold serial dilutions of the allantoic fluid containing the virus were measured for their infectivity titer and performed for Alere™ i Influenza A&B test. The detection limits of the kit were expressed in EID_{50}/test. The results revealed that the kit detected viral RNA of different subtypes of AIVs with the lower detection limit ranged in between $10^{-1.4}$–$10^{2.1}$ EID_{50}/test compared with IC kit. In addition, the Alere™ i Influenza A&B kit detected the recent H5 and H7 HPAIVs within the same detection limits.

Furthermore, the specificity of the kit was also evaluated on 14 avian strains of avian paramyxoviruses (APMV), Newcastle disease viruses (NDV), infectious bronchitis virus (IBV), infectious laryngotracheitis virus (ILTV), and infectious bursal disease virus (IBDV). The Alere™ i Influenza A&B kit did not show any reactivity with these samples (Table 3).

Table 2. Detection of avian influenza viruses by Alere™ i Influenza A&B kit and detection limit of the kit

Viruses	Alere™ i Influenza A&B [a]	Detection limit (EID_{50} /test) [b]
Low pathogenicity avian influenza virus		
A/duck/Tottori/723/1980 (H1N1)	IAV positive	$10^{0.9}$
A/duck/Hokkaido/17/2001 (H2N3)	IAV positive	$10^{-0.4}$
A/duck/Mongolia/4/2003 (H3N8)	IAV positive	$10^{1.4}$
A/duck/Czech/1956 (H4N6)	IAV positive	$10^{-0.9}$
A/duck/Pennsylvania/10218/1984 (H5N2)	IAV positive	$10^{0.6}$
A/duck/Hong Kong/960/1980 (H6N2)	IAV positive	$10^{1.4}$
A/seal/Massachusetts/1/1980 (H7N7)	IAV positive	$10^{0.6}$
A/turkey/Ontario/6118/1968 (H8N4)	IAV positive	$10^{1.1}$
A/turkey/Wisconsin/1966 (H9N2)	IAV positive	$10^{1.6}$
A/chicken/Germany/N/1949 (H10N7)	IAV positive	$10^{-0.1}$
A/duck/England/1/1956 (H11N6)	IAV positive	$10^{-0.4}$
A/duck/Alberta/60/1976 (H12N5)	IAV positive	$10^{-1.4}$
A/gull/Maryland/704/1977 (H13N6)	IAV positive	$10^{0.1}$
A/mallard/Astrakhan/263/1982 (H14N5)	IAV positive	$10^{-1.4}$
A/duck/Australia/341/1983 (H15N8)	IAV positive	$10^{-0.9}$
A/duck/Hokkaido/WZ/82/2013 (H16N3)	IAV positive	$10^{1.8}$
High pathogenicity avian influenza virus		
A/duck/Vietnam/HU5-1571/2016 (H5N1)	IAV positive	$10^{2.1}$
A/chicken/Kumamoto/1-7/2014 (H5N8)	IAV positive	$10^{2.0}$
A/chicken/Hokkaido/002/2016 (H5N6)	IAV positive	$10^{1.9}$
A/duck/Japan/AQ-HE29-22/2017 (H7N9)	IAV positive	$10^{2.0}$

[a] Reactivity against $10^{6.0}$ 50% egg-infective dose (EID_{50}) of virus.

[b] Detection limits of the kit were indicated by EID_{50} of viruses.

Table 3. Reactivity of Alere™ i Influenza A&B kit with other avian viruses

Viruses	Strain name	Alere™ i Influenza A&B
APMV	NDV/LaSota	Negative
	NDV/B1 Hitchner	Negative
	APMV-2/Chicken/Yucaipa/1956	Negative
	APMV-3/Turkey/Wisconsin/1968	Negative
	APMV-4/Duck/Mississippi/320/1975	Negative
	APMV-5/Budgerigar/Japan/TI/1975	Negative
	APMV-6/Duck/Hong Kong/199/1977	Negative
	APMV-7/Duve/Tennessee/4/1975	Negative
	APMV-8/Goose/Delaware/1053/1976	Negative
	APMV-9/Duck/New York/22/1978	Negative
	APMV-10/Penguin/Falkland Island/324/2007	Negative
IBV	B-42	Negative
ILTV	NS-175	Negative
IBDV	Saga	Negative

Abbreviation: APMV, avian paramyxovirus; NDV, Newcastle disease virus; IBV, infectious bronchitis virus; ILTV, infectious laryngotracheitis virus; IBDV, infectious bursal disease virus.

Detection of virus genes in swabs and tissue homogenates of chickens experimentally inoculated with H5N6 HPAIV

The applicability of the kit for the diagnosis of H5N6 HPAIV infection in the chickens was evaluated and compared with the IC kit, ESPLINE® A INFLUENZA. For experimental infection, all chickens started to show clinical signs on 1–1.5 days post-infection (dpi), and the last chicken died on 2.5 dpi. Overall, 34 swabs and 20 tissue samples were collected from chickens that were experimentally inoculated with A/chicken/Hokkaido/002/2016 (H5N6) (Table 4 and 5), and virus titration and diagnostic tests using the Alere™ i Influenza A&B kit and the IC kit were performed.

Regarding tracheal and cloacal swab samples, virus titers varied in the range from under the detection limit to $10^{6.0}$ EID_{50}/ml and $10^{6.3}$ EID_{50}/ml, respectively, at 0.5 and 2.5 dpi. The Alere™ i Influenza A&B kit detected viral RNA from all chickens between 0.5 and 1.5 dpi (Table 4). Moreover, the Alere™ i Influenza A&B kit and the IC kit detected the genes or the proteins of IAV from the swabs with virus titer over than $<10^{0.8}$ EID_{50}/ml and $10^{3.3}$ EID_{50}/ml, respectively. For the tissue homogenates, the total of 20 tissue samples were collected from the 4 dead chickens and tested using the Alere™ i Influenza A&B kit and the IC kit. Both kits showed all positive results for IAV in the tissue samples on experimental infection with H5N6 HPAIV, and the virus titer ranged between $10^{7.0}$ and $10^{9.6}$ $TCID_{50}$ per gram tissue (Table 5).

Table 4. Comparison of virus detection from swab samples of experimentally inoculated chickens with H5N6 HPAIV

Day post-infection	Chicken 1 [a]		Chicken 2		Chicken 3		Chicken 4	
	Tracheal	Cloacal	Tracheal	Cloacal	Tracheal	Cloacal	Tracheal	Cloacal
0.5	- / - / <0.8	- / - / <0.8	- / - / <0.8	- / - / <0.8	+ / - / <0.8	- / - / <0.8	+ / - / 1.3	- / - / <0.8
1.0	+ / - / 2.0	- / - / <0.8	+ / - / 1.8	- / - / <0.8	- / - / <0.8	- / - / <0.8	+ / - / 2.0	- / - / <0.8
1.5	+ / + / 5.3	+ / - / 3.6	+ / - / 4.6	+ / - / 3.3	+ / + / 3.3	+ / - / 4.0	+ / + / 4.8	+ / + / 4.6
2.0	+ / + / 4.6 †	+ / - / 3.8 †	+ / + / 6.0 †	+ / + / 3.0 †	+ / + / 4.6 †	+ / + / 4.6 †	+ / + / 5.3	+ / + / 3.3
2.5	ND, ND, ND	ND, ND, ND	ND, ND, ND	ND, ND, ND	ND, ND, ND	ND, ND, ND	+ / + / 4.3 †	+ / + / 6.5 †

[a] Result of Alere™ i Influenza A&B kit, ESPLINE kit and EID_{50} titer (log_{10} EID_{50}/ml) were indicated, for example (+/ - / 2.0). †: Chicken died. +, viral antigen or RNA detected; –, viral antigen and RNA have not detected; ND, sample is not determined by the assay, because the chicken was died at previous day of post infection.

Table 5. Comparison of virus detection from tissue homogenates of experimentally inoculated chickens with A/chicken/Hokkaido/002/2016 (H5N6)

Chicken ID	Sampling date (dpi)	Detection from tissue homogenates [a]				
		Brain	Trachea	Lung	Kidney	Colon
1	2.0†	+ / + / 7.3	+ / + / 8.3	+ / + / 9.6	+ / + / 9.0	+ / + / 8.0
2	2.0†	+ / + / 8.3	+ / + / 7.3	+ / + / 8.8	+ / + / 9.3	+ / + / 8.8
3	2.0†	+ / + / 7.0	+ / + / 9.3	+ / + / 9.3	+ / + / 8.3	+ / + / 8.3
4	2.5†	+ / + / 7.6	+ / + / 7.0	+ / + / 8.3	+ / + / 8.8	+ / + / 8.3

[a] Result is indicated Alere™ i Influenza A&B kit, ESPLINE® kit and $\log_{10}$ $TCID_{50}$/g, respectively. †: The tissues were collected at different dpi when the chicken died. ID number of each chicken is identical with Table 3. +; viral antigen or RNA detected. dpi; days post infection.

For the applicability in the field, the performance of the Alere™ i Influenza A&B kit was evaluated using totally 224 swabs including tracheal and cloacal swabs. In detail, 134 swab samples were collected from apparently healthy chickens, and 90 swab samples were collected from experimentally infected birds; chickens, Muscovy ducks, and domestic ducks with HPAIV mentioned in Table 2. Results indicated that the kit does not detect viral RNA of IAV from these tracheal and cloacal swabs as the result without a non-specific detection in the field swab samples. From these results, to compared with virus isolation method, the sensitivity and specificity of the Alere™ i Influenza A&B kit for detecting HPAIVs were 100% and 99.3%, respectively (Table 6).

Table 6. Comparison of sensitivity and specificity of Alere™ i Influenza A&B kit and virus isolation method

Alere™ i Influenza A&B	**Virus isolation (Tracheal and cloacal swab)**		**Sensitivity (%) (95% CI)** [a]	**Specificity (%) (95% CI)** [a]
	Positive	Negative		
Positive	78	1	100 (100%)	99.3 (99.3 ± 0.01%)
Negative	0	145		

[a] The data are analyzed by Cohen Kappa statistically. CI, confidence interval.

Discussion

Virus isolation is the recommended method for the diagnosis of AI to provide an accurate result with high sensitivity. In recent years, more rapid tests including PCR-based methods have been developed and used for the diagnosis. The Alere™ i Influenza A&B assay provides isothermal nucleic acid amplification for the qualitative detection by NEAR [23] and differentiation of both influenza A and influenza B viruses within 15 min in human samples without specific laboratory equipment and techniques other than the Alere i device [21,22]. The Alere™ i Influenza A&B kit is targeting on the PB2 gene of influenza A and PA gene of influenza B virus (Alere Scarborough, Inc, 2015); and these target regions are highly conserved in AIVs of all 16 HA subtypes [6], but not in other avian viruses such as APMV, IBV, ILTV, and IBDV. In addition, the device qualitatively measured fluorescently labeled target RNA during NEAR described by Bell *et al.* [25]. In the present study, the Alere™ i Influenza A&B kit was evaluated for its applicability to detect AIVs as the kit has not been previously evaluated using animal samples. The kit detected representative strains [HA subtypes (H1–H16), NA subtypes (N1−N9)] of LPAIV and HPAIV with the lower virus titer, and consequently, the detection limit was expressed in the range of $10^{-1.4}$–$10^{2.1}$ EID_{50}/test (Table 2). This detection limit was similar as that in other RNA detection methods including RT-qPCR, loop-mediated isothermal amplification (LAMP) and nucleic acid sequence-based amplification (NASBA) [25,33].

To further assess the performance of the Alere™ i Influenza A&B kit, samples from the chickens experimentally inoculated with A/chicken/Hokkaido/002/2016 (H5N6) were tested. The kit was used for viral detection in swab samples collected from birds since these samples are frequently used to detect AIV in the field and are easily prepared without risk of contamination. The kit detected viral RNA of IAV on tracheal swab at 0.5 day and on cloacal swab at 1.5 days post-infection (Table 4), suggesting that the kit has the capacity to detect AIV infection from the upper respiratory organ as fast as RT-qPCR reported previously [25]. In one swab sample, the kit showed positive result, albeit the virus isolation was under the detection limit. It is possible that viral RNA in the sample was detectable by the kit although the virus protein and infectious virus particle were undetectable by IC kit and virus isolation, respectively. For the kit specificity, the kit did not show non-specific results with AIV-free swab samples which were collected from healthy chickens raised in farms and in an animal facility (Table 6). These results suggest that the Alere™ i Influenza A&B kit can be used to screen samples for H5N6 HPAIV infection in chickens at various stages of infection.

Most countries adopt a stamping-out policy to eradicate high pathogenicity avian influenza (HPAI) in poultry within a short time period. To accomplish this strategy, rapid detection and accurate identification of HPAI cases are critical activities to control the disease spread. In countries, like Japan, which has only experienced sporadic outbreaks of LPAI and HPAI, screening tests on samples from dead poultry with suspected AIV infection can further accelerate control measures and decision making. Therefore, the IC kit, which detects nucleoprotein (NP) of AIVs, is authorized in Japan and is applied to dead birds for screening test in farm-side by local veterinarians. In the fact, HPAI outbreaks have been successfully eradicated in Japan without the transmission of HPAIV to the surrounding farms [34-36]. In present study, the Alere™ i Influenza A&B kit was evaluated which has comparable performance to that of RT-qPCR-based assays [17,18,25,32]. Moreover, the kit could provide the benefits of simple and rapid detection of IAVs in the field without laboratory facility, particularly in far-off areas and in other developing countries where the diagnostic laboratory is not enough trained. However, the assay has limitations in that false positives may occur in samples [22,25], this test performance was not conducted in the fields to check actual condition of the kit usage, and cost per each test is higher compared with virus isolation or antigen detection kit [37]. To clarify these points, further development and assessments on the Alere™ i Influenza A&B kit are needed for both human and animal purpose.

In conclusion, the reactivity of the Alere™ i Influenza A&B kit was analyzed against LPAIVs, HPAIVs, and other avian viruses. Moreover, the kit was evaluated on the samples from experimentally inoculated chickens with A/chicken/Hokkaido/002/2016 (H5N6) and healthy chickens from the field. These results indicate that the Alere™ i Influenza A&B kit has a potential for the detection of AIVs, including H5 and H7 HPAIVs. Furthermore, the kit is easy to handle and suitable to use on site, therefore, the kit is the applicable screening tool in the field for rapid diagnosis of AIV infection in chickens. In addition, to improve practical usage of the kit in bird species, the detection of specific subtypes such as H5 and H7 AIVs can increase the importance of this kit in the veterinary field, regarding early detection of these AIVs with high potential for virus spillover to human.

