Corporation obtaining approval, the name of its representative, and the address of its main office

Name of Entity: Intervet K.K.
Name of Applicant: Yukihiro Takemoto, CEO and President
Address: 2-3-7, Hirano-cho Chuo-ku Osaka City

## Approved Type 1 Use Regulation

| | |
|---|---|
| Name of the type of living modified organism | Herpesvirus of turkey strain HVT-NDV/F transfered with the Newcasle disease virus F protein gene (NDV-F, Meleagrid herpesvirus 1 (Herpesvirus of turkey, Turkey Herpesvirus, Marek's disease virus serotype 3) |
| Contents of Type 1 Use of Living Modified Organism, etc. | [1] Transport and storage (including the transport and storage of inoculated animals carrying viable live genetic recombination vaccine.)<br>[2] The Law on Securing Quality, Efficacy and Safety of Products Including Pharmaceuticals and Medical Devices (Act No. 145 of 1960. Hereinafter called the "Law on Pharmaceuticals and Medical Devices.") A use in accordance with a protocol prepared based on a notification of clinical trial plan to be submitted based on paragraph 2 of Article 80-2 of the law and Article 7 of the Ordinance on the Performance Criteria of Clinical Tests of Pharmaceuticals for Animals (MAFF Ordinances No. 75 of 1997) if a test (hereinafter called "clinical trials") is performed in order to collect data related to the results of a clinical trial for inclusion among data submitted pursuant to the provisions of paragraph 3 of Article 14<br>[3] Use according to a written application for approval based on paragraph 1 of Article 14 of the Law on Pharmaceuticals and Medical Devices (excluding acts that fall under [4]).<br>[4] Inoculation (vaccination for chicken)<br>[5] Disposal of post-inoculation instrument and use residue in accordance with the infectious industrial waste disposal standard under Article 12-2 of Disposal of Waste Management and Public Cleansing Act (Act No. 137 of 1970)<br>[6] Disposal other than [5] (including the disposal of animals inoculated with viable live gene recombination vaccine.)<br>[7] Incidental actions to [1]-[6] |
| Methods of Type 1 Use of Living Modified Organism, etc. | — |

# Overview of the Biological Diversity Risk Evaluation Report

## I. Information collected for biological diversity risk evaluation

### 1. Information concerning the host or its taxonomic species

(1) The taxonomic positioning and distribution circumstances in the natural environment

a. The taxonomic position, scientific name (genus and species), Japanese name

Scientific name: *Meleagrid Herpesvirus 1*

Genus: Mardivirus

Species: *Meleagrid herpesvirus 1*

Japanese name: Shichimencho Herpesvirus (hereinafter called “HVT”)

English name: Herpesvirus of turkey, Turkey Herpesvirus, Marek’s disease virus serotype 3

b. Host strain name

HVT PB1 strain

The virus used as a parent strain of the gene-recombinant microorganism is HVT, the PB1 strain used as a strain for manufactureing Marek's disease live vaccine. ThePB1 strain is isolated from blood of healthy turkey flock in Norforlk, the U.K. around 1969 (Literature 1) and is not atenuated by subculturing wild strain.

c. Living condition in the natural environment in and outside Japan

HVT is derived from healthy turkeys and in general, widely distributed among domestic turkey. There is an example of isolationfrom wild turkeys. Synonim of HVT is the Marek's disease virus serotype 3 (hereinafter called “MDV3”) and antigenically similar to the Marek's disease virus serotype 1 (hereinafter called “MDV1”) and the Marek's disease virus serotype 2 (hereinafter called “MDV2”). MDV1 is pathogenic while MDV2 and HVT (MDV3) are nonpathogenic.

(2) History and the current status of use

Marek's disease is a disease in chickens characterized by lymphoid tumor formation and neuropathy caused by infection of pathogenic MDV1, and is one of the most important diseases in the chicken industry. Live vaccine is a means effective for prevention of Marek's disease. Nonvirulent MDV1, nonpathogenic MDV2, and HVT are used as live vaccine.

Host virus PB1 strains were used from 1970 to 2009 as for strains producing Marek's disease live vaccine sold outside of Japan. The live vaccine was approved in 45 countries or regions. Only after 2001, at least 2.2 billion turkeys' strains were sold. HVT live vaccines from different strains (FC 126 strains) have been used since 1970s in Japan, too.

(3) Physiological and ecological (biological) characteristics

b. Basic characteristics

An animal holding HVT in the natural world is a turkey. The turkey is classed in the family of

Phasianidae in the taxonomic order of Galliformes (according to the World Bird List of the International Ornithological Congress. The Japanese taxonomic name is in accordance of CHECK-LIST OF JAPANESE BIRDS (7th Revised Edition)). Birds other than the turkey with experimentally confirmed cases of HVT are the chicken (Literature 1), quali (Literature 2) and ring-necked pheasant (Attachment 1), and those that are not be infected are the duck (Attachment 2), pigeon (Attachment 2), and bobwhite (Attachment 1).

The duck, which is classified in the toxonomic order of Anseriformes, and the pigeon, which is classified in the toxonomic order of Columbiformes, are thought not to be infected because they are not close in systematology. The bobwhite is classed in the family of Odontophoridae in the taxonomic order of Galliformes and is thought not to be infected because it is different in systematology.

The chicken, quali, and ring-necked pheasant, which are infected with HVT, are in the family Phasianidae, and the green pheasant, copper pheasant, rock ptarmingan, which are also in the family Phasianidae, may display susceptibility to HVT. The pheasant, a closely related species of ring-necked pheasant, is thought to display the same susceptibility as the ring-necked pheasant.

The turkey, the original animal holding HVT, is naturally infected when it is farmed in a region where HVT exists, and by 10-week-old, all individuals in a group become carriers of the virus and antibody (Literature 3). If the turkey is inoculated with HVT, all inoculated individuals are affected and all of those living with them are affected, too (Literature 4). There is a small amount of data on the HVT susceptibility of other birds of the Phasianidae family. All inoculated individual chickens are affected, but those living with them are not affected or only some are if any (Literatures 5, 6, 7, and 8, and Attachment 3). There is no data on the rate of infection of quali. When inoculated, quali produces antibodies and infection is established. In addition, HVT is used as Marek's disease vaccinate in a quail farming as well as in a chicken farming (Literature 2). Only HVT inoculated individuals among ring-necked pheasants carry the virus. It is suggested that the susceptibility of HVT is low (Attachment 1). Based on the knowledge above, the turkey is originally an animal holding HTV and the susceptibility of other birds of the Phasianidae family is low. Even if birds of the Phasianidae family such as the green pheasant, copper pheasant, and rock ptarmingan have high susceptibility to HVT, their susceptibility is thought to be lower than the turkey. Therefore, even if a part of individuals are infected, it is not likely that the infection spreads and is kept in the group.

In inoculation tests of primates such as the cynomologous, rhesus macaque, bonnet macaque, and marmoset with FC 126 strains and pathogenic MDV JM strains, no abnormality was found in clinical manifestations and hematology tests. In addition, primates above are thought not to be infected with the virus because no neutralizing antibody is produced (Literatures 10 and 11).

About 200 hundred people who have an opportunity of being exposed to HVT including a mis-injection case and who have no such opportunity were examined to find whether to retain antibody against HVT by the fluorescent antibody method. Six were positive but were not found to

have a correlation with HVT exposure, and no neutralizing antibody positive person was found from fluorescent antibody carriers (Literature 10).

HVT has about 160-kbp double-stranded DNA genome (Literature 9). The genome is enclosed in a 90-100 nm diameter capsid. Capsid is made up of 162 hollow-centered capsomeres arranged as an icosahedron. Capsid is enclosed in an envelope, and particles budding from core lose envelopes and their size is around 150-170nm. Particles mature in cytoplasmic, become enveloped and 220-250-nm particle (Fig.1, Literature 7).

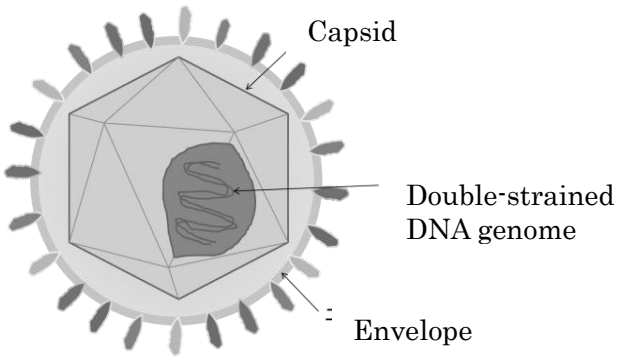


Fig. 1 HVT Particle Pattern Diagram

HVT genome organization is same as other Alphaherpesvirinae and similar to MDV1, MDV2 or herpes simple virus Serotype 1 (hereinafter called HSV1) (Fig.2). The genome constitutes 6 regions, the Unique Long (UL) and Unique Short (US) regions, and their respective repeated sequences are positioned outside. Terminal Repeat Long (TRL) and Internal Repeat Long (IRL) positioned on both sides of UL region have the same nucleotide sequence reversed. Repeated sequences on both sides of the US region have the same configuration (Literature 9).