Brief summary

Diagnosis of AI is performed by standard methods such as virus isolation, gene detection, and antigen detection. Virus isolation using embryonated chicken eggs is the most sensitive method that can provide decisive evidence of the infection. However, virus isolation is time-consuming and requires a laboratory with high biosecurity. Molecular diagnosis methods, such as RT-cPCR, RT-qPCR, and gene sequencing allow rapid and sensitive characterization of influenza viruses. Therefore, molecular diagnosis methods are widely used for both surveillance and diagnosis of AI; however, these methods require sample processing such as RNA extraction and PCR reaction with temperature-sensitive reagents at the laboratory. The Alere™ i Influenza A&B is a novel isothermal nucleic acid amplification kit that can detect and differentiate between influenza A and B viruses in human specimens in approximately 15 min. In the present study, the performance of the Alere™ i Influenza A&B kit was evaluated for its ability to detect avian influenza viruses in chickens. The kit was able to detect representative AIVs (HA subtypes H1–H16, including the recently isolated H5 and H7 HPAIVs), and the detection limit of the kit for these viruses varied between $10^{-1.4}$–$10^{2.1}$ EID50 per test, which is higher than the analytical sensitivity of the antigen detection IC kit ESPLINE® A INFLUENZA. In experimentally infected chickens inoculated with HPAIV A/chicken/Hokkaido/002/2016 (H5N6), viral RNA was detected in the tracheal and cloacal swabs by this kit. These results indicate that this kit has the potential to be used as a rapid screening test of influenza A virus infection in chickens.

Chapter II

Establishment of a mouse- and egg-adapted strain for the evaluation of vaccine potency against H3N2v influenza virus in mice

Introduction

Humans are periodically infected with influenza viruses that cause seasonal epidemics, making it a public health burden. Human influenza caused by influenza A viruses (IAVs) are predominantly existing among the human population and seasonal influenza with acute respiratory signs causes illness in humans. Currently, H1N1 and H3N2 subtype viruses are circulating in the human population [38,39]. It is well known that all IAVs originate from waterfowl, which are natural reservoirs of the viruses, and transmitted IAV to birds and mammals, including pigs and humans. The genetic analysis illustrated those past pandemic viruses were circulating in the pig population and were transmitted to humans directly or after genetic reassortment [39,40].

In 2011, swine-origin influenza H3N2 viruses sporadically affected the human population by direct contact with pigs in Indiana, the United States of America (USA), resulting in human infections with non-human H3N2 virus as called variant H3N2 (H3N2v) [41,42]. The documentation reported 439 cases of human infection with H3N2v viruses in 13 states presently, and the largest outbreak with 321 human cases was recorded during 2011–2012 [43]. Even though case reports of human infections with these H3N2v viruses decreased recently, there are still public health concerns for H3N2v infection in humans. This is because these viruses are antigenically distinct from the seasonal H3N2 influenza viruses, and vaccines currently available against human seasonal H3N2 viruses are not cross-reactive to H3N2v viruses [44,45]. Accordingly, the majority of the population is still susceptible to H3N2v viruses [42,46]. These facts suggested that vaccine strategies against the H3N2v virus infection, which raises concerns on potential future pandemic, should be established immediately.

Ferrets were susceptible to H3N2v viruses without any adaptations. The viruses replicated well and showed moderate pathogenicity in the host animals [47,48]. Accordingly, the evaluation of certain vaccines against H3N2v viruses was conducted in ferrets as well as their original host species, pigs, resulting in different levels of effectiveness in preventing their infections [42,48]. Alternatively, these viruses were poorly replicated in mice [42]. No mouse model for H3N2v vaccine evaluation has been developed until presently. Another serious concern for vaccine development for H3N2v viruses is their poor replication potential in chicken embryos [49].

The present study aims to develop a suitable virus strain from an H3N2v virus, A/Indiana/08/11 (H3N2)v [Ind/08/11], for the evaluation of egg-based vaccine against H3N2v virus infection in mice, by assessing viral growth in mice and chicken embryos, and immunogenicity of H3N2v whole-virus (WV) particle vaccine in mice.

Materials and Methods

Viruses and cells

Human influenza virus, Ind/08/11 was kindly provided by Dr. Richard Webby, the St. Jude Children Research Hospital, TN, USA. Madin-Darby canine kidney (MDCK) cells were maintained in minimal essential medium (MEM, Nissui Pharmaceutical), supplemented with 0.3 mg/ml L-glutamine (Nacalai Tesque), 100 U/ml penicillin G (Meiji Seika Pharma), 0.1 mg/ml streptomycin (Meiji Seika Pharma), 8 mg/ml gentamicin (Takara Pharmaceutical), and 10% fetal calf serum (FCS, Sigma-Aldrich) to propagate influenza viruses. Virus stocks were propagated in MDCK cells at 35°C under 5% CO_2 or in 10-day-old chicken embryos at 35°C, for 48 hr. The infectious fluids were stored at −80°C until use.

Virus titration

Ten-fold serial dilutions of viruses in serum-free MEM were inoculated into MDCK cells, and the cells were incubated in serum-free MEM supplemented with 5 μg/ml trypsin acetylated (Sigma-Aldrich) at 35°C under 5% CO_2 for 72 hr. The presence of the cytopathic effect (CPE) of the infected cells was judged, and 50% tissue culture infective dose ($TCID_{50}$) was calculated by the Reed and Muench method [31]. For viruses exhibiting unclear CPE into MDCK cells, virus titration was performed by immunoperoxidase (IPX) staining assay with slight modification from Kameyama *et al.* [50], using polyclonal chicken antisera against A/Hong Kong/4801/2014 (H3N2) and anti-chicken IgG horseradish peroxidase-conjugated antibody (Bethyl Laboratory, TX, USA). For the evaluation of the result of the IPX assay, the reactivity of viral antigen was recorded as negative (–) and positive (1+ to 4+), and the virus titers were introduced by calculating $TCID_{50}$.

Blind passages of Ind/08/11 virus in mice and chicken embryos

Five 8-week-old female BALB/c mice (Japan SLC, Shizuoka, Japan) were intranasally inoculated with $10^{5.2}$ $TCID_{50}$/50μl of Ind/08/11 at the first passage under intraperitoneal administration of an anesthetic reagent [51]. At 3 days of post-challenge (dpc), mice were euthanized using thiopental sodium (Nipro ES Pharma, Osaka, Japan), and their lungs were collected. Lung homogenates were prepared using a Multi-Beads Shocker (Yasui Kikai) to make 10% (w/v) of suspension in viral transport medium. The pooled homogenates were inoculated into MDCK cells, and the supernatants of them were further passaged in mice. The virus obtained from blind passages in mice was 10-fold serially diluted and

inoculated into the allantoic cavities of two 10-day-old chicken embryos. All the embryos were incubated at 35°C for 48 hr, and allantoic fluids of them were collected separately. In each dilution, pooled allantoic fluids were directly inoculated into two 10-day-old chicken embryos for the following passage. Virus titers and hemagglutinin (HA) titers were monitored throughout all egg passages. Finally, mouse- and egg-adapted Ind/08/11 (Ind/08/11 ma-P7/e-P3) was obtained through seven times passage in mice and three times passage in chicken embryos.

Characterization of a developed strain

For the detailed characterization of Ind/08/11 ma-P7/e-P3, amino acid substitutions in the HA protein were determined, and growth potentials were assessed in chicken embryos and mice. The amino acid substitution in the HA of the developed strain was identified by nucleotide sequencing of the genes as previously described [52]. Briefly, the RNA extracted using TRIzol LS Reagent (Life Technologies, Carlsbad, CA, USA) was reverse-transcribed using the Uni12 primer and M-MLV reverse transcriptase (Promega, Madison, WI, USA). The full-length HA segment was amplified via polymerase chain reaction using gene-specific primer sets and Ex Taq polymerase (Takara Bio, Shiga, Japan). Direct sequencing of the HA gene segment was performed using a BigDye Terminator version 3.1 Cycle Sequencing Kit (Life Technologies, Carlsbad, CA, USA) and a 3500 Genetic Analyzer (Life Technologies, Carlsbad, CA, USA). Amino acid substitutions in the HA proteins between Ind/08/11 and Ind/08/11 ma-P7/e-P3 were analyzed using GENETYX network version 12.0 (Genetyx Co., Tokyo, Japan) by determining amino acid position referring to the H3 numbering system [53].

In order to assess growth potentials, Ind/08/11 or Ind/08/11 ma-P7/e-P3 with $10^{2.0}$ $TCID_{50}$/0.1 ml was inoculated into 10-day-old chicken embryos and incubated at 35°C. Allantoic fluids were collected at 0, 12, 24, 36, 48, and 72 hr of post-inoculation (hpi) to assess the viral growth expressed by $TCID_{50}$ in MDCK cells and HA titers.

To assess the growth potentials of the viruses in mouse lungs, eight 8-week-old female BALB/c mice were intranasally challenged either with $10^{5.2}$ $TCID_{50}$/30 µl of Ind/08/11 or $10^{5.0}$ $TCID_{50}$/30 µl of Ind/08/11 ma-P7/e-P3 under anesthesia. At 3 dpc, four mice in each group were euthanized, and their lungs were collected for making 10% (w/v) of lung homogenates to investigate viral growth. The body weights of the other mice were monitored daily for weight loss and clinical signs until 14 dpc.

Assessment of antigenicity in the developed strain

For the vaccine preparation, the candidate vaccine strain, Ind/08/11 ma-P7/e-P3, was propagated in 10-day-old chicken embryos. After inactivation of infectious allantoic fluid with 0.1% formalin at 4°C for 7 days, the virus was purified using the methods of ultracentrifugation and sedimentation through a sucrose gradient, as modified from Kida *et al*. [54]. The total protein concentration was measured using the Pierce™ BCA Proteins Assay Kit (Thermo Fisher Scientific, Waltham, MA, USA).

Five 4-week-old mice were administered with the inactivated vaccine containing 100 µg of purified Ind/08/11 ma-P7/e-P3 by subcutaneous injection, and double doses were given with two-week interval. Blood samples were collected from the tail vein of mice after 2 weeks of the second immunization.

Neutralizing antibody (NT) titers in mouse serum samples were measured using the serum neutralization test (SNT). All serum samples were treated by heating at 56°C for 30 min and were mixed with 100 $TCID_{50}$ of the virus, then incubated at room temperature for 1 hr. This mixture was inoculated onto MDCK cells and incubated at 35°C under 5% CO_2 for 1 hr. After washing unbounded viruses by serum-free MEM, the cells were subsequently incubated with serum-free MEM containing 5 µg/ml acetylated trypsin. After the incubation for 72 hr, CPE was monitored, and the NT titer of each serum sample was determined as the reciprocals of serum dilution resulting in 50% inhibition of CPE of 100 $TCID_{50}$ of the virus.

Potency test of H3 vaccine against Ind/08/11 in mice

The potency test of the inactivated vaccine prepared by purified viruses was conducted based on the previous study [55]. Briefly, the inactivated vaccine containing 4, 20, and 100 µg of purified Ind/08/11 ma-P7/e-P3 was administered subcutaneously twice with a two-week interval into 10 4-week-old female BALB/c mice in each dose group. Phosphate buffered saline (PBS) was administered as containing without purified viruses as a control study. Blood samples were collected from the tail vein after two weeks of each immunization, and NT titer in the serum was measured by the SNT test. After the second blood correction, all the mice were challenged intranasally with $10^{5.0}$ $TCID_{50}$/30 µl of Ind/08/11 ma-P7/e-P3 strain under the anesthesia. At 3 dpc, the 5 mice from each group were euthanized to collect their lungs for measuring viral growth. The other 5 mice were monitored daily for their weights and clinical manifestations until 14 dpc.

Statistical analysis

The Student's *t*-test was applied to confirm differences in the body weight of mice, virus recovery, and the NT titer between the two groups. A p value less than 0.05 was considered significant.

Ethics statement

All animal experiments were performed in the animal biosafety level 3 (ABSL-3) facility at the Faculty of Veterinary Medicine of Hokkaido University, and were approved by the Institutional Animal Care and Use Committee of the Faculty of Veterinary Medicine, Hokkaido University (Approval number: 16-0105 and 21-0016). All experiments were conducted per the guidance of this committee. The Faculty of Veterinary Medicine, Hokkaido University has been accredited by the Association for Assessment and Accreditation of Laboratory Animal Care International (AAALAC International) since 2007. In all the mouse experiments, the humane endpoint was determined as 70% of body weight at the challenge.

Results

Establishment of a candidate vaccine strain Ind/08/11 ma-P7/e-P3 and its growth in mice and chicken embryos

Even though the maximum titer ($10^{5.2}$ $TCID_{50}$/head) of Ind/08/11 was intranasally inoculated into mice, none of the challenged mice died, and the viruses were only recovered from lungs of 2 out of 5 mice with virus titers less than $10^{4.0}$ $TCID_{50}$/g (Table 7). To select the virus that sufficiently replicates in the mouse lung, the virus was passaged seven times in mice. Furthermore, mouse-adapted Ind/08/11 was serially passaged three times in chicken embryos to improve virus replication in eggs. The growth ability of two viruses, Ind/08/11 and Ind/08/11 ma-P7/e-P3, were compared and assessed in chicken embryos. Ten-day-old chicken embryos were inoculated with these viruses with $10^{2.0}$ $TCID_{50}$/0.1 ml, and viral growth was monitored at different time points (Figure 1). The virus titers of the passaged virus reached $10^{8.1}$ $TCID_{50}$/ml at 48 hpi, whereas those of the parental virus were at most $10^{4.9}$ $TCID_{50}$ /ml at 72 hpi. In addition, the viral growth with significant HA titers was observed from 36 hpi in the passaged virus, whereas no detectable HA activity was observed in the parental virus. The results indicated that Ind/08/11 ma-P7/e-P3 showed higher replication capacity in chicken embryos compared with the parental virus.

Moreover, mice were challenged with Ind/08/11 or Ind/08/11 ma-P7/e-P3 to compare their replication ability in mice. For the challenge study, 4 mice were inoculated with $10^{5.0}$ $TCID_{50}$/head of Ind/08/11 ma-P7/e-P3, and other 5 mice were inoculated with $10^{5.2}$ $TCID_{50}$/head of Ind/08/11 (the maximum dose in the MDCK cell culture). At 3 dpc, viral growth in the lungs of the challenged mice was assessed (Figure 2). The parental Ind/08/11 virus was recovered from 2 out of the 5 mice, and the virus titers ranged between $10^{2.7}$ and $10^{3.3}$ $TCID_{50}$/g. On the other hand, Ind/08/11 ma-P7/e-P3 virus was recovered from all 4 mice with significantly higher titers in their lungs as an average titer $10^{5.5}$ $TCID_{50}$/g. All the mice survived after the challenge for the 14 days (Figure 3). Based on these results, a challenge strain was successfully established for the evaluation of the Ind/08/11 ma-P7/e-P3 vaccine, although it is sublethal in mice.