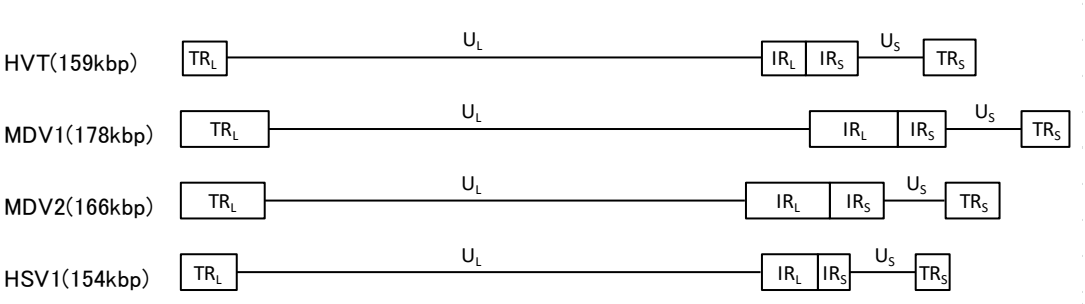


Fig. 2 HVT Genomic Organization

According to the information on the whole nucleotide sequences of HVT genome (the Accession Number assigned by DNA Data Bank of Japan (DDBJ): AF291866), the number of the Open Reading Frames (hereinafter called “ORF”) is 397, and 99 effective genes is coded. Of these, 76 genes are preserved in MDV1, too, and 71 genes in MDV2, 65 genes in HSV1 (Literature 9). The homology of amino acid found in proteins expressed from reserved genes is 36-82 % in case of MDV1 and 34-81 % in case of MDV2 (Literature 9). The whole nucleotide sequences of HVT genome is based on the analysis of FC126 strains, which are different from the host virus PB1 strains, but a partial base sequence of the host virus PB1 strains (the Accession Number assigned by DNA Data Bank of Japan (DDBJ): M84473) has a 98.9 % homology to FC126 strains. In

addition, both FC126 strains and the host virus PB1 strains are vaccine strains derived from healthy turkeys, subculture of wild nonpathogenic strains from the beginning of isolation.

b. Conditions for inhabitable or raising (proliferation) environment

An animal holding HVT in the natural world is a turkey. HVT is widely distributed among the flock of turkeys. There is an example of isolation from wild turkeys (Literature 8). HVT is used as an avian vaccine in Japan, too. Many chicken flocks are thought to have it, but it is not separated from chickens that do not use the vaccine (Literature 7). The host virus PB1 strains proliferated from chicken embryonic cell at 30 to 40 deg C and did not at 25 to 45 deg C (Attachment 4). PB1 strain did not proliferated in cells derived from human beings, green monkeys, dogs, cats or cattle (Attachment 5).

c. Predatory or parasitic

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d. Reproduction or proliferation form

As HVT is cell associated, living cell is essential for its viability and it can nether be reproduced nor viable in the extracellular environment. Still, there are exceptions. Because HVT is matured in the feather follicle, HVT-infected turkey's emulsion on the skin and the feather follicle are infectious (Literatures 6 and 7). Therefore, excretory substance from the skin is thought to be an infectious agent.

As for an infectious route, it is confirmed that HVT is airborne infection (dust infection) to chickens and turkeys (Literatures 4, 5, and 6). Virus DNA was detected from dusts from isolators of infected chickens (Literature 18). HVT, which is matured and excreted to outside of the body with dander, is thought to be an infectious agent. There are cases where airborne infection (dust infection) to turkeys is propagated from a farm, which is about 90 m away (Literature 7) and where the infection to chickens from a cage where inlets and exhaust ports are connected (Literature 6). If a turkey group is exposed to HVT, a whole group is infected in a few weeks. In rare case of chickens, however, only a part of individuals are rerely infected (Literatures 6, 7, and 8). General reviews (Literatures 7 and 8) describe HVT is transmitted by contact. This is based on an observation that the infection is caused by cohabitation. The data that proves whether the infection is caused by contacting skins and mucosa or whether HVT is orally infected through excreted virus-infected feed and water is not included, and the infection by contact is not fully verified. In addition, virus excretion from oral cavity or feces was not recognized (Attachments 6 and 7), droplet and droplet nuclei infections may be a cause. In addition, it was confirmed that there is no vertical transmission (Literatures 7 and 8). When HVT is inoculated into chickens, early individuals develop viremia 6 days later and most 3-4 weeks later. Cell-associated virus proliferates in lung, thymus, bursa of Fabricius, spleen, and other lymphatic tissues and spread to other internal organs by infecting leukocytes 1-4 days after inoculation.

Cell-free virus is detected from feather follicle a few weeks after inoculation, and rarely after this

period (Literature 7). The HVT-infected leukocyte is a cell other than a macrophage or B lymphocyte. It seems to be a T lymphocyte (Literatures 8 and 12). After infecting chickens, HVT proliferates and infects various organs, and after that, transfers to a latent infection. The latent infection means a condition where no infectious particle is produced. Confirmed latent internal organizations are spleen, thymus, bursa of Fabricius, wing plexus, sciatic nerve, epithelial cell, feather follicle, and others (Literature 13). The proliferation in the feather follicle shows a few-week transient course (Literature 7 and 8). The spread of HVT infection in chickens are limited to only a part of individuals because the infection lasts long but the excretion lasts a short time.

Fig. 3 shows a replicative cycle of herpesvirus. The herpesvirus attaches cell surfaces through glycoprotein on the envelope surface. In the attached virus, envelops are fused with cell membranes directly or endocytosis and capsid intrudes into cytoplasm. At this time, release tegument materials inside the envelope into cytoplasm. The capsid is transferred to nuclear pores through microtube and virus genome is emitted into nucleus.

The virus genome is reproduced and copied in the nucleus. The virus genome reproduced as polymer is cut into a single length and integrated into the capsid. The capsid is primarily enveloped and goes out into the space between the inner and outer membranes of the nuclear membrance when it is budded from the inner coat of the nuclear membrance. When particles move into cells, the primary envelop is lost. Particles mature in the cytoplasm. After obtaining tegument materials in cytoplasms, the capsid is secondarily enveloped when it is budded in a vesicle of the Golgi network. By this process, matured particles are positioned in vesicle inside a cell. By fusing vesicles with cell membrane, matured particles are emitted outside cell (Literatures 14 and 15).

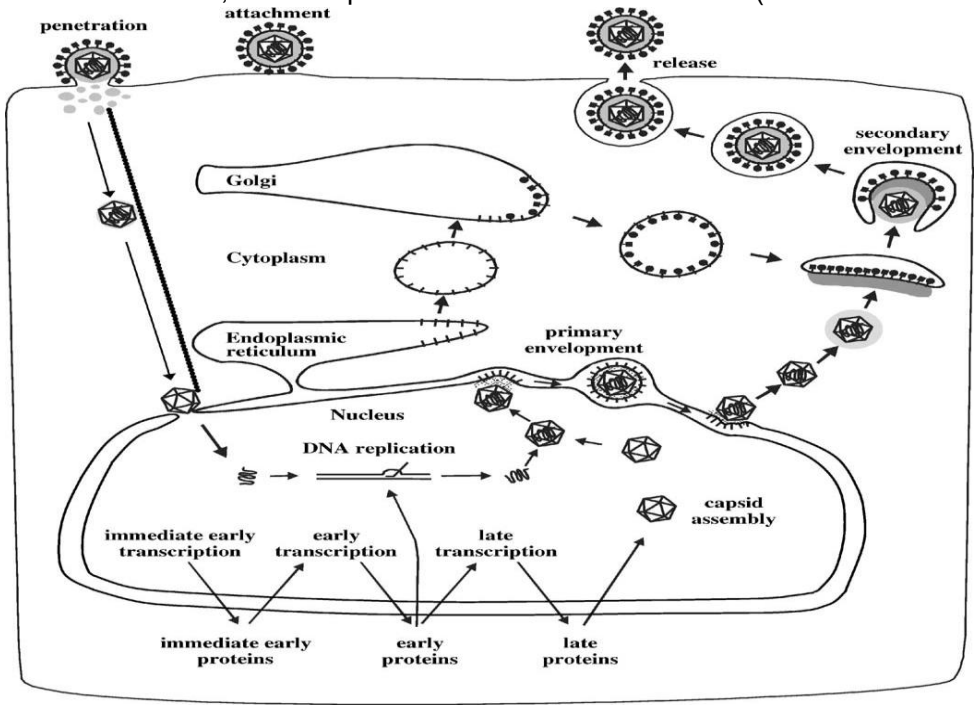


Fig. 3 Replicative Cycle of Herpesvirus
(Cited from 106:167-180 of "Budding events in herpesvirus morphogenesis" Mettenleiter T. C.,2004 Virus Research)

A hybrid between extremely closely-related species in Alphaherpesvirinae (herpes simplex virus types 1 and 2, equine herpes virus (EHV) types 1 and 4, and bovine herpesvirus types 1 and 5) is known, but no hybrid between low homology viruses is known (Literatures 16 and 17). As HVT, which has gene composition different from MDV1 or MDV2 and exhibits low homology, is considered not to recombine with these viruses.

As for homologous recombination (HR) between strains in the same virus species, it has recently become clear that the HR often occurs within many species of Alphaherpesvirinae and that this plays an important role in the virus evolution. This shows a possibility of homologous recombination in HVT if there is a chance of coinfection with different strains, but there is little chance of coinfection in the outdoor infection beause there is no example of HVT isolation from unvaccinated chickens (Literature 7) and there is no wild animal holding HTV in Japan. In addition, if homologous recombination should occur between strains, it is considered that there is no fear of problem such as acquiring new pathogenicity because HVT is nonpathogenic.

Generally speaking, herpesvirus genome in the period of latent infection is maintained as circular extrachromosomal genetic factor (episome).

It is known that in case of Epstein-Barr virus (HHV4), human herpesvirus 6 (HHV6), and MDV1 in herpesvirus, viral genome is incorporated into its chromosome of infected animals and the virus can be replicated from the infected animals' chromosome, while there is no known example that viral genome is incorporated into chromosome of the infected animals in case of other herpesvirus including HVT (Literature 19).

e. Pathogenicity

HVT is deprived from healthy turkeys and is not generally pathogenic. HVT vaccine strains subculture wild strains and are not attenuated. HVT does not show tumorigenic potency to turkeys, but when turkey cocks are infected, they may be troubled in fertilization (Literature 8).

When HVT is inoculated into chickens, they generally do not express symptom and give no immune system disorder. If, however, an excess amount is inoculated, atrophy of bursa Fabricii and thymus and mild nerve inflammation are found. HVT may be an initiating factor (IF) of autoimmune disease because HVT is related to a peripheral nerve disorder and autoimmune vitiligo in particular lines of chickens (Literature 8). However, chickens that are found to have peripheral nerve disorders are limited to ADOL experimental line (Literature 20) susceptible to Marek's disease virus and Smith line (Literature 21) chickens, which are experimental model animals for vitiligo. These lines of chickens are particular lines used for experiments, and those generally raised do not exhibit symptoms.

If HVT is inoculated to qualis, neither symptom nor macroscopically and histogenetically, tumor involvement was found (Literature 2).

If HVT was inoculated to ring-necked pheasants (close related to Japanese pheasants), viremia was found in some individuals, but no tumor involvement was macroscopically found (Attachment

1).

HVT does not exhibit pathogenicity to chickens, qualis, and ring-necked pheasants other than lines for particular experiments. If other birds of the Phasianidae family are infected, they do not exhibit pathogenicity, and it is thought that the infection does not influence on individual survival and reproduction.

f. Productivity of toxic substances

There is no report for HVT to produce toxic substances.