Table 7. Virus recovery from mouse lungs during blind passages of Ind/08/11 (H3N2)v

Viruses [a]	Challenge titer (log_{10} $TCID_{50}$/head)	Virus recovery from lungs at 3 dpi (log_{10} $TCID_{50}$/g)				
		1	2	3	4	5
ma-P1	5.2	3.7	4.0	<u><1.8</u>	<u><1.8</u>	<u><1.8</u>
ma-P2	5.8	<u><1.8</u> [b]	3.3	3.0	<u><1.8</u>	<u><1.8</u>
ma-P3	4.8	4.0	3.3	3.5	2.6	<u><1.8</u>
ma-P4	3.7	5.8	6.8	4.8	<u><1.8</u>	3.0
ma-P5	5.2	4.6	4.5	4.3	4.8	4.5
ma-P6	6.3	6.0	6.3	6.3	6.0	6.0
ma-P7	7.5	6.0	5.8	6.6	6.3	6.5

[a] ma-Pn: mouse-adapted viruses generated from the "n" passage.
[b] Underlined lung homogenates were not involved the virus inoculum for next passage.
dpi; days of post-infection.

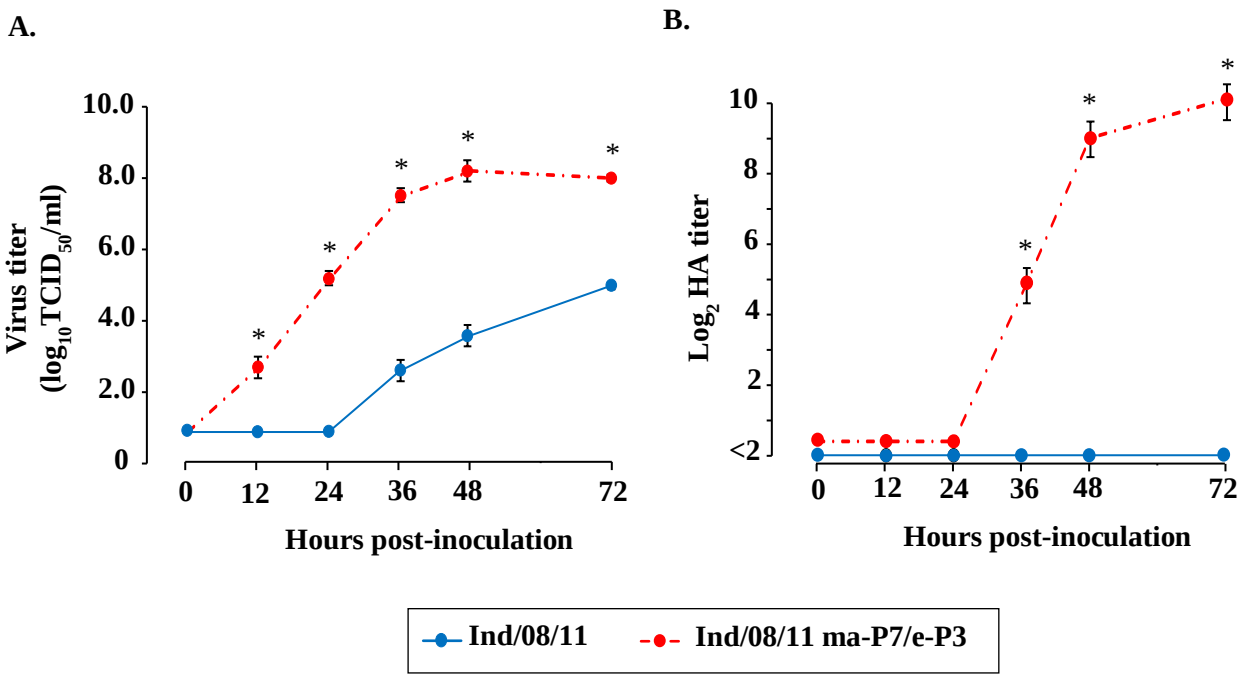

Figure 1. Growth kinetics of the parental and passaged viruses in chicken embryos.

The viral growth was assessed using three chicken embryos inoculated with the viruses of $10^{2.0}$ $TCID_{50}$/0.1 ml for 72 hpi. The viral growth was quantitatively indicated by $TCID_{50}$ in MDCK cells (A) and by HA titer using chicken red blood cells (B) at each time point. The blue line and dash-dotted red line represent Ind/08/11 and Ind/08/11 ma-P7/e-P3, respectively. Asterix indicates a significant difference between the Ind/08/11 and Ind/08/11 ma-P7/e-P3 groups ($p < 0.05$) at each time point.

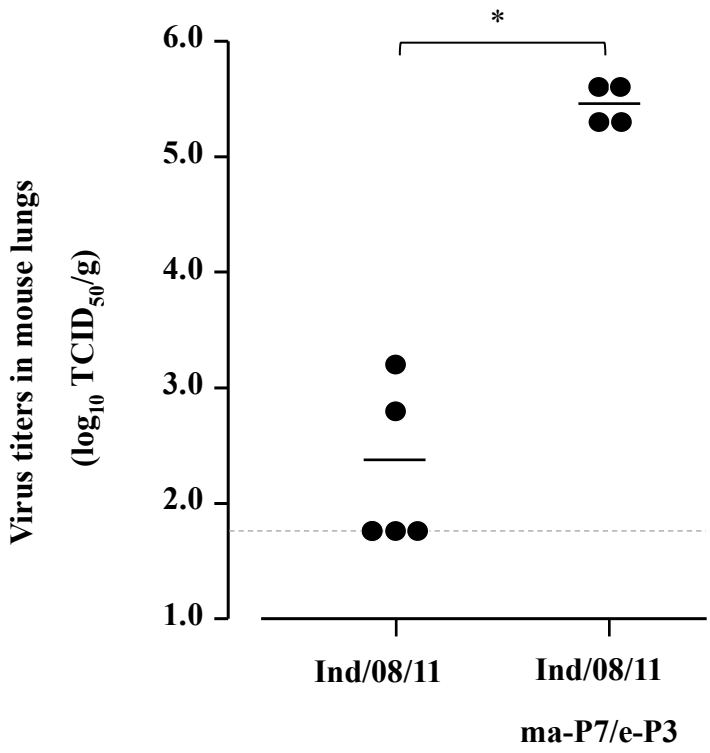

Figure 2. Virus recovery of Ind/08/11 (H3N2)v viruses after serial passages.

Lungs of mice inoculated with Ind/08/11 or Ind/08/11 ma-P7/e-P3 were collected at 3 dpc. Virus titers of each mouse lungs was expressed as dots and average virus titer in the group are indicated with bars. The dashed line indicates the lower detection limit of the virus titration ($10^{1.8}$ $TCID_{50}$/g). Asterix shows a significant difference ($p < 0.05$) under the hypothesis that the titer below than lower limit is set as $10^{1.8}$ $TCID_{50}$/g, which may provide the highest significant titer in the group.

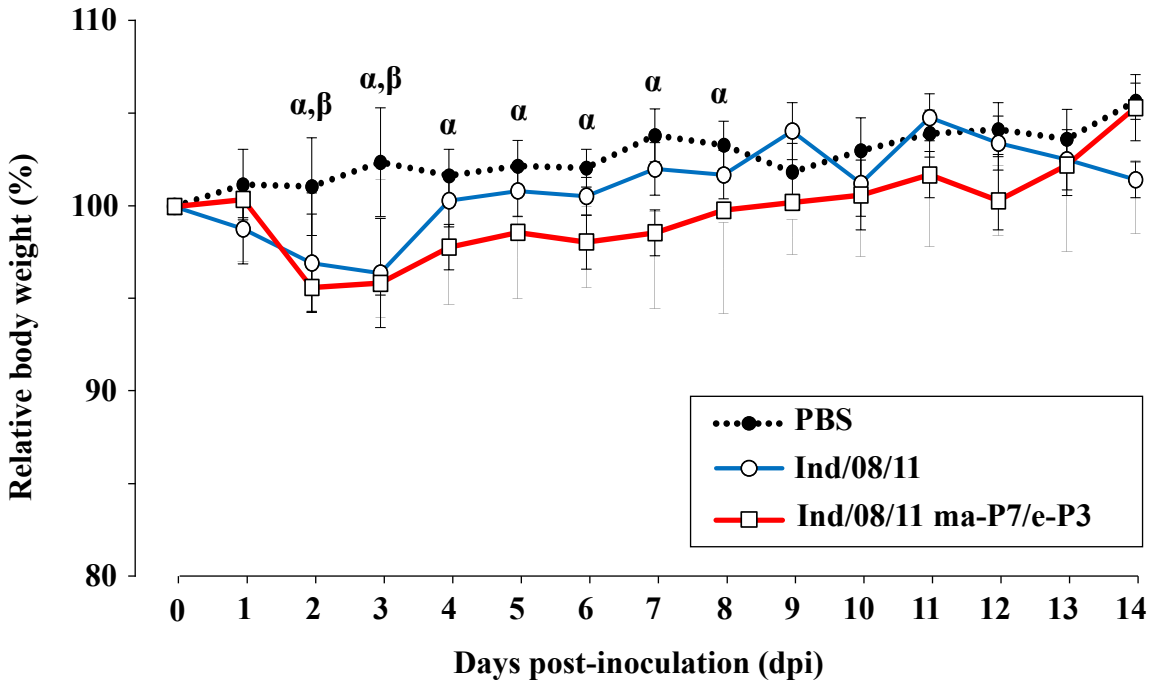

Figure 3. Body weight changes of mice challenged with Ind/08/11 or Ind/08/11 ma-P7/e-P3 viruses.

To assess the growth of these viruses in mice, mice (n = 8/group) were intranasally challenged with undiluted cell suspension collected from infected MDCK cells with pooled lung homogenates. Body weight was monitored throughout 14 days of post-inoculation and the average of body weight changes from day 0 was indicated. Lines with the circle and square is represent the mice groups challenged with different viruses, respectively. The dashed line indicates the control group. α and β: significant difference between the PBS and Ind/08/11 group challenged with $10^{5.2}$ $TCID_{50}$/30 µl and Ind/08/11 ma-P7/e-P3 group with $10^{5.0}$ $TCID_{50}$/30 µl groups in a day ($p < 0.05$), respectively.

Amino acid substitutions on Ind/08/11 ma-P7/e-P3 and antigenicity analysis

During these blind passages, three amino acid substitutions, A138T, N165K, and V226A, were observed in the HA protein (Table 8). Of these, two amino acid substitutions, A138T and N165K, were recognized on the head domain of the HA protein, and the other substitution, V226A, was found on the receptor-binding site of the HA protein. Antigenic correlation of Ind/08/11 ma-P7/e-P3 with the parental Ind/08/11 strain was assessed by the SNT test using mouse sera after immunization with Ind/08/11 ma-P7/e-P3 (Table 9). Two to 4-fold differences were observed between titers against the parental and passaged viruses in each serum. This indicated that after serial passages in mice and chicken eggs, the antigenicity of Ind/08/11 ma-P7/e-P3 changed slightly, but not remarkably. Overall, the viruses derived from serial passages in mice and chicken embryos potentially replicated well in both, and this Ind/08/11 ma-P7/e-P3 virus was used as a candidate strain for the vaccine preparation and challenge studies.

Table 8. Amino acid changes in HA protein throughout the passages of Ind/08/11 (H3N2)v virus

Viruses	Amino acid in corresponding position of HA protein		
	138 [c]	165	226
Ind/08/11	A	N	V
Ind/08/11 ma-P1 [a]	•	•	•
Ind/08/11 ma-P3	T	•	•
Ind/08/11 ma-P5	T	K	•
Ind/08/11 ma-P7	T	K	•
Ind/08/11 ma-P7/e-P3 [b]	T	K	A

[a] ma-Pn: mouse-adapted viruses generated from the "n" passage.
[b] ma-Pn/e-Pn: mouse- and egg-adapted viruses generated from the "n" passage.
[c] numbers of amino acid position were calculated according to H3 numbering system.

Table 9. Neutralizing antibody titers of immunized mice against two virus strains

Vaccine strain	Ind/08/11 [a]		Ind/08/11 ma-P7/e-P3	
	Titers in each mouse	GM	Titers in each mouse	GM
Ind/08/11 ma-P7/e-P3	80, 160, 160, 160, 160	139	160, 320, 320, 640, 640	368

Abbreviations: Ind/08/11, A/Indiana/08/2011 (H3N2)v; Ind/08/11 ma-P7/e-P3, mouse- and egg-adapted A/Indiana/08/2011 (H3N2)v after seven passages in mice and three passages in chicken embryos; GM, geometric mean. [a] Titration of neutralizing antibody using Ind/08/11 was assessed by IPX staining assay.

Potency of the vaccine against H3N2v virus in mice

The vaccine was prepared from Ind/08/11 ma-P7/e-P3 virus and injected twice into mice in three different doses to conduct the potency test. Table 10 shows NT titers of the serum samples collected from immunized mice against homologous strain. The induction of neutralizing antibodies was observed in a dose-dependent manner. After the second immunization, NT titers induced with 100 μg of the vaccine were ranged between 160 and 640 (geometric mean (GM) titer, 403). Alternatively, in the 20-μg groups, the GM of induced antibody titers was 202. NT titers in serum collected from the 4-μg group ranged from 40 to 80, and GM titer was 66.

Then, the mice were challenged with $10^{5.0}$ $TCID_{50}$/head of homologous strain. Five mice were euthanized at 3 dpc, and their lungs were collected for assessing viral growth in mice (Figure 4). In the PBS group, the challenge virus was recovered at a relatively high titer ($10^{5.5}$–$10^{6.3}$ $TCID_{50}$/g) at 3 dpc, whereas no virus was detected in the mice immunized with the highest dose of the vaccine. Additionally, viral growth was apparently suppressed in the mice immunized with 4 or 20 μg of the vaccine. All the challenged mice, even immunized or non-immunized, showed a significant reduction in body weight compared with the no challenge group (Figure 5). Among them, the significant differences in body weight were observed between the groups of the 100 μg and PBS from 4 to 8 dpc, and the bodyweight of the mice in the 100-μg group more quickly recovered than those in other groups. In the 20-μg group, body weights decreased until 3 dpi and slightly recovered; this was significantly different at 7 and 8 dpi compared with those of the PBS group. The same pattern of the bodyweight change was observed in the 4-μg group, although a slightly severe reduction of body weight was observed compared with those of the 20-μg group. Overall, the vaccine prepared from Ind/08/11 ma-P7/e-P3 induced enough immunity in mice with decreasing the impact of disease caused by the homologous challenge strain.

Table 10. Neutralizing antibody titers of immunized mice in three different doses against Ind/08/11 ma-P7/e-P3

Vaccine dose (µg)	Titers in each mouse	GM
4	40, 40, 80, 80, 80, 80, 80, 80, 80, 80	66
20	80, 160, 160, 160, 160, 320, 320, 320, 320, 320	202
100	160, 320, 320, 320, 320, 640, 640, 640, 640, 640	403
PBS	<20, <20, <20, <20, <20, <20, <20, <20, <20, <20	<20

GM, geometric mean.

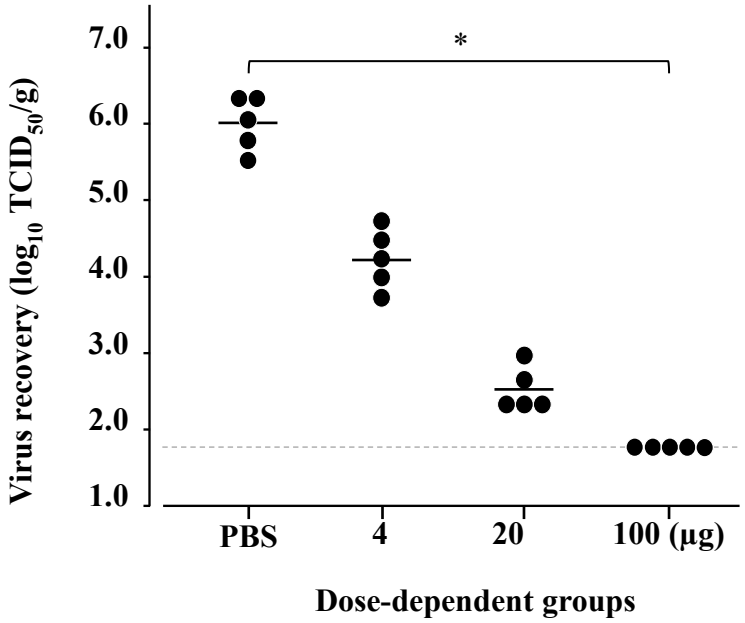

Figure 4. Virus recovery from the lungs of immunized mice after the challenge.

Four mice in each vaccine-dose group were challenged with $10^{5.0}$ $TCID_{50}$/30 µl of the homologous strain, Ind/08/11 ma-P7/e-P3, after two times of subcutaneous vaccination with 4, 20, and 100 µg of the vaccine strain, as well as without antigen (PBS), respectively. The virus in lungs was titrated from the mice in each group at 3 dpc. Virus titers of individual lung sample is express as dots and geometric mean of virus titers in the group are indicated with the bar. The dashed line indicates the lower detection limit of the virus titration ($10^{1.8}$ $TCID_{50}$/g). Asterix indicates the significant difference ($p < 0.05$) under the hypothesis that the titer below than lower limit is set as $10^{1.8}$ $TCID_{50}$/g, which may provide highest considerable titer in the group.

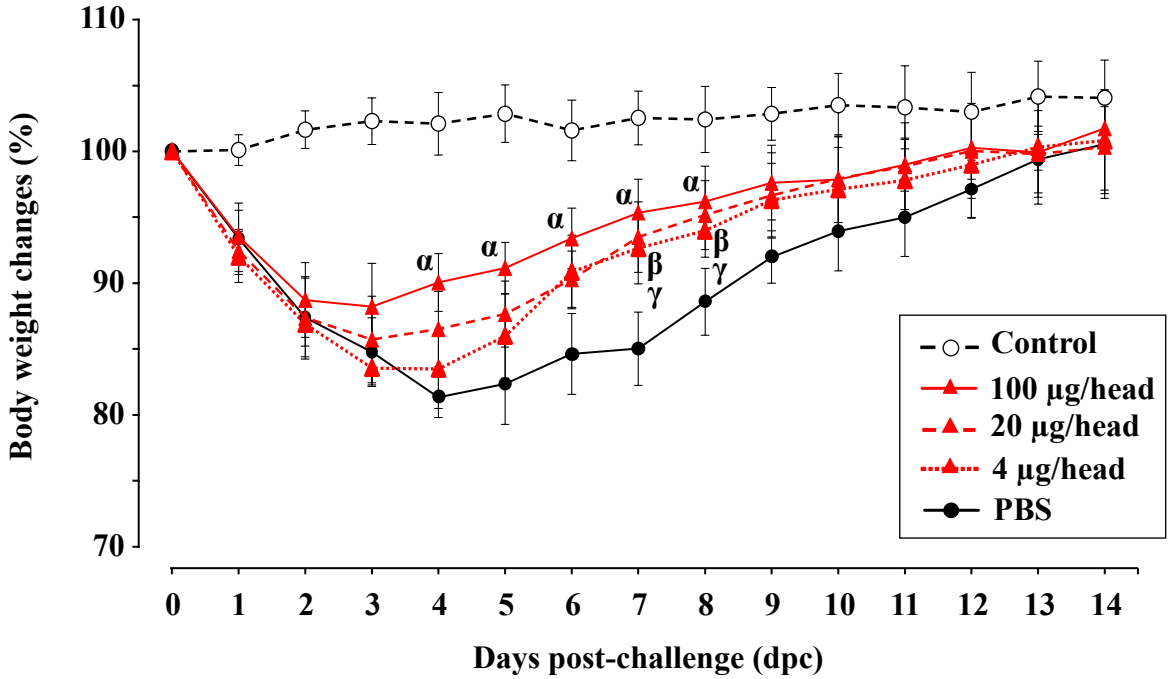

Figure 5. Body weight changes of immunized mice after virus challenge.

Five mice in each vaccine-dose group were challenged with $10^{5.0}$ $TCID_{50}$/30 μl of the homologous strain, Ind/08/11 ma-P7/e-P3, after two times of subcutaneous vaccination with 4, 20, and 100 μg of the vaccine strain, as well as without antigen (PBS), respectively. Body weight of the mice was monitored daily for 14 days after the challenge. An average of the body weight proportion on each post-challenge day to day 0 indicates with open (no challenge) or closed points with lines (vaccinated in different doses). In the figure, α, β, and γ indicate the significant difference from the PBS group to the 100-, 20-, or 4-μg dose groups ($p < 0.05$), respectively.

Discussion

Vaccination is one of the strongest measures to control transmissible diseases, including influenza. This is by suppressing the impact of disease manifestation by inducing acquired immunity in the host. In previous studies, a failure to confer protective immunity against H3N2v virus on naïve host was confirmed by low cross-reactivity of it to recently available influenza vaccines in humans [56]. It should be essential to establish specific vaccine strains for H3N2v viral infections and their evaluation models. The pathogenicity of H3N2v viruses in different animal models, including mouse, ferret, and pig, had been assessed in previous studies [42,57]. The H3N2v viruses caused moderate pathogenicity in ferrets with slight body weight loss, whereas they showed low pathogenicity with limited replication in mice. With these findings, there was a concern raised to develop adequate virus strains that can replicate in mice to evaluate vaccine efficacy *in vivo* settings. Indeed, parental Ind/08/11 was recovered from only 2 out of 5 mice inoculated (Figure 2). Alternatively, candidate strains for vaccine production should be antigenically close to circulating viruses and confer protective immunity against them. Generally, influenza vaccines were produced in chicken eggs [55,58,59]. However, viruses isolated from humans do not grow efficiently in embryonated chicken eggs, which may a negative impact on vaccine development. In this study, to overcome these deficiencies, establishment of a candidate vaccine using the strategy of serial passages was carried out, resulting in higher yields of viruses in eggs (Figure 1).

In this study, the H3N2v virus replicated more efficiently in mice than the parental virus after ten passages (Figure 2). Due to multiple passages in mice and embryonated chicken eggs, amino acid substitutions were confirmed at three positions in HA proteins of Ind/08/11 viruses: A138T, N165K, and V226A. Two amino acid substitutions (A138T and N165K) were in the head domains of the HA protein near antigenic sites A and B [53]. The mutation N165K was previously reported to contribute to viral replication in mouse lung [60,61]. Interestingly, the mutation N165K was also observed in the independent egg adaptation study of the Ind/08/11 strain conducted by Barman *et al*. [49]. The other amino acid substitution of V226A was recognized at position 226, which should be a critical determinant for host adaptation [62] and contributed to high affinity to host cells [63]. The single adaptive mutations at the positions 156, 186, and 228 that were found during the previous egg adaptation studies led to antigenic changes [45,64], thereby resulting in ineffective antibody induction to protect from viral infection. Though an apparent antigenic change in egg-adapted H3N2v was not confirmed in comparison with the parental Ind/08/11

in this study, further clarification of characteristics of egg-adapted H3N2v is essential to assess the potential of antigenic change, identify the molecular basis of other seven segments, and determine a role of a genetic difference which enhances the growth in embryonated chicken eggs and provides host adaptation.

The present study confirmed the high immunogenicity of the whole-virus particle (WV) vaccines using mouse models. The previous study demonstrated that the WV vaccine had higher immunogenicity than currently available ether-split vaccines [65]. However, current technological advance provides novel platforms for the vaccine, i.e., mRNA, plasmid, viral vector, etc. [66]. Additionally, the use of adjuvant or other immune modulators that stimulate innate immunity is discussed to enhance vaccine efficacy [67]. Mouse models should be initially employed for the evaluation of these platforms in the previous studies. It is therefore important to evaluate each platform by comparing its efficacy with the WV vaccine using mouse models. This established model significantly contributes to the future preparation of developing an effective vaccine for humans against H3N2v infection, under a concept of better response for a potential pandemic.

Brief summary

Sporadic spreads of swine-origin influenza H3N2v viruses were reported in humans, resulting in 439 human infections between 2005 and 2018 in the USA. Thus, an effective vaccine is needed to better control a potential pandemic for these antigenically distinct viruses from seasonal influenza. In this study, a candidate vaccine strain with efficient growth capacity in chicken embryos was established through serial blind passaging of Ind/08/11 in mice and chicken embryos. Three amino acid substitutions, A138T, N165K, and V226A, were found in the HA proteins of the passaged viruses without a major change in the antigenicity. This mouse- and egg-adapted virus was used as a vaccine and challenge strain in mice to evaluate the efficacy of the H3N2v vaccine in different doses. Antibodies with high neutralizing titers were induced in mice immunized with 100 μg of inactivated WV particles, and those mice were significantly protected from the challenge of homologous strain. These findings indicated that the established strain in the study is useful for vaccine study in mouse models.

Chapter III

Efficacy of oral vaccine against classical swine fever in wild boar and estimation of the disease dynamics in the quantitative approach

Introduction

Classical swine fever (CSF) is considered one of the most devastating emergent, multisystemic viral diseases of pigs and is a notifiable disease by the World Organization for Animal Health (OIE) [68]. This disease is caused by classical swine fever virus (CSFV) which belongs to the *Pestivirus* genus within the *Flaviviridae* family, and CSFV is an enveloped virus carrying a positive-sense single-stranded RNA genome approximately 12.3 kb in length with one large open reading frame flanked by an uncapped 5'- untranslated region (UTR) and an adenosine-uridine rich 3'-UTR region. Based on 5'-UTR and E2 encoding regions, CSFV is genetically classified into three genotypes (1, 2, and 3) and variable sub-genotypes (1.1–1.7, 2.1–2.3, and 3.1–3.4) [5]. Susceptible and reservoir hosts of CSF are swine species, including domestic pigs and wild boar. The forms of CSFV infection in host animals are highly variable depending on the virus and host factors, such as the virulence of the strain and host immune responses, which differ by the age of the host and presence of secondary infection. The forms are classified as acute, chronic, and persistent infections [69,70].

In September 2018, CSF reemerged in Japan 26 years after the last CSF case. The first CSF outbreak was reported in domestic pigs in Gifu prefecture, in the central area of Japan. Soon after, wild boars infected with CSFV were found in an area near the first case of CSFV detection in pigs. As of the end of June 2021, a total of 3,050 wild boars were confirmed to be infected with CSFV in 23 prefectures. The infections were caused by a moderately virulent CSFV strain genetically belonging to genotype 2.1, which is closely related to recent CSFV strains isolated in China (2015) and Korea (2017–2019) [71,72]. Overall, the detection of infected host animals was delayed due to the CSFV infections being caused by moderately virulent strains, which resulted in the presence of asymptomatic clinical signs and silent infection in the susceptible hosts, playing a potential role in the CSF spread in the population. Since the CSF cases were confirmed in wild boars, control measures were implemented such as setting up fencing to restrict wild boar movement, increasing the capturing of wild boars, and disinfection of affected areas wherein wild boars were found as captured and dead. To control the CSF spread at the field, the commercial bait vaccine (Pestiporc Oral, IDT Biologika GmbH, Dessau-Rosslau, Germany), an attenuated CSF vaccine based on C-strain, was applied in wild boars since March 2019. However, the initial CSF outbreaks led to a situation in which CSFV infections in wild boars were continuously detected, and the affected areas of wild boars were expanded.

The surveillance and implementation of oral vaccination were applied to control CSFV infection in wild boar populations; but CSF-affected area was expanded to the neighboring prefectures; the effect of oral vaccination in wild boars was unclear. Therefore, the present study aims to evaluate the vaccine efficacy against CSFV among the wild boar population, for first four vaccinations in two seasons which were implemented continuously with an interval of several weeks until August 2019. Under the aim, disease diversities of wild boars based on virological assessments were assumed according to the antigen and antibody status in each individual and the disease dynamics of CSFV infections based on the epidemiological analysis were visualized in the studied area, to understand the role of wild boar in CSFV infections during circulating in the population and improve CSF control measures in wild boars.

Materials and Methods

Wild boar data and data sources

Following the confirmation of the initial CSF outbreaks in Gifu prefecture, the investigation of CSF in wild boars began. Hunters and local people who found captured and dead wild boars sent the carcasses to the Livestock Hygiene Service Centers in each area. Under this large-scale surveillance, local veterinarians collected samples from a total of 1,443 wild boars between September 2018 and August 2019, and the biological information of sampled wild boars was recorded. The ages of wild boars were estimated according to the body length of the individual animal; wild boar with the body length of less than 80 cm, between 80 and 100 cm, and more than 100 cm, were categorized as piglet of less than 1 year old, young wild boar of 1–2 years old, adult wild boar of more than 2 years old, respectively [73]. According to the criteria, 540 individuals were less than 1 year old, 360 individuals were 1–2 years old, and 534 individuals were more than 2 years old; however, nine individuals were not categorized as any of the three. A total of 754 (52.3%) and 682 (47.3%) wild boars were classified as male and female, respectively (Table 11). Sixteen wild boars with unknown information about age or/and gender were not classified into any of the categories.