An amino acid sequence which is predicted from a whole nucleotide sequence of HVT (the Accession Number assigned by DNA Data Bank of Japan (DDBJ): AF291866) was retrieved in Allergen Database for Food Safety (ADFS) (http://allergen.nihs.go.jp/ADFS/index.jsp). UL36 Large tegument protein shows 25 % similarity of derived from cockroach allergen, Bla g 1.02(37 % or more is positive). No other protein suspected to be allergic was coded (Attachment 8). HVT is used for hens and meat-type chickens as Marek's disease vaccine for 40 years or more. As no report on a problem in raising and developing wild plants could be found, no toxic substance is thought not to be produced.

g. Other information

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2 Information regarding the preparation of living modified organism

(1) Information concerning donor-nucleic acid

a. Configuration and Origins of Components

① LTR promoter

cDNA of the long terminal repeat derived of the Schmidt-Ruppin avian sarcoma virus of subgroup D (SR-ADS-D) strains. The number of bases is about 600 bp (Attachment 9).

② F Protein Gene

cDNA of F protein gene derived from 30 strains of the Newcastle disease virus (hereinafter called “NDV”) clone (vaccine strains). The number of bases is about 1800 bp (Attachment 9).

b. Component functions

① LTR promoter

It has functions that promote the expression of F protein gene connected to a downstream. LTR of a Retrovirus has functions initiation of the reverse transcription of virus genome, integration of infected cells into chromosome, regulation of transcription of infected cells from genome (Literature 23).

② F Protein Gene

Fusion protein (F protein) of NDV has membrane fusion in the co-existence with hemagglutinin-neuraminidase protein (Literature 24).

The virus penetrate into cells by the fusion between viral envelope and cell membrane. In addition, the formation of syncytium starts by the fusion of cells. The membrane fusion is essential to establishment of infection, and antibodies against F and NH proteins are known for preventing viral infection (Literature 25).

(2) Information concerning vector

a. The name and origin

pGEM-3Z (Fig.1 of Attachment 10 and Literature 26)

Modified plasmid derived from Escherichia coli and improved to use for gene cloning. Commercially available as universal vector.

b. Characteristics

The number of nucleotide of pGEM-3Z is 2743bp and the nucleotide sequence is registered in the Accession Number:X65304.3 assigned by DNA Data Bank of Japan (DDBJ). The sequence to transfer plasmid to other strains is not included. A sequence for HVT homologous recombination was used as a backbone of the cloning vector pVEC04.

The vector is a cloning vector with ampicillin resistance genes, multicloning site, LacZ, and Sp6/T7 promotor. The Characteristics are: the clone retaining the insertion can be screened by blue/white selection; and inserted genes can be used for in vitro transcription.

(3) Method for the preparation of living modified organism

a. Overall structure of nucleic acid transformed into the host

The cloning vector pVEC04(Fig.2, p.2 of Attachment 10) was created by introducing the HVT homologous sequence (Figs 3 and 4, p.3-4, Attachment 10), LTR promoter (preparation by pRSVcat) and new cloning sites to pGEM-3Z cloning site (Fig. 5, p.5 of Attachment 10 and Literature 27).

F protein gene was cloned from cDNA synthesized from genome RNA of NDV harvested from embrionated eggs inoculated with NDV (Fig. 6, p.6, Attachment 10, Literature 27). F protein gene was finally cloned in the BglII site of pVEC04 and resulted plasmid was named pNDV04 (Fig.7, p.7, Attachment 10 and Literature 28). The nucleic acid to be transfect into a cell is prepared from plasmid pNDV04, digested by restriction enzyme, and used in the straight-chain form (Attachment 10 Fig.8, p.8). The nucleic acid to be transfected into a cell includes HVT homologous

region (839bp), LTR (about 600 bp), F protein gene (about 1800 bp including a noncoding region), and HVT homologous region (164bp) in order from the upstream side (Total 3472bp).

b. A method to transform nucleic acid into the host

Total DNA extracted from primary chick embryo cell infected with HVT and pNDV04 in the straight-chain form are subjected to a cotransfection to the primary chick embryo cell (Literature 27).

The gene inserted to the cloning site downstream of LTR promoter was introduced into HVT genomes by using HVT homologous sequence to be placed outside.

c. The development progress of living modified organism

The virus expressing F protein was selected in the indirect fluorescent antibody method. NDV hyperimmunized chicken serum was used for a primary antibody and Fluorescein Isothiocyanate (hereinafter called “FITC”) labelled anti-chicken IgG rabbit serotype for a secondary antibody. Infected chick embryo primary cells with the virus collected from a plaque randomly selected and confirmed the existence or absence of fluorescent. The presence or absence of fluorescence was confirmed by limiting dilution of the virus where fluorescence was found, and the dilution was repeated until the fluorescence was found in 95 % or more homogeneous mixture (Literature 27). The isolate #04-44-13, which possess F protein gene, further repeated subcultures in a primary chick embryo cell and created the manufacturing master seed. For the manufacturing master seed, HVT-F 011299 is displayed and the U.S. manufacturing site (Intervet Inc. 29160 Intervet Lane, Millsboro, DE 19966-4217) managed it. The name of the recombinant virus was HVT-NDV/F strain.

(4) The state of existence of nucleic acid transferred into cells (host body) and the stability of phenotypic expression by the nucleic acid.

a. The state of existence of transferred nucleic acid in gene-recombinant microorganisms.

The donor-nucleic acid is incorporated into the host's genome and is located in the BglII site (Figs. 10 and 11, p. 10-11, Attachment 10) in US10 homologous gene in US region (Fig.9, p. 9, Attachment 10) (Fig.4).


Fig. 4 Insertion Site of Donor-Nucleic Acid in HVT-NDV/F Strain

LTR: LTR promoter derived from Rous sarcoma virus (RSV)

NDV/F：Newcastle disease virus fusion (F) protein gene

b. The stability of expression of the objective gene in the host

After subculturing HVT-NDV/F strain in cultured cell, restriction enzyme cleavage pattern near the insertion site of donor-nucleic acid (Southern blot) and nuleotide sequences were confirmed. For the master seed virus (hereinafter called “master seed”) and the subculture for 11 passage, neither loss nor reconfiguration is found in the donor-nucleic acid (Attachment 11) and no difference was recognized in the base sequence (Attachment 12). In addition, the Southern blotting technique found after subculture of HVT-NDV/F strain by chickens, no difference was recognized between HVT-NDV/F strain subcultured for 2 generations from the master seed and HVT-NDV/F strain subcultured from 8 generations in chickens (Attachment 13).

The expression of F protein gene was checked in the fluorescent antibody method and the immunoprecipitation method using the monoclonal antibody against F protein. In the former, F protein was expressed in any of the master seed, virus subcultured for 11 passage in cultured cell, and virus subcultured from 5 passage in chickens (Attachment 11), and in the latter, like F protein in NDV, sedimentation of about 60 kDa of protein was found in the master seed and virus subcultured for 5 passage in cultured cell (Attachment 14).

Study have made whether pathogenicity was found in HVT-NDV/F strain back passaged in chickens. One-day chicken (5 passage) inoculated with HVT-NDV/F strain subcultured for 4 passage in chicken did not show Marek's disease symptoms during 8-week observation period, and at a necropsy 8 weeks after inoculation, no gross pathology of Marek's disease was found (Attachment 11). The ability of HVT-NDV/F strain to provide the immunity to Marek's disease and Newcastle disease was confirmed by chicken challenge studies, and it was found out that even vaccines manufactured by subculture for 11 passage in cultured cell from the master seed could provide effective protection (Attachment 15).

Therefore, the genetic stability of HVT-NDV/F strain is considered to be high.

(5) Detection of living modified organisms (LMOs), identifying methods, their sensitivity and reliability

HVT-NDV/F strain can be detected in the indirect fluorescent antibody method by using anti-NDV-F protein monoclonal antibodies. The fluorescent staining is carried out on 8-well chamber slide. Sensitivity was studied by using final product of vaccine as isolated HVT, specific fluorescence could be detected up to 400-time dilution. At that time, the infection value per inoculation dose 0.1 mL was 12 PFU. In this method, a cultured chick embryo cells are specifically dyed when HVT-NDV/F strain is inoculated to a chicken. On the other hand, the cells are not dyed when HVT is inoculated to a chicken or in case of non-inoculated chicken (Attachment 16).

(6) Difference from a host or its taxonomic species.

a. Difference in characteristics between gene-recombinant microorganisms and hosts used for their preparation or organism species they belong to

[1] Proliferation style (the existence or absence of viremia and new intact virus)

HVT-NDV/F strain in chick embryo primary cells are proliferated in the range of 30-40 deg C, not at 25 or 45 deg C like the host virus (Attachment 4).

HVT-NDV/F strain in the bodies of inoculated chickens were proliferated in many organs including liver, spleen, kidney, lung, thymus, and bursa Fabricii by 1 week after inoculation and the virus was detected from feather follicles in a few weeks like host virus. On the other hand, no virus was detected from the oral cavity and cloaca of an infected chicken (Attachments 6 and 7).

② Genetic characterization

As for HVT-NDV/F strain, F protein expression cassette was inserted into the Us region of the host virus (Fig.4 and Fig. 10, p.10, Attachment 10). Genes ORF2 (US10 homolog) of uncertain function were at the insertion site but were destroyed in the middle due to insertion (Attachment 10 Fig.11, p. 11). OPF2 functions are unknown and the destruction of ORF2 does not change phenotypes,

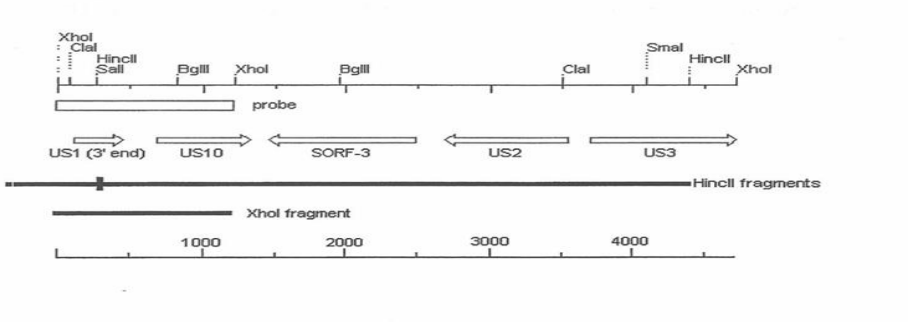


Fig 5 Restriction Enzyme Map around the Donor-Nucleic Acid Insertion Site
Arrows show ORFs and their directions. The lower bar shows bp.
The figure shows parent (PB1 strain) before insertion and the donor-nucleic acid was inserted into *Bgl*II site of US10 homolog.