The corresponding dates and location names of wild boars were used for epidemiological analysis and the geographical coordinates (latitude and longitude) of each wild boar were described through the Geocoding search system [74]. Geographical information about the dissemination of vaccine baits in the prefecture was used to investigate vaccine efficacy in wild boars.

Table 11. Summary of wild boar samples collected in Gifu prefecture between September 2018 and August 2019

Age group	Total	Gender			Status	
		Male	Female	Unknown	Captured	Dead
≤1 year	540	271	266	3	478	62
1–2 years	360	176	181	3	328	32
>2 years	534	302	231	1	463	71
Unknown	9	5	4	0	9	0
Total	1,443	754	682	7	1,278	165

Wild boar samples

All wild boar samples of Gifu prefecture were collected under the national surveillance program authorized by the Ministry of the Environment and Ministry of Agriculture, Forestry, and Fisheries (MAFF), Japan. Between September 2018 and August 2019, blood, body fluids (ascites, heartwater, and pleural effusion), and tissue samples (tonsil, spleen, and kidneys) were collected. Using these wild boar samples, prefectural Livestock Hygiene Service Centers have conducted the gene-based detection of CSFV using conventional reverse transcription-polymerase chain reaction (RT-cPCR) according to the standard method [75] and antibody detection using enzyme-linked immunosorbent assay (ELISA) [76], then results were reported to the central government. According to results obtained using tonsil samples, 486 individuals (38%) out of 1,278 captured wild boars were positive for CSFV by RT-cPCR, and 132 individuals (80%) out of 165 dead wild boars were positive for CSFV by RT-cPCR. From ELISA results, 448 of the captured wild boars (35%) and 51 of the dead wild boars (31%) were antibody-positive. Further quantitative analyses described below were conducted under the Agreement of "Chu-oh Kaho No. 18-21" between the Livestock Hygiene Service Centers and Hokkaido University, which was issued on 15 October 2019, as secondary usage of the samples. Besides that, serum samples collected from other 77 wild boars in Mie prefecture were used for evaluation of real-time reverse transcription-polymerase chain reaction (RT-qPCR) and RT-cPCR.

RT-qPCR for the detection of CSFV from the serum

Viral RNA was extracted from 50 µl serum or body fluid sample using a MagMax™-96 AI/ND Viral RNA isolation kit (Applied Biosystems, Thermo Fisher Scientific). RT-qPCR reactions were carried out in 20 µl volumes to amplify the 5' UTR of CSFV using the gene-specific primers CSF 100F and 192R, and the CSF Probe 1 [77]. In the reaction, 2 µl of RNA template was used, and RT-qPCR was carried out with a One Step PrimeScript RT-PCR Kit (Perfect Real Time) (TaKaRa Bio) according to the manufacturer's instructions. The temperature profile was 5 min at 42°C and 10 sec at 95°C (reverse transcription), followed by 45 cycles of 15 sec at 95°C, 30 sec at 58°C, and 30 sec at 72°C (PCR amplification). The fluorescence was detected during the annealing step in a LightCycler® 480 instrument.

Luciferase-based serum neutralization test

All serum and body fluid samples were diluted in phosphate-buffered saline (pH 7.2) with Tween 20 (final concentration, 0.15%). This mixture was treated by heating at 56°C for 30 min [78]. Serum neutralization test (SNT) using a luciferase-based recombinant virus was performed according to a protocol previously described [79]. The neutralizing antibody (NT) titers of antibodies against vCSFV GPE$^-$/HiBiT were calculated according to luciferase activity measured in the culture supernatants using Nano-Glo HiBiT lytic detection system (Promega, Madison, WI, USA). A cutoff value for the SNT was calculated based on the average number of the mock-infected 96-well plates having five times the standard deviation of tested wild boar sera. The NT titer of each serum was expressed as the highest serum dilution showing complete neutralization of vCSFV GPE$^-$/HiBiT.

Regression analysis of antibody detection

As SNT is the standard method to quantify NT titers of antibodies against CSFV infection, sample-to-positive (S/P) values of ELISA were compared with titers of SNT for the determination of the correlation of these values. The analysis was performed using the linear regression method in R program [80].

Temporal trend analysis of antibody responses in the wild boar population

The commercial bait, an attenuated CSF vaccine based on C-strain, was applied to control CSF spread since March 2019 [10]. Vaccine baits were disseminated twice per season in the designed areas according to the guidance of the MAFF [81]. The study period involved two seasons with four vaccinations: 1st vaccination, 24–29 March; 2nd vaccination, 7–11 May; 3rd vaccination 10–16 June; and 4th 20–24 August in 2019. Based on the quantitative detection of antibodies against CSFV, the NT titers in wild boar sera were analyzed weekly in a temporal trend analysis according to the calculation of a GM titer. In addition, the proportion of seropositive animals per animals was expressed to show the dynamics of estimated diverse groups and immune responses of wild boars on a weekly base. The geographical analysis was conducted by overlaying the map of oral vaccine dissemination to the locations of wild boar captured or found as dead, which were categorized as vaccinated, to assess the geographical efficacy of bait vaccine dissemination. For the analysis, an area with a 2.5 km diameter was set up and recognized as the vaccinated area associated with the habitat area of wild boars in the field [82].

Classification of quantitative values

Based on the quantitative values obtained from RT-qPCR and SNT, the investigated wild boars in the present study were classified speculatively into groups using the criteria involving the combination of the degrees of the presence of CSFV and antibody. Critical ranges of the value were applied to the criteria under the classification of individual group and had a different cycle threshold (Ct) value of RT-qPCR and NT titer. The Ct value of 40 and NT titer of 1:8 were fixed as the threshold of CSFV-gene detection and antibody detection, respectively. With the quantitative analysis of values, Groups A, B, C, D, and E, were described among the wild boar population, and the value ranges were expressed respectively regarding the quantitative classification: Ct values less than 30 and NT titers less than 8, Group A; Ct values less than 30 and NT titers between 8 and 32, Group B; Ct values between 30 and 40 and NT titers equal or less than 128, Group C; Ct values equal or more than 30 and NT titers more than 128, Group D; Ct values more than 40 and NT titers between 8 and 128, Group E; and Ct values equal or more than 40 and NT titers less than 8, no infection.

Multi-distance spatial cluster analysis and Kernel density analysis

To calculate the maximum distance of wild boars classified in the groups, a multi-distance spatial cluster analysis tool in ArcGIS software version 10.6.1 (ESRI, Redlands, CA, USA) was used according to the guidelines provided on the designer's website and the previous studies [10,83,84]. A common transformation of Ripley's K function was used in the analysis. For an analysis of the spatial pattern of the group, observed K values were compared to the Expected K values of a completely random spatial distribution of CSF detection with 999 simulations, which is equal to a confidence level of 99.9%. The Diff K values contain the Observed K values minus the Expected K values. The Expected K values producing the highest Diff K values were set as the maximum distance for the relationship between CSF detections in wild boars in Gifu prefecture. Kernel density, a nonparametric estimator for simplifying the spatial level of consequent events, especially for Groups A and B, was estimated using the Kernel density tool of ArcGIS 10.6.1. From the processing of Ripley's function, radiuses of 21.5 km and 11 km were obtained from the calculation for Group A and Group B, respectively. These radiuses were applied as the maximum distance for a significant spatial relationship between CSF detections.

Ethics statements

All wild boar samples were collected in the prefectures under the national surveillance program authorized by the Ministry of the Environment and MAFF, Japan as one of the control measures for CSF in wild boar. According to the request from the central government, the Livestock Hygiene Service Centers conducted CSF diagnosis on the wild boar samples by RT-cPCR and ELISA tests and reported the results to the central government. In the present study, the research activities were performed using these wild boar samples under the agreement of “Chu-oh Kaho No. 18-21”, which was issued on 15 October 2019, between the Livestock Hygiene Service Centers and Hokkaido University as secondary usage of the samples. The BSL-3 facility of the Faculty of Veterinary Medicine, Hokkaido University is permitted to store and use CSFV by the MAFF, Japan in accordance with the Act on Domestic Animal Infectious Diseases Control (Permission number: 19).

Results

Quantitative detection of viral RNA in wild boar serum

A total of 1,443 serum and body fluid samples were collected between September 2018 and August 2019 under large-scale surveillance in Gifu prefecture (Table 11). At the prefectural level, tonsil samples were selected and tested for regular CSF diagnosis according to the standard method approved by the MAFF, Japan. Tonsil samples of 1,443 wild boars provided the status of the presence or absence of specific genes of CSFV using RT-cPCR. In this study, due to the limitation of a homogenate of tonsil samples, viral RNA was extracted from the serum or body fluid sample of wild boars and was quantified by RT-qPCR using CSF specific primers and a probe. A Ct value of 40 was applied for the cutoff to distinguish CSFV positive and CSFV negative. Moreover, after the evaluation of RT-qPCR using serum samples, the result indicated that RT-qPCR with the cutoff had high sensitivity and specificity at 100% and 98.4% to RT-cPCR, respectively (Table 12).

Detection of antibodies against CSFV

All of the 1,443 sera and body fluids were used for the detection of antibodies against CSFV using ELISA in Livestock Hygiene Service Centers of Gifu prefecture to screen the antibody response. To confirm the results, these wild boar samples were further investigated for the quantification of neutralizing antibody titers against CSFV by SNT. As SNT is the golden standard method, the initial dilution of the sample was 1:2, and the presence of antibody response would be judged by this criterion. However, with certain factors such as high toxicity of serum samples in the cell culture, SNT had a 2-fold serial dilution initiated from 1:8. Based on the neutralization antibody titers quantified by SNT, the S/P values of ELISA were compared for a conformability of the tests (Figure 6). The correlation coefficient between the average of S/P values and titers of SNT was calculated as 0.81.

Table 12. Comparison of RT-cPCR and RT-qPCR using wild boar sera collected in Mie prefecture, Japan (n = 77)

Assays		**RT-cPCR**		**Total**
		Positive	Negative	
RT-qPCR	Positive	16	1	17
	Negative	0	60	60
Total		16	61	77

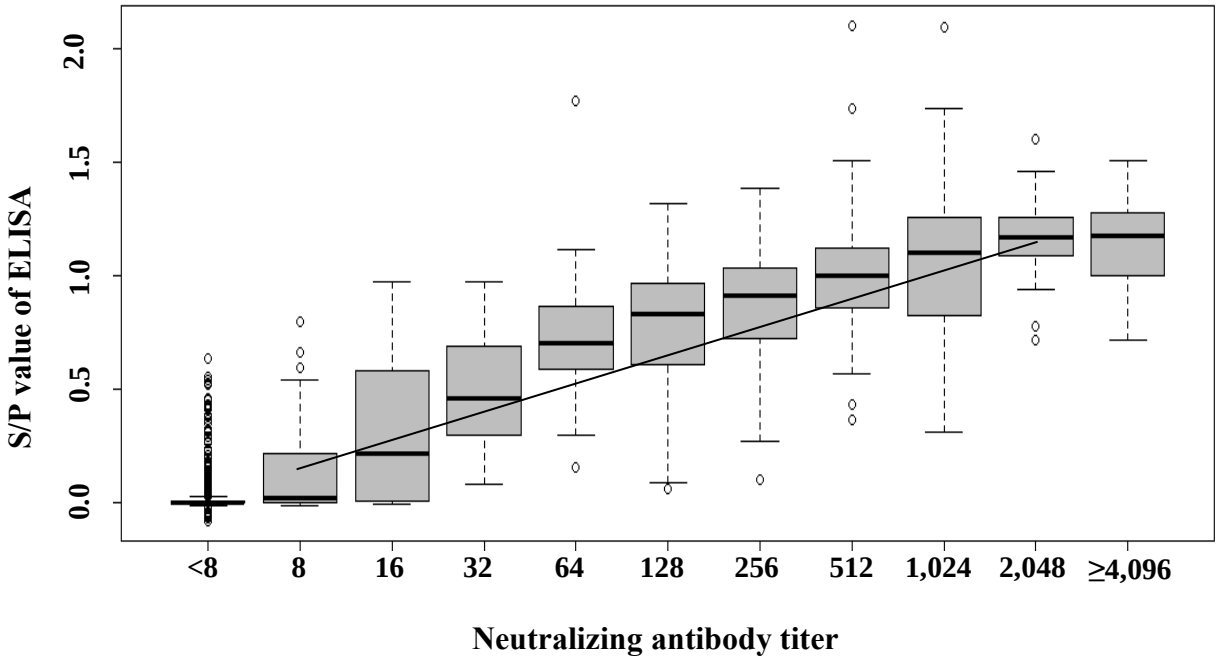

Figure 6. Quantitative association of wild boar serum samples between NT titers and S/P values of ELISA.

Antibody responses of 1,443 wild boar sera were expressed as the NT titers of luciferase-based SNT and S/P values of ELISA and were plotted. The value of the correlation coefficient ($R^2 = 0.81$; $p < 0.001$) indicated a statistically significant linear correlation.

Temporal trend analysis of antibody response in wild boar during with- and without-vaccination period

In the temporal analysis, the period of the present study was divided into two parts: before (from September 2018 to February 2019) and after (from March 2019 to August 2019) oral vaccination (Figure 7), and a total of 598 and 568 wild boars were involved in each part, respectively. In context, among 1,166 wild boar samples, 8.5% (n = 51) and 48% (n = 271) antibody-positive wild boars were detected before and after oral vaccination, respectively (Table 13). Before oral vaccination implementation, wild boars developed low neutralizing antibody titers as a GM titer of SNT up to 90. On the other hand, NT titers dramatically increased from the 1st vaccination and further implementation of oral vaccinations resulted in high GM titer as approximately 560 NT titers. Moreover, induction of neutralizing antibody, which was observed in each vaccination period, was likely to be enhanced by the vaccination; 37 of GM titer between the 1st and 2nd vaccination, 57 of GM titer between the 2nd and 3rd vaccination, 24 of GM titer between the 3rd and 4th vaccination, 90 of GM titer in the 4th vaccination. In the context, the proportion of antibody-positive wild boar after initial vaccination indicated that the rate of wild boars with antibody response among the total population per week was kept as a high rate.

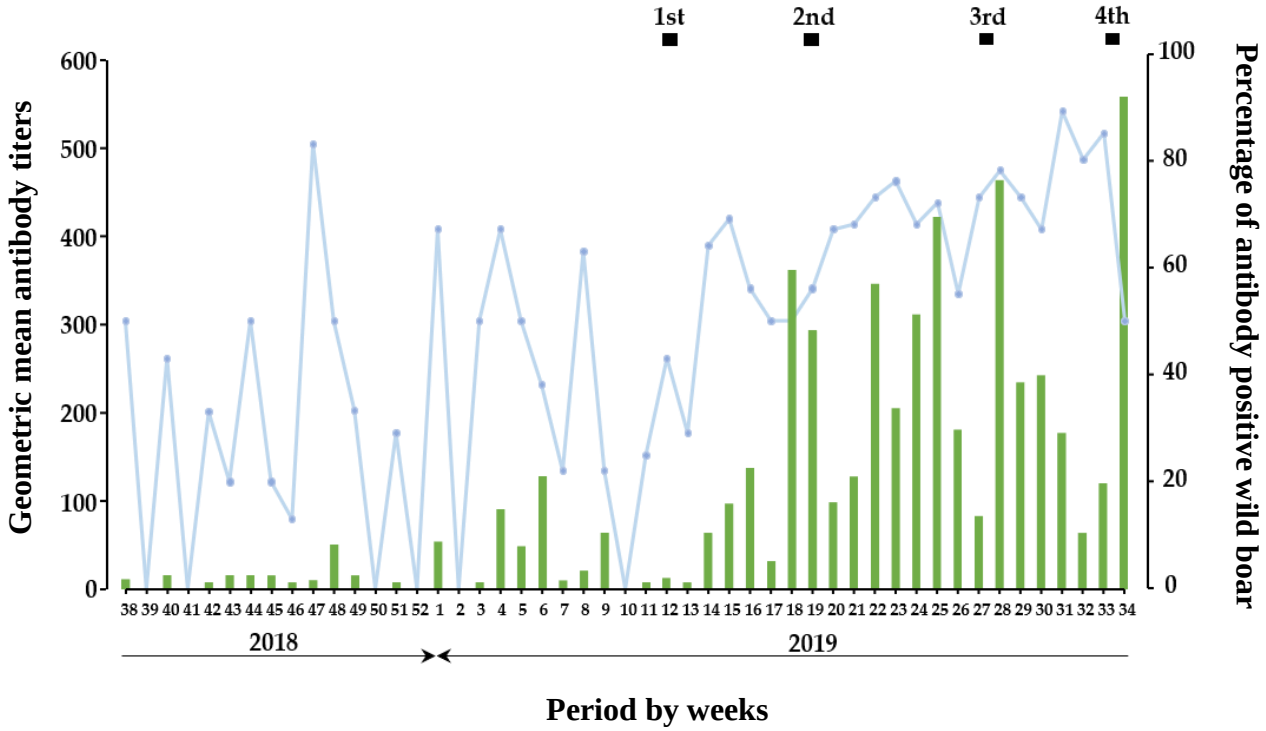

Figure 7. Prevalence of antibodies against CSFV in wild boar.

In temporal trend analysis, results on wild boar samples (n = 1,166) were combined and analyzed as a week base. The green bar indicates the GM titer of SNT in wild boar sera per week, and the blue line indicates the percentage of antibody-positive wild boars in each week. The black box indicates the duration of oral vaccination.

Table 13. The summary for results of RT-qPCR and SNT using wild boar samples collected in Gifu prefecture, Japan (n = 1,166)

Assays	Total sample	Before-oral vaccination			After-oral vaccination		
		n [a]	Positive	Negative	n	Positive	Negative
RT-qPCR	1,166	598	110	488	568	172	396
SNT	1,166	598	51	547	568	271	297

[a] Number of wild boar samples

Divergent quantitative classifications among wild boar population in Gifu prefecture

In the present study, the quantitative analysis of the detection of viral genes and antibodies against CSFV was applied to clarify the details of the immunized or diseased status of wild boars. For the quantitative analysis of CSFV detection, 1,166 out of 1,443 wild boar samples were selected regarding the volume of samples, and viral RNAs were quantified by RT-qPCR targeting the 5'UTR region of CSFV [77]. In the test, 282 samples were diagnosed as CSFV positive, and 884 samples were diagnosed as CSFV negative (Table 14). Moreover, the comparison of RT-cPCR and RT-qPCR using wild boar samples, serum and tonsil were showed low sensitivity and specificity than the same comparison in the only serum samples, which should be due to the difference of sample type (Table 12). Quantitative ranges of the value were applied to the criteria under the classification of individual group and had a different Ct value of RT-qPCR and NT titer. Ct value of 40 and a NT titer of 1:8 were fixed as the cutoff values to distinguish quantitative values for the analysis. Additionally, value ranges were applied regarding quantitative classifications (Table 15). According to the criteria, approximately 14% of wild boars (n = 168) were classified to Group A. About 2% (n = 23) of the population was classified to Group B. In addition, the proportions of wild boars in Group C and D were approximately 7% (n = 81) and 16% (n = 187), respectively.

Table 14. Comparison of RT-cPCR and RT-qPCR using wild boar samples collected in Gifu prefecture, Japan (n = 1,166)

Assays		**RT-cPCR (Tonsil)**		**Total**
		Positive	Negative	
RT-qPCR (Serum)	Positive	266	16	282
	Negative	199	685	884
Total		465	701	1,166

Table 15. Quantitative classification of the antigen and antibodies against CSFV

Groups	Cutoff values		Total	Age				Status	
	RT-qPCR	SNT		≤1	1–2	>2	Un-known	Captured	Dead
Group A	<30	<8	168	57	45	66	0	130	38
Group B	<30	8–32	23	11	5	7	0	16	7
Group C	30–40	≤128	81	18	22	41	0	54	27
Group D	≥30	>128	187	24	62	101	0	185	2
Group E	≥40	8–128	83	15	27	41	0	78	5
No infection	≥40	<8	624	329	130	156	9	606	18
Total			1,166	454	291	412	9	1,069	97

Proportional distribution of diversity of wild boars

The comparison of proportions among the groups of wild boars indicated that wild boars in Group A dominantly presented in the period before vaccination but decreased to a certain level after the implementation of the initial vaccination (Figure 8). On the other hand, Group B was observed occasionally before vaccination but periodically observed in the vaccination period. Proportions of Group A and B were 11.3% and 0.7%, respectively before vaccination, and slightly increased to 17.4% and 3.0%, respectively after vaccination. The proportion of Group C was still stable for both before- and after-vaccination periods. Moreover, the proportion of wild boars classified in Group D slightly increased for all periods of after-vaccination compared with before-vaccination. The proportion of wild boars classified in Group E was apparently higher in the periods of after-vaccination compared with ones of before-vaccination.

Efficacy of oral vaccine for wild boar

According to the data source, bait vaccines were disseminated in the surrounding areas of the urban region where most of the wild boars were found with CSFV until February 2019 (Figure 9) and were periodically disseminated more widely (Figure 10). After the implementation of the first vaccination, the number of antibody-positive wild boar increased, and the development of neutralizing antibody titers progressively increased to approximately 560 of geometric mean (GM) titer (Figure 7). In the analysis, 83 (7%) wild boars were classified in Group E, implying potential wild boars with vaccine response, likely to be protected against CSFV infection overall vaccination periods. However, five out of these wild boars were found to be dead in a field, which could have been caused by other factors such as hunger. The numbers of wild boars with potentially protective immunity against CSFV infection were 26, 35, 19, and 3 out of 568 wild boars in the 1st, 2nd, 3rd, and 4th vaccination periods, respectively. By age group, 15, 27, and 41 wild boars were categorized as vaccine response with age ranges of less than 1 year old, 1–2 years old, and more than 2 years old, respectively (Table 15). Moreover, the geographical analysis showed that 14 wild boars (17%) were found outside of the oral vaccine disseminated area, throughout the entire vaccination periods.

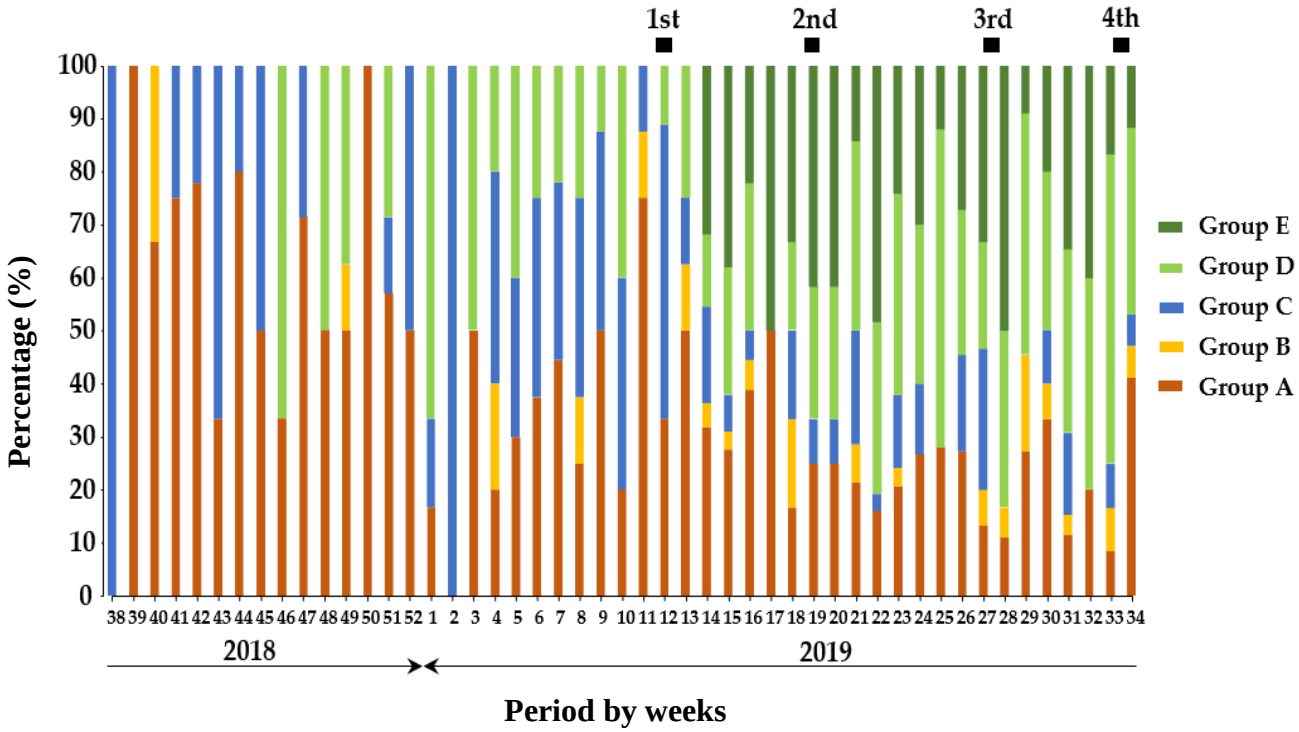

Figure 8. The proportion of wild boars based on the degree of antigen and antibodies against CSFV.

In the temporal trend analysis, the results of quantitative analysis on wild boar samples (n = 1,166) were combined and analyzed weekly. The dark green, green, blue, orange, and red colors indicate the classifications, Groups A, B, C, D, and E in the wild boar population, respectively. The black box indicates the duration of oral vaccination.

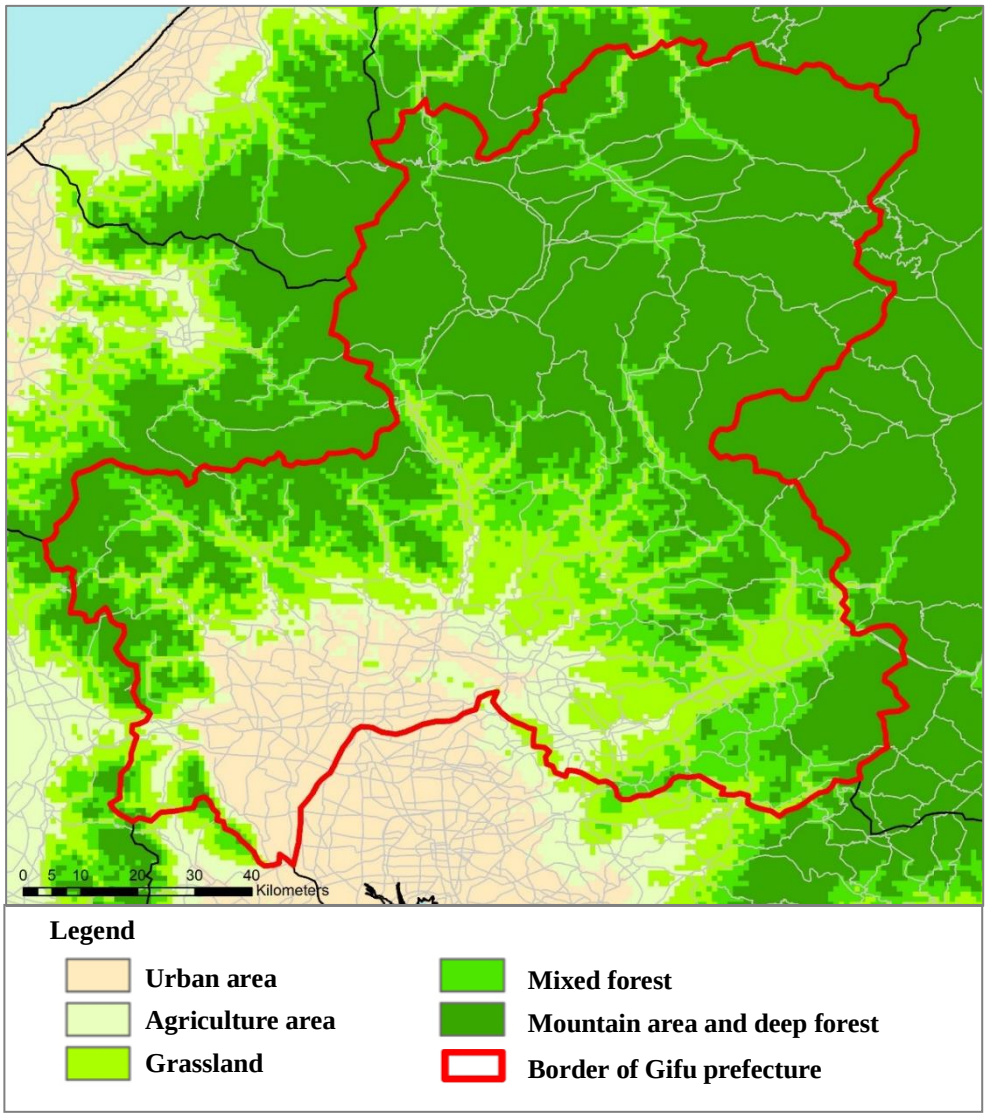

Figure 9. Geographical map of Gifu prefecture.

The map indicates the natural structure of the landscape and localization of the urban area in Gifu prefecture. Areas of urban, agriculture, grassland, mixed forest, and mountain and deep forest are highlighted by light gold, light green, green, dark green, and darkest green colors, respectively. A red line highlights the prefectural border between neighboring prefectures.