③ Pathogenicity

The host virus showed chickens neither tumorigenic potency nor pathogenicity (Literature 29). Like the host virus, HVT-NDV/F strain showed chickens neither tumorigenic potency nor pathogenicity (Literature 17).

HVT-NDV/F strain includes sequences derived from retrovirus. Retrovirus incorporates virus genomes into chromosomes of infected cells in the process of proliferation (provirus), and this process is essential for malignant transformation of infected cells. Two LTR sequences and virus-derived integrase at both ends of retrovirus genomes are necessary for provirus (Literature 24).

Only one LTR sequence is introduced into HVT-NDV/F strain and two sequences are not placed at both ends. It seems that HVT-NDV/F strain genes are not integrated into chromosomes or malignant transformation of inoculated chickens.

④ Productivity of toxic substances

HVT-NDV/F strain have expression of F protein of NDV. NDV is a pathogen of zoonotic infection and produces conjunctivitis symptoms to human beings, but it has not been reported that F protein is toxic.

As a result of retrieval of ORF in the donor-nucleic acid used for the recombination, ORF (1662bp), which codes the target protein, was retrieved as the longest ORF, and in addition to that, 18 pieces of short ORF (117-543 bp) were retrieved (Fig.12, p.12, Attachment 10). Translated peptides for 6 frames of donor-nucleic acid including short ORF were retrieved in the Allergen Database for Food Safety (ADFS), and F protein showed 27 % homology to allergen substances derived from turban shell (in the FAO/WHO method, 37 % or more is positive). In addition, no other allergic suspected substance was retrieved (Attachment 18). The possibility that protein is actually translated from short ORFs cannot be denied. It has been confirmed in pathogenicity confirmation tests (Attachment 11) that subculture for 5 passage in chickens did not restore pathogenicity. It seems that neither toxic substances were produced nor unintended nature was not expressed. Besides, vaccinates for chickens including HVT-NDV/F have been sold overseas since 2007 and 3.45 billion doses or more have been used. There has been no report that inoculated chickens, the surrounding natural environment, or people who ate the chicken meat have experienced any problem and the safety has been confirmed.

⑤ Infectivity

(Tissue affinity and persistent infectivity)

In infected chickens, HVT-NDV/F strain was detected from liver, spleen, kidney, lung, thymus, bursa Fabricii, and feather follicle, but not from oral cavity and cloaca like host virus PB1 strain (Attachments 15 and 16). The host virus PB1 strain havs persistent infection for a long time and the antibody is retained through life (Literature 8). The persistent infection of HVT-NDV/F strain was confirmed by collecting virus from peripheral blood lymphocytes (PBL) by 2 years after inoculation (Attachment 19).

Birds that can be infected with HVT-NDV/F strain are like those with host virus: in addition to turkeys, chickens, qualis, and birds of the Phasianidae family are and pigeons and ducks are not (Attachment 2). HVT-NDV/F strain in cells derived from human beings, green monkeys, dogs, cats, rabbits, and cattle did not proliferate like host virus PB1 strain (Attachment 5).

⑥ The presence/absence of likelihood of endogenous virus activation and the provision of pathogenicity

There is no report that HTV activates endogenous virus. In addition, there is no similar report for RSV and NDV. Therefore, it is thought that like host virus PB1 strain, there is no possibility for HVT-NDV/F strain to activate endogenous virus and provide pathogenicity.

To manufacture vaccines using HVT-NDV/F strain, chick embryo cells are used. The endogenous avian leukosis virus (ALV) and the endogenous avian virus (EAV) are known as particle producing and endogenous retrovirus derived from chick embryo cells (Literature 32), and it is thought that these viruses are contaminated into live vaccines for chickens manufactured by using chick embryo cells.

There is a report that in the past, exogenous ALVs were contaminated into live vaccine for chickens manufactured from chick embryo cells and that the pathogenicity to chickens was shown (Literature 33). In addition, there is a report that the reticuloendotheliosis virus (REV) was cantaminated into live vaccines for chickens manufactured from duck embryo cells (Literature 34).

Howerver, little is known about an example of pathogenicity to chickens caused by the endogenous virus of live vaccine for chickens manufactured by chicken embryo cells. Normally, endogenous retrovirus does not exhibit pathogenicity to hosts. The vaccine using HVT-NDV/F strain applies to chickens. As the animal is the same species as cells for manufacturing, it is thought that the endogenous avian leukosis virus (ALV) and the endogenous avian virus (EAV) do

not exhibit pathogenicity to the applied target animals.

⑦ Excretion and cohabitation infectivity from inoculated animals

Like host virus PB1 strain, HVT-NDV/F strain exhibit the cohabitation infectivity (Attachment 3). In a few weeks after inoculation, infectivity was found in feather follicles, but not in oral cavity and cloaca (Attachments 16 and 17). Like host virus PB1 strain, virus DNA was detected from dander of a host infected with HVT-NDV/F strain (Attachment 20). By the 2nd -5th week after inoculation, the excretion of virus from dander of inoculated chickens was found (Attachments 21 and 22). Inoculated chickens, however, did not excrete the virus from dander again even if stress is added by feed and water restriction after the excretion period (Attachment 20). Like host virus PB1 strain, HVT-NDV/F strain were not detected from eggs (Attachments 23 and 24). HVT-NDV/F strain was not collected from fece excreted by the infected chicken (Attachment 25).

⑧ Survival capability in the natural world

HVT-NDV/F strain is a virus associated with cell and cannot survive 1 week or more in rain water, mud water, clotted blood, and serum (Attachment 26). If HVT-NDV/F strain was prepared and stands at the room temperature, virus was detected by 48 hours later but not 1 week later (Attachment 27). In cell-free HVT-NDV/F strain excreted in dander, the infectivity titter was found to be lower 1 week later. At 4 deg C, the infection value is below detection limits 4 weeks later at room temperature (Attachment 28).

⑨ Crossbreeding

As for the possibility of the crossbreeding of HVT with MDV1 and MDV2, because it is not known about crossbreeding between low homologous viruses (Literature 16), it is thought that no crossbreeding will occcur between serotypes.

As for the possibility of homologous recombination among HVT strains, because there are little opportunities for co-infection through outdoor infection, there is little likelihood for the homologous recombination (HR) among strains. HVT-NDV/F strain does not change characteristics from the host virus' and there is no likelihood for crossbreeding among serotypes or strains.

⑩ Inactivate

HVT-NDV/F strain was inactivated by alcohol, chlorine, cationic detergent, and lime hydrate. (Attachment 29).

In addition, HVT-NDV/F strain is inactivated by artificial gastric juice (Attachment 30).

b. Chatacteristics such as colony formation and color formation, which allow for the identification from the host such as a gene-recombinant microorganism

Unlike a normal HVT, the plaque of HVT-NDV/F strain is specifically dyed by the fluorescent antibody method using Monoclonal antibody that recognizes F protein (Attachment 14).

3 Information regarding the Preparation of Living Modified Organism

Newcastle disease exhibits strong infectivity with respiratory symptoms, diarrhea, and nervous symptoms and is an economically important disease. NDV pathogenicity greatly depends on strains, which are categorized as velogenic (highly virulent), mesogenic (intermediate virulence), or lentogenic (nonvirulent) types. In spite of ages in weeks and managed state, most clinical symptoms depend on pathogenicity of prevailing pathogenicity. Symptoms vary from 100 % death rate to only mild respiratory symptom. Most recognizable economic losses in egg-laying hens are lowering in an egg-laying rate and quality of egg shell and egg albumen. The reduction in the fertility rate and hatching rate have been reported.

Marek's disease is a quickly spreading neoplastic disease. Its clinical symptoms are various. In many cases, affected birds lose weights and exhibit paralytic symptoms in wings and legs, and tumor formation. The mortality rate of unvaccinated chickens is about 5-50 %, and usually, 10-week to 20-week old chickens develop the disease. MDV1-infected chickens carry virus and keep on excreting for life. Virus is infected from oral cavity or respiratory tract. The infectivity of dander from feather follicles of chicken infected with Marek's disease lasts for a few months.

Live or inactivate vaccine is used to prevent Newcastle disease. Live vaccine has an advantage of administration through drinking water and water spray while inactivate vaccine must be injected one by one.

In rare cases, however, a respiratory symptom may occur. Usually, the Newcastle disease vaccine must be given several times. The oil adjuvant type of inactivate vaccine maintains immunity for long, but due to the residual adjuvant component, shipping is prohibited for a certain period of time after injection.

To prevent Marek's disease, HVT, nonvirulent MDV1 and MDV2 are used as vaccines. HVT is widely used for long because its validity is high and easily used with another product. Cell free viruses and viruses associated with cell are in the market, but MDV1 and 2 vaccines are limited to products of virus associated with cell. Vaccine should be inoculated to 18-day old embryonated egg or subcutaneously and intramuscularly at day old chicken. Generally, these vaccines provide lifelong protection.

Conventional vaccines required several time inoculations for Newcastle disease (Fig. 5). The characteristic of HVT-NDV/F strain is, like Marek's disease, only one vaccination can give immunity to Newcastle disease. However in a region where Newcastle disease is prevalent, it is recomend to combine with ND live vaccine once before onset immunity. Other advantages are it has no risk of respiratory symptom and there is no shipping restrictions due to residue. In addition, the effect of HVT used as a vector can prevent Marek's disease, too. At present, Newcastle and Marek's diseases vaccinates are inoculated to almost all chickens at large chicken farms. The use of Newcastle disease vaccine with HVT as vector virus seems to contribute to reduce the labor and cost by reducing the number of vaccine doses and improve the productivity by reducing the stress of chickens. The information on the product was shown in Attachment 31.

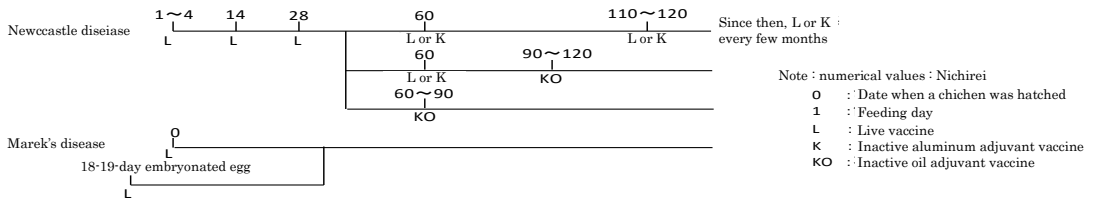


Fig. 5 Newcastle and Marek's Diseases Vaccine Program Recommended by the Japanese Society of Poultry Diseases (JSPD)

(1) Content of use

[1] Transfer and storage (including the transport and storage of inoculated animals carrying viable live genetic recombination vaccine.)

[2] The Law on Securing Quality, Efficacy and Safety of Products Including Pharmaceuticals and Medical Devices (Act No. 145 of August 10, 1960. Hereinafter called the “Law on Pharmaceuticals and Medical Devices.”) A use in accordance with a protocol prepared based on a notification of clinical trial plan to be submitted based on paragraph 2 of Article 80-2 of the law and Article 7 of the Ordinance on the Performance Criteria of Clinical Tests of Pharmaceuticals for Animals (MAFF Ordinances No. 75 of 1997) if a test (hereinafter called ”clinical trials”) is performed in order to collect data related to the results of a clinical trial for inclusion among data submitted pursuant to the provisions of paragraph 3 of Article 14

[3] Use according to a written application for approval based on paragraph 1 of Article 14 of the Law on Pharmaceuticals and Medical Devices (excluding acts that fall under [4]).

[4] Inoculation (to chickens)

[5] Disposal of post-inoculation instrument and use residue in accordance with the infectious industrial waste disposal standard under Article 12-2 of Disposal of Waste Management and Public Cleansing Act (Act No. 137 of 1970)

[6] Disposal other than [5] (including the disposal of animals vaccinated with viable live gene recommbination vaccine.)

[7] Incidental actions to [1]-[6]

(2) Method of use

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(3) Information gathering method after the start of Serotype 1 use by a person who applies for approval

—

(4) Measures to prevent any adverse effect on biological diversity if such effect may be achieved.

Refer to p.28 Emergency Measure Plan.

(5) Results of uses in loboratory or the environment analogous to the type 1 use are expected.

In rearing in an isolator, like the host virus PB1 strain, HVT-NDV/F strain were horizontally transmitted to cohabiting chickens at the low rate (Attachment 3).

However, in case of rearing at a laboratory closer to field conditions, HVT-NDV/F strain was not horizontally transmitted to cohabiting chickens (Attachments 17 and 32). Similarly, they were not horizontally transmitted to pigeons or ducks. However, turkeys are horizontally transmitted (Attachment 32). HVT-NDV/F strain is already used in the field of abroad, but no effect on the surrounding environment has been reported.

(6) Information concerning uses outside Japan

The plain vaccine “Innovax-ND” including HVT-NDV/F strain is authorized in the U.S., Canada,

Columbia, Costa Rica, Jordan, and Thailand (Table 1).

Bivalent combined vaccine “Innovax-ND-SB” including HVT-NDV/F strain and MDV2 (SB-1 strain) is authorized in the U.S., Canada, Columbia, Azerbaijan, Mexico, Panama, Peru, Thailand, and India (Table 2). Since 2007, 3.45 billion or more doses of the vaccine including HVT-NDV/F strain has been used.

The risk assessment attached when Innovax-ND and Innovax-ND-SB were authorized in the U.S. is shown in Attachment 33. The environmental evaluation document released by the Canadian Food Inspection Agency (CFIA) after the authorization on Innovax-ND-SB in Canada is shown in Attachment 34.

Table 1 Innovax-ND Sales Results by Country (Unit: thousand doses)

| Sales year | the U.S. | Canada | Trinidad and Tobago | Jordan | UAE | Oman | Lebanon | Iraq | Thailand | Total |
|---|---|---|---|---|---|---|---|---|---|---|
| 2010 | 7,640 | | | | | | | | | 7,640 |
| 2011 | 290,214 | 2,880 | 8,300 | 13,400 | | | | | | 314,794 |
| 2012 | 310,196 | 5,840 | 0 | 52,400 | 9,600 | 2,450 | 4,900 | 1,200 | 22,454 | 409,040 |

Table 2 Innovax-ND Sales Results by Country (Unit: thousand doses)

| Sales year | the U.S. | Canada | Trinidad and Tobago | Mexico | Colombia | Bolivia | Peru | *Venezuela* | Thailand | Azerbaijan | Total |
|---|---|---|---|---|---|---|---|---|---|---|---|
| 2007 | 32,096 | | 100 | | | | | | | | 32,196 |
| 2008 | 162,132 | | 5,775 | | | | | | | | 167,907 |
| 2009 | 307,317 | 750 | 40,660 | 6,085 | | | | | | | 354,812 |
| 2010 | 432,844 | 4,437 | 40,947 | 124,978 | 11,409 | 1,200 | | | | 15,000 | 630,815 |
| 2011 | 415,038 | 753 | 31,796 | 184,763 | 72,207 | 0 | 7,787 | | 38,005 | 4,900 | 755,249 |
| 2012 | 514,525 | 0 | 45,500 | 110,838 | 98,744 | 0 | 11,724 | *10,420* | 105,200 | 4,900 | 901,851 |

(7) Information on Kinetics inside the Body of Inoculated Animal

a. Information concerning genetic live gene recombinant vaccine fate inside the body of vaccinated animals

In case of the intramuscular or subcutaneous inoculation into a chicken, by 21 days later, HVT-NDV/F strain is collected from liver, spleen, gonad, lung, thymus, bursa Fabricii, peripheral blood lymphocyte (PBL), and feather follicle, but neither from cloaca nor mouthwash (Attachments 15 and 16). In addition, no infectious virus was detected from feces, either (Attachment 25).

In case of inoculation of the inside of the egg, the infectious virus from feather follicles was

collected by 1-5 week old, in case of inoculation at 1-day old, the virus was collected by 2-5 week old, but was not in 6th and 9th week (Attachments 15, 16, 20, 21, and 22). As for persistent infection, virus was reisolated from the peripheral blood lymphocytes (PBL) by the 104th week after inoculation(the second year) and the persistency were confirmed (Attachment 19).

b. Information concerning the existence or absence of spredding of genetic modefied vaccine from inoculated animal bodies and their excreta, blood, body fluid, egg, etc.,

In a cohabitation rearing among chickens inoculated with HVT-NDV/F strain, non-inoculated chickens, and turkeys, horizontal transmission was found. It seems that chickens inoculated with HVT-NDV/F strain excrete the strain. In breeding chickens in an isolator, a part of individuals were found to be horizontally infected (Attachment 3), but in breeding at a laboratory, the horizontal infection was not found (Attachments 17 and 32). The virus was separated from leukocyte, liver, spleen, kidney, gonad, lung, thymus, bursa Fabricii, feather follicle, and dander of inoculated animals, but not from oral cavity, cloaca, fece or egg (egg yolk and white) (Attachments 15, 16, 19. 21, 22, and 25).

If blood, muscle, liver, cardiac, and muscular stomach of chickens inoculated with HVT-NDV/F strain were preserved at 4 deg C, the virus was detected 24 hours later in the liver, but not 48 hours later. In other sites, the virus was not detected 24 hours later (Attachment 35). In addition, if the sites were preserved at -20 deg C, the virus was not detected in all inspected sites 24 hours later (Attachment 35). In case of exposure to mud water, rain water, serum, and clotted blood, the virus was detected until 4 days later, but not 1 week later (Attachment 26).

A part of infectivity of the virus excreted in dander remained 4 weeks later at 4 deg C, but no infectivity was found 4 weeks later at room temperature (Attachment 28).

Based on these factors, the virus associated with cells in the vaccine and chicken bodies could not survive in the environment for 1 week or longer. It is difficult for the cell free virus excreted in dander to survive in the environment for 4 weeks or longer.

c. Information concerning the presence/absence of likelihood of vertical transmission of the genetic recombination vaccine in inoculated animals

As a result of inspection of the inside of eggs or eggs of breeding hens subcutaneously inoculated with HVT-NDV/F strain, the virus was detected. !!!

(Attachments 23 and 24). HVT-NDV/F strain was not excreted into the egg, and it seemed there was no possibility of vertical transmission.

d. Information concerning the presence/absence of likelihood of propagation to wild animalsand plants

HVT-NDV/F strain did not proliferate in cells derived from mammals such as human beings, green monkeys, dogs, cats, rabbits, and cattle.

(Attachment 5). Chickens, pigeons, and ducks inoculated with HVT-NDV/F strain were not infected even if they were kept cohabitationally, but turkeys were

(Attachment 32). In addition, pigeons and ducks that were directly inoculated with HVT-NDV/F strain were not infected (Attachment 2). HVT-NDV/F strain did not horizontally transmit among chickens (Attachments 17 and 32) or only a part of individuals were infected (Attachment 3). The

characteristics shown above were not changed from the host virus PB1 strain (Attachments 5, 32, 2, and 3). It seems that the possibility of the transmission of HVT-NVD/F strain to wild animals and plants is similar to that of the host virus PB1 strain.

In addition, as qualis and ring-necked pheasants show susceptibility to HVT, other birds of the Phasianidae family may have susceptibility to HVT. Furthermore, it seems that pigeons, crows, sparrows, and other wild birds, which may enter farms, will not be infected with HVT because they do not belong to the Phasianidae family. Japanese minks, raccoon dogs, martens, and other wild animals, which prey chickens, are mammals and will not be infected with HVT. It seems that human beings will not be infected with HVT as a result of antibody tests including miss-injection cases.

Historically, HVT has been safely used as live vaccine for chickens in Japan for about 40 years. The propagation to wild animals and plants including birds of the Phasianidae family has not been reported (Attachments 36 and 37). It seems that the possibility of propagating HVT-NDV/F strain to birds of the Phasianidae family is similar to the host virus PB1 stains.

e. Other necessary information

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II. Overview of adverse effect on biological diversity risk assessment for each item

1 Characteristics to decrease other microorganisms

(1) Identification of wild animals and plants that may be influenced

The characteristics of decreasing microorganisms in HVT itself, a taxonomic species of the host, have not been reported. The turkey is a primary animal holding HVT. It seems that other birds of the Phasianidae family have low sensibility, and it is inferred that even if a part of individuals are infected, it is not likely that the infection spreads and is kept. In addition, as there are little opportunities for coinfection in the outdoor infection and HVT is non-pathogenicity, it is considered that there is no fear of problem such as acquiring new pathogenicity. The recombinant microorganism expresses F protein of Newcastle disease, but the toxicity of F protein has not been reported. It seems that HVT-NDV/F strain has not changed the characteristics of decreasing other microorganisms from the host virus PB1 strain.

Therefore, no wild animals and plants, which may be influenced resulting from characteristics of decreasing other microorganisms, were identified.