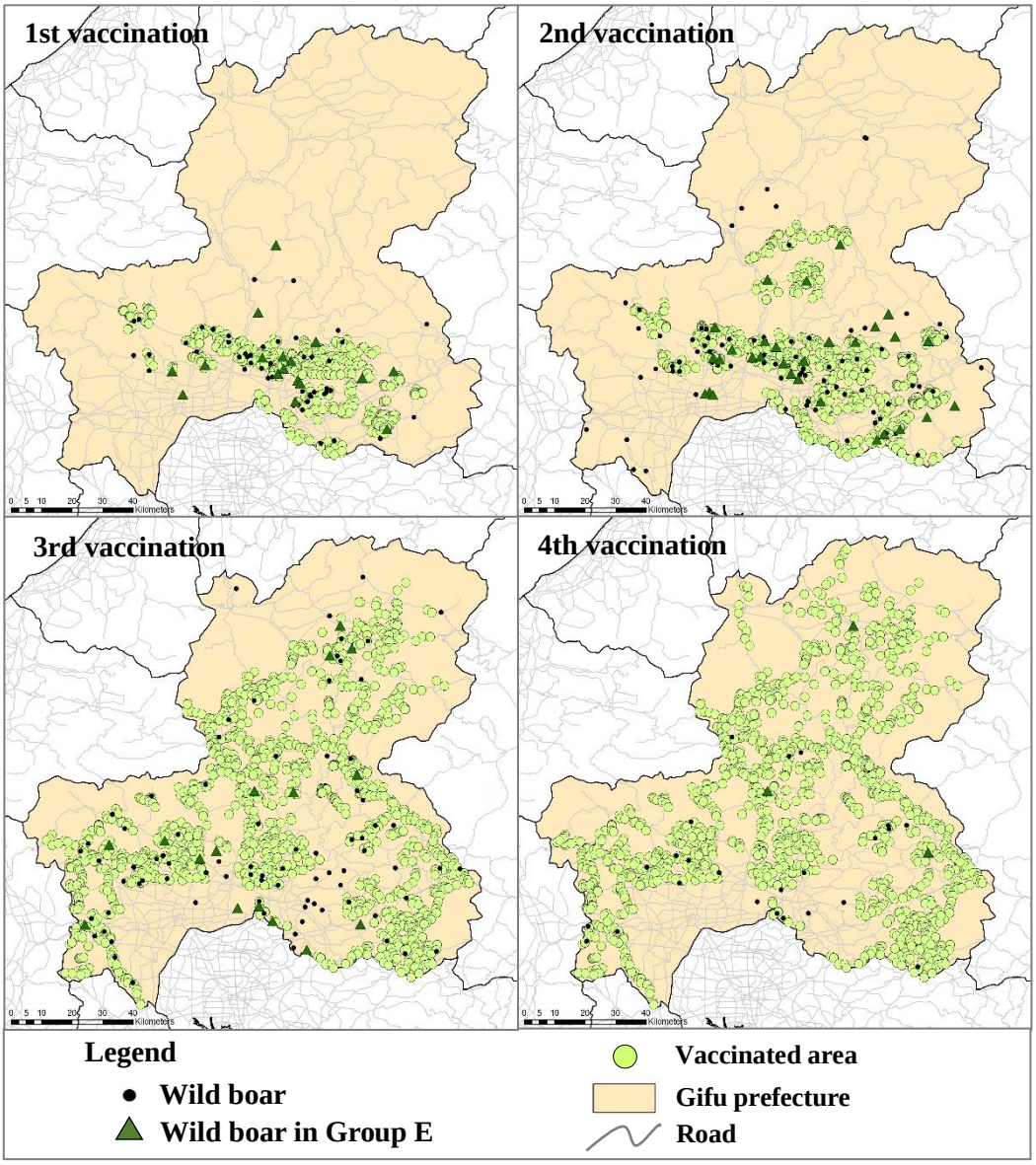

Figure 10. Geographical dissemination of bait vaccine and its response in wild boar

The study period was divided into four regarding each vaccination to visualize the oral vaccination period and the coverage of the vaccination. On the map, black dots indicate the location of the sampled wild boar. The dark green triangles indicate the location where the wild boars in Group E were found in the quantitative analysis. The individual location of dissemination of vaccine baits is indicated by light green circle and the area has been designated as a wild boar habitat zone with an approximately 2.5 km radius.

In addition, wild boars classified in Group A were dominantly observed as 27% among total wild boars in the 1st vaccination period, and the other groups were low proportion between 3 and 23%. Between the 2nd and 4th vaccination periods, the dominant wild boar group was Group D, having an apparent increase as ranged between 28 and 34%. Moreover, the proportion of wild boars in Group A periodically decreased until the 4th vaccination, having the range between 10 and 19%, and this trend was also observed in wild boars in Groups B and C throughout the periods. Taken together, it was suggested that continuous vaccination events contributed to the induction of protective immunity among wild boar population.

Kernel density estimation analysis

The Kernel density estimation was applied to Groups A and B to visualize the spatial distribution of wild boars. It was indicated that wild boars in Group A were initially found in the southern part of the prefecture with a high density and were eventually disseminated in the entire prefecture (Figure 11). In Group A (n = 168), 74% of wild boars were found in areas at a high density (Level 4–6), and 16% were found in areas with low density (Level 1–3). The analysis on wild boars classified in Group B indicated that a total of 23 wild boars were in several areas that had several hot spots with variable density; 70% of them were located in six areas with higher density (Level 3–4), and 30% of wild boars were located in five areas with lower density (Level 1–2) (Figure 12).

To investigate the relationship between Groups A and B with time sequence in terms of CSFV infection, the occurrence of CSFV infections in both groups by every three-month period was overlaid with the strata map of the kernel density analysis (Figure 13). Recognition of wild boars in Group A was limited only in high-density areas, but there were none of the wild boars in Group B during the first period (between September 2018 and November 2018). In the second (between December 2018 and February 2019) and third periods (March 2019 and May 2019), wild boars in Groups A and B existed in the areas with high density and had been gradually disseminated within the equilibrium. Between June 2019 and August 2019, both groups were widely scattered throughout the entire prefecture, and more than half of them were found even outside of high-density areas.

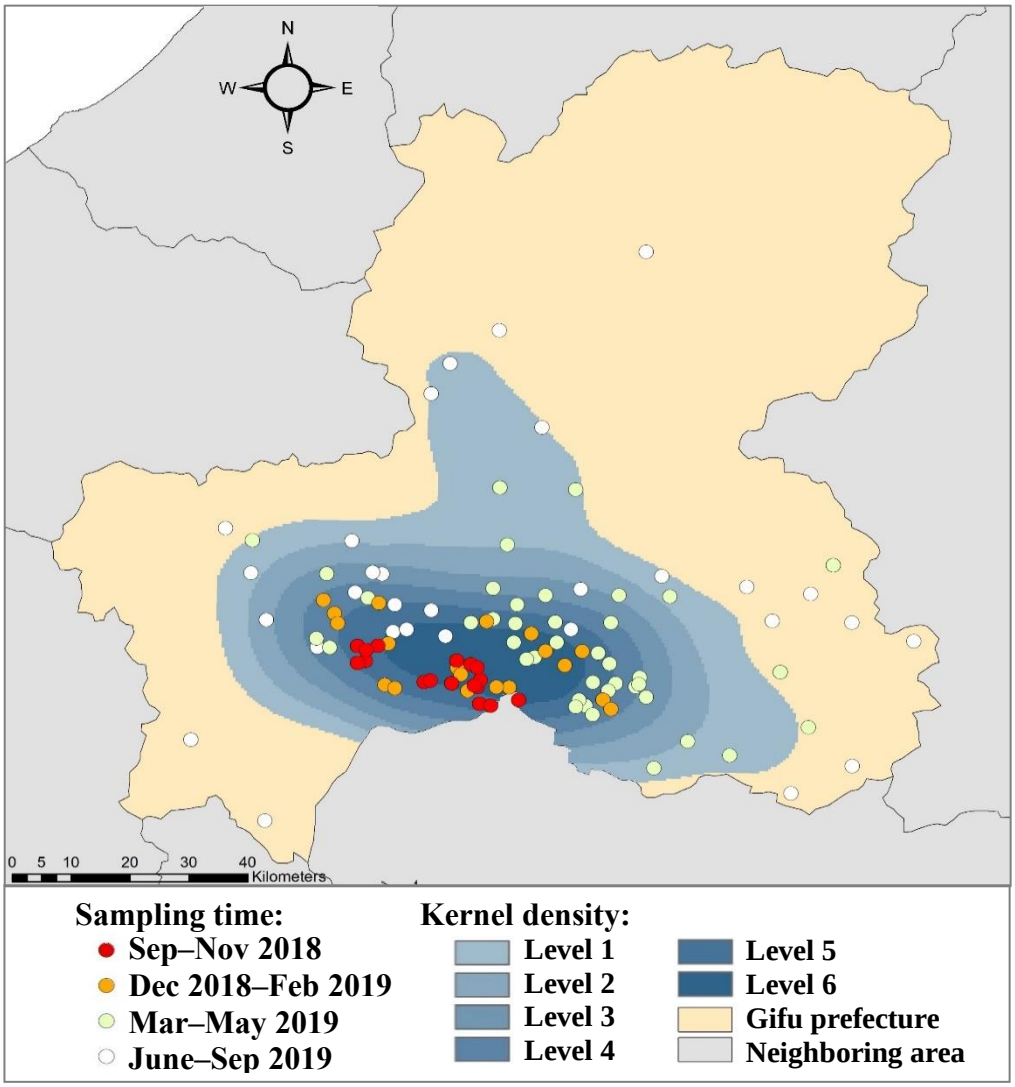

Figure 11. Spatial-time change of wild boar classified in Group A among wild boar population in Gifu prefecture.

The heat map illustrates the estimated kernel density of Group A as likely to wild boar with acute or persistent infection of CSF and there was one epicenter from high density (dark blue, level 4–6) to low density (light blue, level 1–3). The highest density of the infection form was recognized in the southern area of Gifu prefecture. The study period was arranged by 4-time courses, red circle, September–November 2018; yellow circle, December 2018–February 2019; light green, March–May 2019; and white circle, June–August 2019.

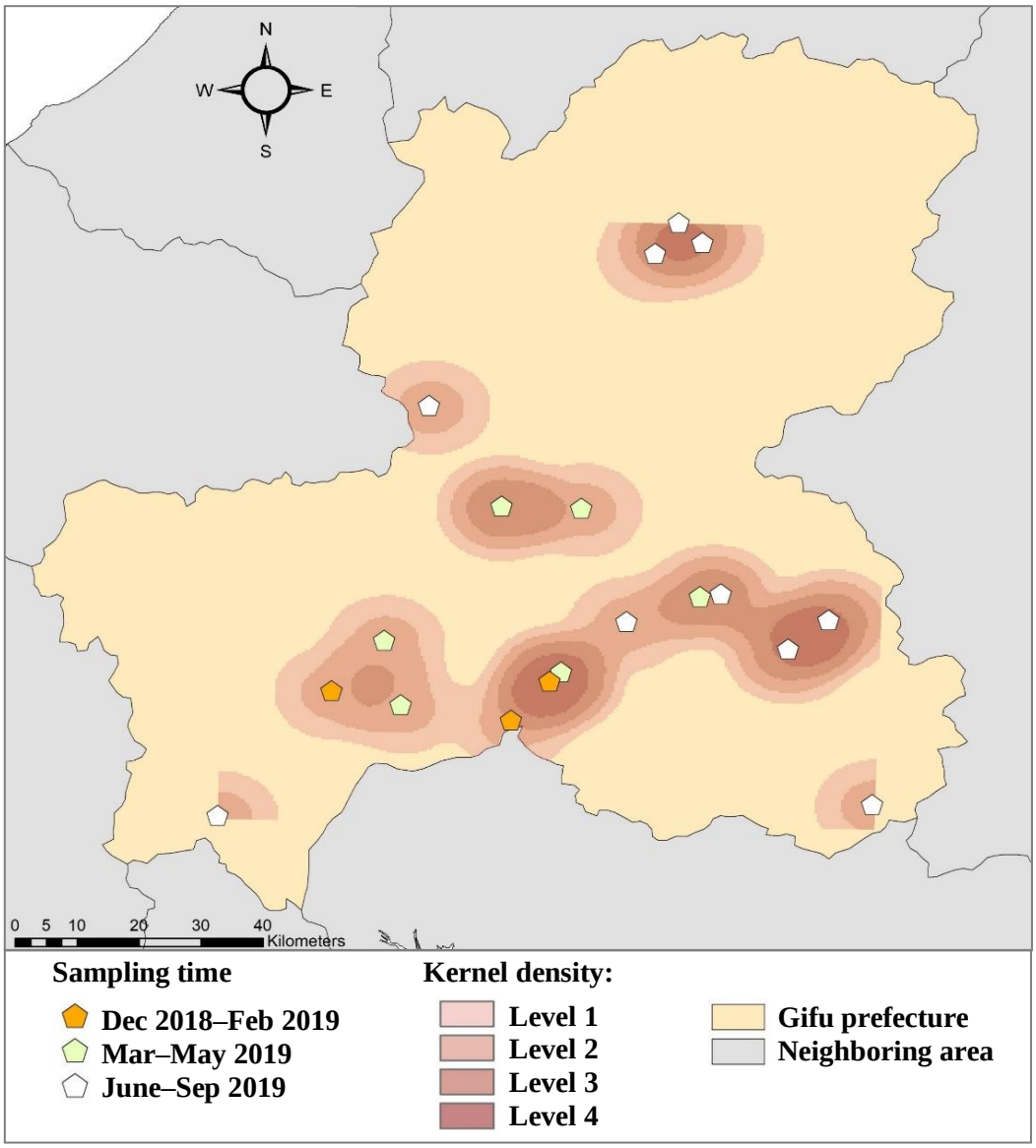

Figure 12. Spatial-time change of wild boar classified in Group B among wild boar population in Gifu prefecture.

The map illustrates the estimated kernel density of Group B as likely to wild boar with chronic infection of CSF and the analysis indicated that it had several hot spots, from high density (dark orange, level 4–6) to low density (light orange, level 1–2). The study period was arranged by 3-time courses, yellow pentagon, December 2018–February 2019; light green pentagon, March–May 2019; and white pentagon, June–August 2019.

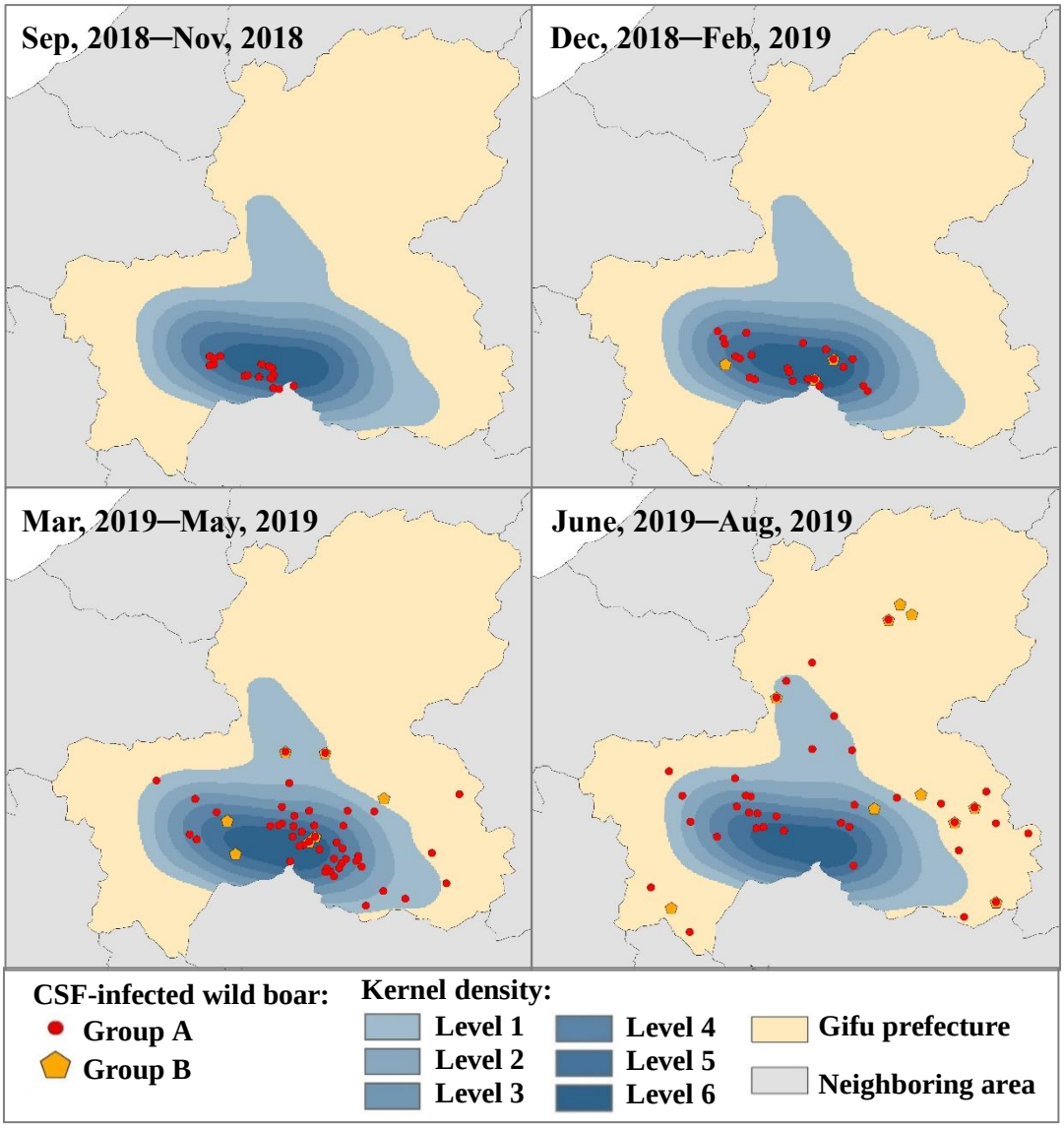

Figure 13. Dynamics of two groups of wild boars classified as a potential natural reservoir of CSFV among wild boar population.

The illustration of Groups A and B was arranged for 4-time courses, as shown on the map. The heat map indicated the estimated kernel density for Group A (red circle) as one epicenter from high density (dark blue, level 4–6) to low density (light blue, level 1–3). Group B (yellow pentagon) was illustrated in the geographical map for the same period.

Discussion

The last notification of a CSF case in Japan was in 1992, and a CSF-free declaration was recognized by OIE in 2007. Based on the unique feature of the country having no bordering countries, the implementation of control measures for disease prevention in Japan is not complicated and without concern of animal movement through the bordering area illegally. As moderately virulent CSFV emerged in wild boars in Gifu prefecture, large-scale surveillance has been implemented since September 2018, and oral vaccination was applied from March 2019 to the present in Japan. The dynamics and risk factors of CSF in wild boars in Japan had been already indicated based on epidemiological data [10,84]. In the present study, the proportions of antibody-positive wild boars and GM titer of SNT were indicated to describe immune status. In addition, the study was highlighted new approaches that RT-qPCR and SNT can provide more precise and quantitative information about the development of immune response and CSFV circulation among the host population to obtain an accurate overview of disease spread in wild boars.

In the present study, the study period involved a time with natural infection of CSFV and with the implementation of oral vaccination. The quantitative analysis revealed that the proportion of antibody-positive wild boars increased continuously during the vaccination period compared with the before-vaccination period, and approximately 48% of wild boars would induce antibody response in the vaccination periods. The increase in antibody-positive rate in the affected area might be attributed to a background of the immune status of wild boar population during natural infection of CSFV between 2018 and 2019. A similar observation is also mentioned in the previous study [85].

As recommended by OIE, tonsil is the best sample for the confirmatory diagnosis of CSF [12]. In the present study, the viral gene in serum and body fluids were quantified by RT-qPCR regarding the ability of the samples from wild boar. Furthermore, quantitative detections of antigen and antibodies against CSFV among the population allow understanding the disease characters, the effectiveness of the vaccine, and progressive changes [76,86]. With this reason, this quantitative analysis involving high-throughput methods suggested that the wild boar population has several characteristics in the infection. The quantitative findings demonstrated that there were wild boar groups with different backgrounds of the amounts of the viral gene and immune response, which may illustrate the diversity of “CSFV infection form” depending on the virulence of the field strain and host factors [5,87,88]. These variable backgrounds were demonstrated in the animal experiment

using pigs and field vaccine study in wild boars [15,85]. The groups are likely to be related with CSFV infections; Groups A, B, C, D, and E are presumed to acute or persistent infection, chronic infection, subclinical infection, recovery, and vaccine response, respectively. Moreover, the CSFV infection caused by moderately virulent strains resulted in multi-infection forms in host animals regarding the difference of age and health condition of wild boar. Among them, wild boars in Groups A and B are assumed to carry out the major characteristic of disease transmission, and these wild boars were potential carriers contributing to the disease maintenance in the field, which was pointed out by European studies [88,89]. As studies described, the risk of carrier pigs with acute or persistent infection, and chronic infection were reported, which highlighted that direct and indirect contacts within the susceptible host could contribute to increasing the risk of CSFV infection and viral transmission among wild boars and domestic pigs [90,91].

Based on these findings of the quantitative detection of antigen and antibody, the temporal analysis of CSFV infections revealed that, in before vaccination, the major infection was assumed acute or persistent infections, and chronic infection (Figure 8). In this assumption, the proportion of wild boars with acute or persistent infection was dominant in this period but decreased by a certain level during vaccination. Interestingly, wild boar with likely chronic infection would be confirmed occasionally before vaccination and was periodically observed in the vaccination period. This would be explained by the evidence that in the vaccinated period, under the assumption that the vaccine response was induced after the implementation of oral vaccination, the profile of groups changed their proportions and the number of antibody-positive wild boars with high NT titers increased (Figure 7–8). On the other hand, the epidemiological analysis suggested that occurrences of potential chronic infection would be overlapped with occurrences of acute or persistent infection in wild boar in the field. It would lead to maintaining of CSFV in the population through long-term infection (Figure 11–13). It means that once the infections occurred widely, the difficulty of CSF control in wild boar was demonstrated similar to previous results [88].

The quantitative approach would describe wild boar status in terms of the vaccination in the CSF affected area. For the efficacy of the oral vaccine, the vaccine response was estimated in 7% of wild boars in the population. Among them, the small proportion of piglets with antibody response indicated that the uptake of the oral vaccine by piglets would be limited. This phenomenon is also indicated in the study of Rossi et al. [89]. That is why it needs to implement long-term vaccination to cover all ages of wild boar, including a new generation of the herd, to prevent vertical transmission. In detail, antibody responses,

including vaccine response and recovery status, were assumed in wild boar. After each oral vaccination, wild boars with an antibody response induced by the oral vaccine were obtained in a proportional range of between 40% and 50% in weekly time points, describing the maintenance of the antibody-positive population in CSF affected areas. This statement demonstrates that the efficacy of the oral vaccine in wild boar supports the results of experimental studies of oral vaccines [85,92]. In terms of antibody response, wild boars assumed as being at a recovery status increased apparently after vaccination from 9% to 26%. Collectively, the increase of the numbers of wild boars with antibody response was likely to be a positive impact of oral vaccination and decrease of CSFV infections in the affected area [93]. However, the limitation is that a clear description of the role of an oral vaccine and the recovery status in terms of CSFV infection was not available in the present study; recent vaccine studies have focused on reliable differentiation of CSF-infected from the vaccinated host [11,85].

Using the criteria based on the quantitative assessment, the disease dynamics (Figure 13) and distribution of wild boars during the vaccination period (Figure 10) suggested that the density of the wild boar population in areas and habitation of wild boar strongly contributed to CSFV spread in the field; these results support previous findings [88]. In this analysis, an area with a 2.5 km diameter was set as the effective area of each bait associated with the habitat area of wild boars in the field [82]. Wild boars with a vaccine response assumed were found in non-vaccinated areas during the oral vaccination period, which may suggest that wild boars should have flexible home ranges of more than a radius of 2.5 km [94]. It was also reported that wild boars are able to shift between habitat types of forest and open areas according to availability of food resources; sows with piglets may change habitat areas because of their sensitivity to human disturbance, and wild boars, especially solitary male wild boars, can have a larger habitat area than females and may re-visit that area at any time [95]. These findings strongly suggested that under a flexible strategy of oral vaccination based on a predictive study, continues vaccinations should contribute to the induction of protective immunity and maintain its level in the population for the long term.

In the quantitative analysis, there is a limitation on the classification of wild boars shown antibody response by either vaccine response or recovery status in CSFV infection. As studies have indicated that depending on virulence of CSFV, the host species would show a variety of clinical signs, however, parallel antibody response is an indicator of progressive CSFV infection or effectiveness of the vaccine in the host [76,86]. To clarify this point,

antigen presence and antibody response should be monitored quantitatively at least twice with a time interval.

Brief summary

CSFV in the wild boar population has been spreading in Japan, alongside outbreaks on pigs, since CSF reemerged in September 2018. The vaccination using oral bait vaccine was initially implemented in Gifu prefecture in March 2019. In the present study, antibodies against CSFV in wild boar were assessed in 1,443 captured and dead wild boars in Gifu prefecture. After the implementation of oral vaccination, the increase in the proportion of seropositive animals and their titer in wild boars were confirmed. Quantitative analysis of antigen and antibodies against CSFV in wild boar implies potential disease diversity in the wild boar population. Animals with status in high virus replication (Ct < 30) and non- or low-immune response were confirmed and were sustained at a certain level after initial oral vaccination. Through continuous vaccination periods, the increase in seroprevalence among wild boars and the decrease in CSFV-positive animals were observed. The epidemiological analysis based on the quantitative virological outcomes could provide more information on the efficacy of oral vaccination and dynamics of CSF in the wild boar population, which will help to improve the implementation of control measures for CSF in countries such as Japan and neighboring countries.

Conclusion

Currently, there is a necessity for sustainable efforts to improve disease control for transboundary diseases (TDs) in humans and animals to ensure livestock production and animal health. Poultry and pigs makeup the major livestock but also these species are highly susceptible to transboundary animal diseases (TADs), such as avian influenza (AI) and classical swine fever (CSF). It raises the importance of implementing a better control strategy for the diseases in chicken, human, and pig, respectively. This study focused on the constructive control measures for these TDs, including the rapid and accurate diagnosis, strategy planning for vaccine, and better evaluation system of disease prevention and dynamics.

For avian influenza virus (AIV) infection, wild waterfowl play an important role in virus introduction and spread in poultry farms with low biosecurity levels. Therefore, the early detection of AIV infections mainly caused by high pathogenicity avian influenza virus (HPAIV) is the key action to monitor and prevent the introduction and spread of AIVs in poultry. For this reason, a rapid and accurate diagnostic kit for AI is needed at the local. One of the novel kits was evaluated in this study, which was developed using an isothermal nucleic acid amplification approach for human usage to detect influenza A and B viruses. The findings from this study suggested that the kit can be used for poultry, especially chicken, to detect viral RNA of all subtypes of AIVs, including low pathogenicity avian influenza virus (LPAIV) and HPAIV, with high sensitivity and specificity. Moreover, this kit is applicable for influenza diagnosis not only in the laboratory but also in the field conditions lacking satisfied laboratory facilities and well-trained personnel.

In terms of spillover infection with influenza A virus, the direct and indirect contact between humans and animals increase the risks as regards human health. Under the effort of zoonosis control, the development of an effective vaccine against such non-human influenza viruses in humans is crucial to engage with the one health concept. In this study, the variant H3N2 vaccine was prepared using the strategy of serial passages in mice and chicken embryos to increase vaccine yield, and its protective potency was evaluated in mice. The results obtained that the vaccine can protect against swine-origin H3N2 infection. The model established in this study could be applied for future preparation of an effective vaccine for humans against variant H3N2 infection. The outcomes can be used for better preparation and control of future pandemics.

From the epidemiological perspective, the implication of the surveillance and implementation of oral vaccination were evaluated against CSF in wild boar in Gifu prefecture, Japan. Based on the CSF-confirmed area during the outbreaks, the role of wild boar was assessed under the CSFV spread among the population. Moreover, to provide better understanding about CSFV infection modes in wild boar population, CSFV infection forms were estimated based on the quantitative analysis combined with the virological and epidemiological approaches. It was suggested that wild boars with acute, persistent, and chronic infections were the main actors in carrying and maintaining CSFV among wild boar population. The implementation of the oral vaccine in wild boar led to an increase in the number of individuals with vaccine responses. Overall, for TDs in animals, this study provided quantitative information from the investigations of virological and epidemiological assessments that can be used to improve further surveillance activity and control measures.

In conclusion, the findings obtained in this study provided an important vision for the contribution of TD control focusing on influenza and CSF as representative diseases. Under this highlight, this information is critical to construct comprehensive control measures for the eradication of TDs involving the early detection of existing causative pathogens, effective preventive strategy with vaccine development, and a better evaluation system for preventive actions. These could reduce the burden in animal and human population in terms of TDs control.

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