(2) Evaluation of specific details of influence

—

(3) Evaluation of likeliness of influence

—

(4) A presence or non-presence of any adverse effect on biological diversity

Therefore, it is judged that as far as the use in accordance with the Type 1 Use Regulations is followed, there is no concern of any adverse effect on biological diversity resulting from the characteristics to decrease other microorganisms.

2. Pathogenicity

(1) Identification of wild animals and plants that may be influenced

It is considered that the range of animals, or a taxonomic species of the host, that may be infected with HVT are copper pheasants and Rock ptermigan which belong to the same Phasianidae family as turkeys as well as chickens, quails, and ring-necked pheasants. It seems that pigeons, crows, sparrows, and other wild birds, which may enter farms, will not be infected with HVT because they are not close to turkeys in taxonomy. Japanese minks, raccoon dogs,

martens, and other wild animals, which prey chickens, are mammals and will not be infected with HVT, considering the results of proliferation tests using cells derived from mammals. It seems that human beings will not be infected with HVT as a result of antibody tests including erroneous injection cases.

In poultry and quali farming in Japan, HVT live vaccine is used, and there is no report that shows HVT isolation from non-turkey wild birds. In addition, no HVT has been separated from unvaccinated chickens. HVT does not exhibit pathogenicity to chickens, qualis, and ring-necked pheasants other than lines for particular experiments. If other birds of the Phasianidae family are infected, they do not exhibit pathogenicity, and it is thought that the infection does not influence on individual survical and reproduction.

To the best of our investigations on pathogenicity and susceptibility, HVT-NDV/F strain has not been changed from the host virus PB1 strain.

As shown above, some wildlife species were assumed to be infected with HVT-NDV/F strain, but no wild animals and plants, which may be affected by pathogenicity, were identified. As far as the use in accordance with the Type 1 Use Regulations is followd, it was judged that there would be no concern of any adverse effect on biological diversity resulting from pathogenicity.

HVT-NDV/F strain includes sequences derived from retrovirus, which is known as tumor virus. Retrovirus undergoes the process of provirus in the replication process, and this is essential for transformation of cells. Provirus requires 2 LTR sequences on both ends of retroviral genome and the existence of integrase derived from virus, but only one LTR sequence is introduced into HVT-NDV/F strain and two sequences are not placed at both ends. It seems that HVT-NDV/F strain genes are not integrated into chromosomes of inoculated chickens and that the chickens turn cancerous.

(2) Evaluation of specific details of influence

—

(3) Evaluation of likeliness of influence

—

(4) A presence or non-presence of any adverse effect on biological diversity

Therefore, as far as the use in accordance with the Type 1 Use Regulations is followed, it is judged that there is no concern of any adverse effect on biological diversity resulting from pathogenicity.

3 Productivity of toxic substances

(1) Identification of wild animals and plants that may be influenced

There is no report for HVT, a taxonomic species of the host, to produce toxic substances. HVT-NDV/F strain expressed F protein of NDV. NDV is a pathogen of zoonotic infection and produces conjunctivitis symptoms to human beings, but it has not been reported that F protein is toxic.

As a result of retrieval of ORF of donor-nucleic acid used for recombination, F protein showed 27 % homology to allergen substance tropomyosin derived from turban shell (in the FAO/WHO method, 37 % or more is positive). In addition, no other allergic suspected substance was retrieved. In addition, it has been confirmed in reversion virulence tests that subculture for 5 passage in chickens did not reverse pathogenicity. It seems that no toxic substances are produced or unintended nature is not expressed.

Therefore, no wild animals and plants, which may be influenced resulting from the productivity of toxic substances, were identified.

(2) Evaluation of specific details of influence

—

(3) Evaluation of likeliness of influence

—

(4) A presence or non-presence of any adverse effect on biological diversity

Therefore, as far as the use in accordance with the Type 1 Use Regulations is followed, it is judged that there is no concernr of any adverse effect on biological diversity resulting from the productivity of toxic substances.

4 Property of Horizontal Transmission of Nucleic Acid

(1) Identification of wild animals and plants that may be influenced

HVT has no characteristics of horizontally transmitting nucleic acid in the usual proliferation. A part of Herpesviridae integrate there genome to chromosome of infected animals. HHV4, HHV6, and MDV1 are known for such characteristics. The other viruses of Herpesviridae are not knowen for such characteristics.

As for the possibility of homologous recombination with a related virus, the recombination between serotypes has not been reported though there are many chances of co-infection with MDV1 or MDV2 through the use of multivalent vaccines including HVT in breeding chickens and the field infection. As for the likelihood of homologous recombination with a same virus species, as there are little opportunities for coinfection in the outdoor infection, there is extremely low likelihood for recombination among strain. In addition, if a recombination should occur between strains, it seems that there is no concern of acquiring new pathogenicity because HVT is nonpathogenic. HVT-NDV/F strain only inserts LTR promoter of RSV and F protein genes of NDV to the host virus PB1 strain, and it seems that the characteristic to horizontally transmit nucleic acid is not changed from the host virus PB1 strain.

Therefore, no wild animals and plants, which may be influenced resulting from properties horizontally transmitting nucleic acid, were identified.

(2) Evaluation of specific details of influence

—

(3) Evaluation of likeliness of influence

—

(4) A presence or non-presence of the possibility of adverse effect on biological diversity

Therefore, as far as the use in accordance with the Type 1 Use Regulations is followed, it is judged that there is no concern of any adverse effect on biological diversity resulting from the characteristics that horizontally transmit the nucleic acid.

III Comprehensive Evaluation of the Adverse Effect on Biological Diversity

As for the characteristics of decreasing other microorganisms, HVT, a taxonomic species of the host, has no characteristics to decrease other microorganisms. The recombinant microorganism expresses F protein of Newcastle disease, but the toxicity of F protein has not been reported. It seems that the characteristics to decrease other microorganisms have not been changed from the host virus PB1 stains. Therefore, as far as the use in accordance with the Type 1 Use Regulations is followed, it is judged that there is no concern of any adverse effect on biological diversity resulting from the characteristics to decrease other microorganisms.

As for pathogenicity, HVT is deprived from healthy turkeys and is not generally pathogenic. HVT vaccine strains subculture wild strains and are not atenuated. HVT does not exhibit pathogenicity to chickens, qualis, and ring-necked pheasants other than lines for particular experiments. Even if other birds of the Phasianidae family are infected, they are not supposed to exhibit pathogenicity, and it is thought that the infection does not influence on individual existence and breeding.

Therefore, as far as the use in accordance with the Type 1 Use Regulations is followed, it is judged that there is no concern of any adverse effect on biological diversity resulting from pathogenicity.

As for the productivity of toxic substances, as a result of retrieval of ORF of donor-nucleic acid used for recombination, no allergic suspected substance was retrieved. In addition, it has been confirmed in pathogenicity confirmation tests that subculture for 5 passage in chickens did not reverse pathogenicity. It seems that no toxic substances are produced or unintended nature is not expressed. For this reason, as far as the use in accordance with the Type 1 Use Regulations is followed, it is judged that there is no concern of any adverse effect on biological diversity resulting from the productivity of toxic substances.

As for the characteristics to horizontally transmit nucleic acids, HVT is not known to have characteristics to integrate chromosomes of infected animals. As for the possibility of homologous recombination with a related virus, the recombination between serotypes has not been reported though there are many chances of co-infection. In addition, as for the likelihood of homologous recombination with same virus species, as there are little opportunities for coinfection in the outdoor infection, there is little likelihood for recombination among strain. For this reason, as far as the use in accordance with the Type 1 Use Regulations is concerned, it is judged that there is no concern of any adverse effect on biological diversity resulting from the characteristics that horizontally transmit the nucleic acid.

From the comprehensive evaluation of the above, as far as the use in accordance with the Type 1 Use Regulations is followed, it is judged that there is no concern of any adverse effect on biological diversity.

Abbreviations and technical terms used in this application document.

| Abbreviations and technical terms | Formal names/English names | Explanations |
|---|---|---|
| HVT | Herpesvirus of turkey | Herpesvirus of turkey (HVT) Derived from a healthy turkey that does not have any pathogenicity. |
| MDV1 | Marek's disease virus serotype 1 | Marek's disease virus serotype 1 disease Pathogen of Marek's disease Pathogenic depends on strains and some strains have no pathogenic. |
| MDV2 | Marek's disease virus serotype 2 | Marek's disease virus serotype 2 disease Derived from a healthy chicken that does not cause disease |
| MDV3 | Marek's disease virus serotype 3 | Marek's disease virus serotype 3 disease HVT (Herpesvirus of Turkey) Synonym |
| Genome | Genome | Cell or virus gene information |
| Capsid | Capsid | A protein shell enclosing viral nucleic acid and protein attached to the acid. |
| Capsomere | Capsomere | A subunit of the capsid |
| Envelope | Envelope | A lipoprotein envelope of the virus buds and matures through cell membrane. It may mean an envelope of large virus. |
| HSV1 | Herpes simplex virus 1 | Herpes simplex virus 1 Alphaherpesvirinae Type species of *Simplexvirus*. It may give rise to herpes labialis |
| ORF | Open Reading Frame | Open Reading Frame. Reading frame of genetic code that includes continuous stretch of amino acid codons without being interrupted by any stop codon. A region where protein genetic information may be coded. |
| Tegument | Tegument | A structure between envelope and capsid of Herpesviridae. It includes a transcription factor, nuclease, and protein kinases, and seems to contribute to effective infection structure. |
| Episome | Episome | Genetic factor. Like plasmid, it independently proliferates or is incorporated into chromosome and proliferates. |
| HHV4 | Human herpesvirus 4 | Epstein-Barr virus synonym It is believed to cause Burkitt lymphoma. |
| HHV6 | Human herpesvirus 6 | It causes exanthema subitum that develops during infancy. In some cases, it may be incorporated into chromosomes and genetically propagate. |

| | | |
|---|---|---|
| RSV | Rous sarcoma virus | Rous sarcoma virus (RSV) A subspecies of avian leukosis virus (ALV) group. |
| NDV | Newcastle disease virus | Newcastle disease virus. Newcastle disease pathogen. Pathogenic depends on strains. A zoonosis and an effect on human is conjunctivitis. |
| F protein | Fusion protein | Glycoprotein constituting NDV envelop. It is required for cell fusion at the time of infection in the co-existence of HN protein. |
| Hemagglutinin-neuraminidase protein | Hemagglutinin-neuraminidase protein | Glycoprotein constituting NDV envelop. It is required for cell fusion at the time of infection in the co-existence of F protein. It has hemagglutinin and neuraminidase activity. |
| Syncytium | Syncytium | A coenocyte generated by fusing more than one cell. |
| Co-transfection | Co-transfection | More than one type of foreign genes is introduced into cultured cell lines of eukaryotic cell by using a reagent and an electroporation method. |
| FITC | Fluorescein Isothiocyanate | The fluorescein is a green fluorochrome (maximum fluorescence wavelength is 519 nm). FITC is derivative thereof and used for a signage. |
| Homolog | Homolog | Homologous gene. A gene which is thought to be derived from common ancestors. |
| Cloaca | Cloaca | A general excretory orifice. An organ, which performs functions of rectum, urine orifice, and genital orifice. |
| Provirus | Provirus | A virus genome incorporated into cellular genome and replicated with the cellular genome. |
| Frame | Frame | Reading frame. A genetic code is trinucleotide and codes 1 amino acid. In one DNA sequence, complementary chains in 6 reading frames. |
| ALV | Avian leukosis virus | Avian leukosis virus The avian leukosis virus group has A-J subgroups. The viruses causing lymphocytic leukemia belong to Subgroup A, B, and J. The endogenous virus belongs to Subgroup E. |
| EAV | Endogenous avian virus | Endogenous avian virus. Indirect evidences for particle presence are shown. It is included in genomes of all lines of chickens. |
| REV | Reticuloendotheliosis virus | Reticuloendotheliosis virus. Causes symptoms to chickens such as hypoplasia, abnormal wing secondary feathers (dropout), tumors, etc. |

Reference (Attached as Literature)

Literature 1 “Viraemia and antibody development in chicks following the administration of turkey herpesvirus.” Churchill A.E. *et al.*, 1973, Veterinary Record 92(13):327-334

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Literature 3 “Epidemiology of a herpesvirus of turkeys: Possible sources and spread of infection in turkey flocks” Witter R. L. and Solomon J. J., 1971, Infection and Immunity 4(4):356-361

Literature 4 “Experimental infection of turkeys and chickens with a herpesvirus of turkeys (HVT)” Witter R. L. and Solomon J. J., 1972, Avian Diseases 16(1):34-44

Literature 5 “Horizontal transmission of turkey herpesvirus to chickens. 1. Preliminary observation” Cho B. R. *et al.*, 1971, Poultry Science 50(3):881-887

Literature 6 “Horizontal transmission of turkey herpesvirus to chickens 5. Airbone transmission between chickens” Cho B. R., 1976, Poultry Science 55:1830-1833

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Literature 8 “Nononcogenic turkey and chicken herpesvirus (HVT)” Schat K. A. and Nair V., 2008, in “Disease of Poultry 12th Edition” (Sarif Y. M. ed.) p491-492, Backwell Publishing Professional. Iowa.

Literature 9 “The genome of turkey herpesvirus” Afonso C.L. *et al*., 2001, Journal of Virology 75(2):971-978.

Literature 10 “Public health implications of Marek’s disease virus and herpesvirus of turkeys. Studies on human and subhuman primates” Sharma J. M. *et al.*, 1973, Journal of The National Cancer Institute 51(4):1123-1128.

Literature 11 “Lack of pathogenicity of Marek’s disease virus and herpesvirus of turkey in marmoset monkeys” Sharma J. M. *et al.*, 1972, Journal of The National Cancer Institute 49(4):1191-1197.

Literature 12 “Characterization of non-oncogenic Marek’s disease virus-infected and turkey herpesvirus -infected lymphocytes” Sheck W. S. *et al.*, 1982, Journal of General Virology 63:333-341

Literature 13 “Latent turkey herpesvirus infection in lymphoid nervous, and feather tissue of chickens” Holland M. S. *et al.*, 1998, Avian Diseases 42(2):292-299.

Literature 14 “Budding events in herpesvirus morphogenesis” Mettenleiter T.C., 2004, Virus research 106 : 167-180

Literature 15 “Herpesvirus assembly: An update” Mettenleiter T.C. *et al.*, 2009, Virus research 143 : 222-234

Literature 16 “Recombination in viruses: Mechanisms, methods of study, and evolutionary consequences.” Marcos P-L. *et al.*, 2015, Infection, Genetics and Evolution 30:296-307

Literature 17 “Characterization of interspecific recombinants generated from closely related bovine herpesviruses 1 and 5 through multiple PCR sequencing assays” Maria P.D.M.Z. *et al.*, 2009, Journal of Virological Methods, 161: 75-83

Literature 18 “Recombination of Globally circulating varicella-zoster virus” Peter N, *et al.*, 2015, Journal of Virology. 89(14):7133 -7146

Literature 19 “Herpesviruses and chromosomal Integration” Morissette G. and Flamand L., 2010, Journal of Virology 84(23):12100-12109

Literature 20 “Characterization and experimental reproduction of peripheral neuropathy in Wite Leghorn chickens” Bacon L. D. *et al.*, 2001, Avian pathology. 30(4):487-499

Literature 21 “Herpesvirus Connection in the Expression of Auto immune Vitiligo in Smyth Line Chickens” Ref G. F., *et al.*, 2015, Pigment Cell Res. 14(1):40-46

Literature 22 “Dynamics of Marek’s disease virus and herpesvirus of turkey shedding in feather dander of broiler chickens” Islam A. F. M. F. *et al.*, 2005, Proceedings of the Australian poultry science symposium, 17:105-108

Literature 23 “The Rous sarcoma virus long terminal repeat is a strong promoter when introduced into a variety of eukaryotic cells by DNA-mediated transfection.” Gorman C. M. *et al.*, 1982, Proceedings of National Academy of Science 79(22):6777-6781

Literature 24 “Mutations in the Newcastle disease virus hemagglutinin-neuraminidase protein that interfere with its ability to interact with the homologous F protein in the promotion of fusion.” Deng R. *et al.,* 1999, Virology 253(1):43-54

Literature 25 “The characterization of monoclonal antibodies to Newcastle disease virus” Russell P. H. *et al*., 1983, Journal of General Virology 64(9):2069-2072.

Literature 26 “Technical Bulletin. pGEM-3Z Vector” Promega

Literature 27 “Avian herpesvirus as a live viral vector for the expression of heterologous antigens.” Sondermeijer P. J. A. *et al.,* 1993, Vaccine 11(3):349-358.

Literature 28 “Protection of chickens from Newcastle and Marek’s diseases with a recombinant herpesvirus of turkeys vaccine expressing the Newcastle disease virus fusion protein.” Morgan R. W. *et al.,* 1992, Avian Diseases 36(4):858-870.

Literature 29 “Quantitative pathogenicity of Marek’s disease virus and vaccine virus strains in chicken: Macroscopical aspects” Mass H. J. L. *et al.,* 1978, Avian Pathology 7(1):61-78

Literature 30 Virus Hatsubgan no Atarashii Bunshi Kiko (New Molecular Mechanism of Viral Carcinogenesis) Naosada Maeda and Yasunobu Yoshikai, 2007, Virus 57(2): 159-170

Literature 31 “Retroviral DNA integration.” Hindmarsh P. and Leis J., 1999, Microbiology and Molecular Biology Reviews 63(4):836-843

Literature 32 “Evidence of avian leukosis virus subgroup e and endogenous avian virus is measles and mumps vaccines derived from chicken cell: Investigation of transmission to vaccine recipients.” Tsang S. X. *et al*., 1999, Journal of Virology 73(7):5843-5851

Literature 33 “Isolation and characterization of an adventitious avian leukosis virus isolated from commercial Marek’s disease vaccines” Fadly A. *et al.*, 2006, Avian Disease 50:380-385

Literature 34 “Reticuloendotheliosis group virus pathogenic to chicken isolated from material

infected with turkey herpesvirus (HVT)" Koyama H. *et al.,* 1976, Avian Disease 20(2):429-434

Literature 35 "Differential amplification and quantification of Marek's disease virus using real-time polymerase chain reaction." Islam, A., *et al.* 2004. J. Virol. Methods 119, 103-113

Attachment List

## Attachment 1 HVT Susceptibility of Non-Chicken Birds (Overseas Test)

**Purposes:**

Study the susceptibility of non-chicken birds to HVT (FC 126 strain).

**Method:**

HVT vaccine was inoculated to adult pigeons, 4-week old bobwhites, 1-day old ring-necked pheasants, and 1-day old turkeys and collected blood 3 weeks later. A few birds' blood were pooled and leukocyte was separated and inoculated to 2nd generation chick embryo cells in culture, incubated them for 5 days at 37 deg C to observe plaques.

In the 8th week after inoculation, all birds are euthanized and observed to see if gross pathology on Marek's disease would be approved.

**Result:**

Found the proliferation of virus in turkeys and ring-necked pheasant. However, none of 4 types of birds offered for an experiment presented any clinical symptom and lesions including tumors (Table 1).

Table 1 Viral Isolation per Bird Species and Necropsy Results

| Bird species | Viral isolation[1)] | | Necropsy[2)] | |
|---|---|---|---|---|
| | HVT | Control | HVT | Control |
| Pigeon | 0/10 | 0/2 | 0/20 | 0/12 |
| Bobwhite | 0/10 | 0/3 | 0/23 | 0/15 |
| Ring-necked pheasants | 5/10 | 0/2 | 0/20 | 0/14 |
| Turkey | 7/7 | 0/3 | 0/21 | 0/13 |

1) Positive number/ analyte number (as analytes, a few are pooled)

2) The number of turkeys that show lesions of Marek's disease

**Considerations and Conclusion:**

Even if HVT vaccine diffuses exempt bird species, it is safe because no clinical symptom and lesion are presented.

**Attachment 2**..... **Proliferation of HVT-NDV/F and PB1 Strains in Birds (Overseas Test)**

**Purposes:**

Study the proliferation of HVT-NDV/F and PB1 strains used for production in bodies of birds (pigeons, turkeys, and ducks).

**Method:**

A high dose （$10^{4.1}$PFU） of HVT-NDV/F strain is once intramuscularly inoculated to 10 pigeons (5-7 week old), 10 turkeys (day-old), and 10 ducks (2-day old).

Furthermore, a high dose ($10^{4.0}$PFU) of PB1 strain is once intramuscularly inoculated to 10 ducks, pigeons, and turkeys. Blood is collected from all animals 3 and 4 weeks later, and leukocyte was separated. Leukocyte was inoculated to primary chick embryo cell in culture and plaques were observed.

**Result:**

Directly intramuscularly injected, HVT-NDV/F strain did not proliferate in bodies of pigeons and ducks. Similarly, PB1 strain did not proliferate in bodies of pigeons and ducks. HVT-NDV/F strain proliferated in 7 out of 9 turkeys. PB1 strain, or parent strains, proliferated in 8 out of 10 turkeys (Table 1).

Table 1 Proliferation of HVT-NDV/F and PB1 Strains in Turkeys and Ducks

| Bird species | HVT-NDV/F strain | | PB1 strain | |
|---|---|---|---|---|
| | Week 3 post-inoculation | Week 4 post-inoculation | Week 3 post-inoculation | Week 4 post-inoculation |
| Pigeon | 0/10 | 0/10 | 0/10 | 0/10 |
| Turkey | 4/9 | 6/9 | 4/10 | 7/10 |
| Ducks | 0/10 | 0/10 | 0/10 | 0/9 |

Positive number /inspection number (The analyte where leukocyte cannot be separated and the bacterial contaminated analyte were not included in the inspection number.)

**Considerations and Conclusion:**

The host ranges were compared between HVT-NDV/F strain and PB1 strains used for produce HVT-NDV/F strain. As a result, no change of the host range due to the gene recombination was recognized in the tested species.

Attachment 3 Horizontal Transmission by Cohabitation with HVT-NDV/F and PB1 Strains (Overseas Test)

**Purposes:**

Compared whether HVT-NDV/F and PB1 strains used to the production are horizontally transmitted.

**Method:**

Inoculated $10^{3.4}$PFU of HVT-NDV/F strain or $10^{3.6}$PFU of PB1 strain were inoculated to ten of 1-day SPF chickens by intramuscular injection. Ten controlled chickens were respectively made to live with each group. Collected blood from tested chickens and controlled chickens in the 3rd, 4th, and 6th week after vaccination and examined whether virus existed in blood. In a virus inspection, leukocyte was separated from blood, and a virus was inoculated into cultured primary chick embryo cell to observe plaques.

**Result:**

It was confirmed that all groups of chickens inoculated with HVT-NDV/F or PB1 strain was infected 4 weeks after the inoculation (Tables 1 and 2). It was confirmed that in the cohabitation group, a part of individuals was infected on the 6th week in case of HVT-NDV/F strain (Table 1) and on 4th week in case of PB1 strain (Table 2).

Table 1 Horizontal Infection of HVT-NDV/F Stains

| Group | The number of post-inoculation weeks | | | |
|---|---|---|---|---|
| | 3 | 4 | 5 | 6 |
| Inoculation | 9/10 | 10/10 | 4/8 | 6/10 |
| Co-residence | 0/10 | 0/10 | 0/7 | 2/10 |

Positive number /inspection number (The analyte where leukocyte cannot be separated and the bacterial contaminated analyte were not included in the inspection number.)

Table 2 Horizontal Infection of PB1 Strain

| Group | The number of post-inoculation weeks | | | |
|---|---|---|---|---|
| | 3 | 4 | 5 | 6 |
| Inoculation | 10/10 | 10/10 | 7/9 | 6/10 |
| Co-residence | 0/10 | 2/10 | 3/8 | 3/8 |

Positive number /inspection number (The analyte where leukocyte cannot be separated and the bacterial contaminated analyte were not included in the inspection number.)

**Considerations and Conclusion:**

HVT-NDV/F strain was diffused to cohabiting chickens. This nature has not been changed from PB1 used for production.

## Attachment 4 Study Growth Temperature for HVT-NDV/F Strain

**Purposes:**

Examine the temperature ranges where the proliferation of HVT-NDV/F strain can be proliferated. Similarly, study the temperature range where PB1 strain used for production and check whether the recombination causes any property change.

**Method:**

Inoculate HVT-NDV/F or PB1 strain to primary chick embryo cells at MOI 0.001 and incubate them at 25, 30, 35, 37, 40, and 45 deg C. On 1st, 2nd, 3rd, and 4th days after incubation, cells and culture supernatant are collected. Proliferation was confirmed through CPE observation and realtime RCR. Realtime RCR used the primer that proliferates HVT SORF1 genes and TaqMan probe, referring to methods of Islams and semiquantitatively compared viral DNA amounts. It was judged that there was proliferation if the number of cycles (Ct value) required for amplification.

**Result:**

Both HVT-NDV/F and PB1 strains showed CPE on primary chick embryo cells at 30 through 40 deg C but did not at 25 deg C. At 45 deg C, because cells did not exhibit sheet formation, CPE could not be observed (Table 1).

Both HVT-NDV/F and PB1 strains continuously increased the viral DNA amount at 30 deg C through 40 deg C. At 25 deg C and 45 deg C, up to 2nd day, DNA amount was found to be slightly increased but underwent the change of less than Ct value 4 (Figs 1 and 2). On non-inoculated control, viral DNA was not found to proliferate both before incubation and after 4-day inoculation at 37 deg C.

Table 1 CPE Observation Result at Each Incubation Temperature

| Incubation temperature | Viral cell | | |
|---|---|---|---|
| | HVT-NDV/F strain | PB1 strain | Control |
| 25 deg C | − | − | − |
| 30deg C | + (4th day) | + (4th day) | − |
| 35deg C | + (4th day) | + (4th day) | − |
| 37deg C | + (3rd day) | + (3rd day) | − |
| 40deg C | + (3rd day) | + (3rd day) | − |
| 45deg C | × | × | × |

＋: CPE positive －: CPE negative ×: Could not confirm because cells did not exhibit sheet formation.

( ) is a CPE appearance date

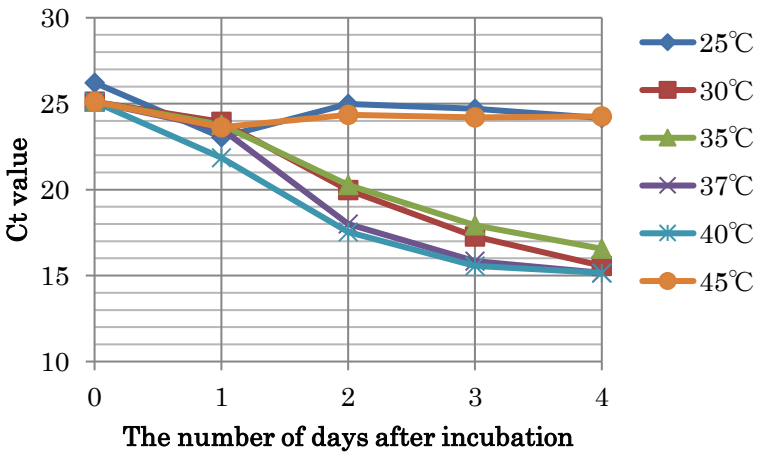



Fig 1. Transition of Virus DNA Amount of HVT-NDV/F Strain at Each Temperature

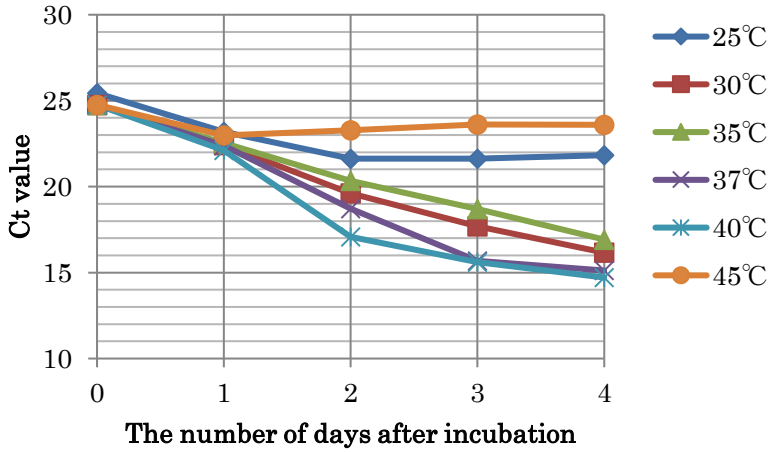



Fig 2. Transition of Virus DNA Amount of PB1 Strain at Each Temperature

**Considerations and Conclusion:**

The temperature range that allows HVT-NDV/F strain to proliferate was 30-40 deg C. At 25 and 45 deg C, virus DNA did not continuously increase and CPE is not observed. It was considered that strains do not proliferate at these temperatures.

No change was recognized in HVT-NDV/F strain from PB1 strains used for the production at the temperature that allows the strain to proliferate.

**Documents for Reference:**

Islam, A., *et al.* 2004. Differential amplification and quantification of Marek's disease virus using real-time polymerase chain reaction. J. Virol. Methods 119, 103-113

## Attachment 5 Proliferation of HVT-NDV/F and PB1 Strain in the Cell Line Derived from Mammal (Overseas Test)

**Purposes:**

Study the proliferation of HVT-NDV/F and PB1 strains used for production in the cell line derived from mammal.

**Method:**

Two 25cm$^2$ flasks of test cells were prepared per virus (Table 1). Into one flask, 200 PFU virus is inoculated and incubated at 37 deg C, subcultured 4 times every 3-5 days (at the time of subculture, the dilution degree is 3-4 times), and CPE was observed. The final subculture is inoculated into 6-hole plate and fluorescent staining was conducted with turkey herpesvirus specific monoclonal antibodies (L78.2).

Table 1 Test Cell Strains

| Cell line name | Derived |
|---|---|
| RK13 | Rabbit's kidney |
| JCK | Calf kidney |
| CRFK | Cat kidney |
| A72 | Dog tumor (tissue unknown) |
| Vero | African green monkey's kidney |
| Hela | Human adenocarcinoma (cancer of the cervix (CC)) |

Use 2nd generation chick embryo cells in culture as positive control

**Result:**

In case of 2nd generation chick embryo cells in culture, plaque was observed on 3rd day after inoculation of HVT-NDV/F or PB1 strain. However, no virus proliferation was detected in any subculture of cell lines derived from rabbits, cattle, cats, dogs, African green monkey, and human beings. If HVT was dyed with specific monoclonal antibodies, no virus plaque was detected from any cultured cell line inoculated with HVT-NDV/F or PB1 strain.

**Considerations and Conclusion:**

HVT-NDV/F strain did not proliferate in case of cultured cells derived from mammal or human beings. This nature has not been changed from PB1 used for production.

## Attachment 6 Biodistribution of HVT-NDV/F Strain and PB1 strains at Hypodermic Inoculation (Overseas Test)

**Purposes:**

Examined Biodistribution of HVT-NDV/F and PB1 strains used for production at hypodermic inoculation.

**Method:**

HVT-NDV/F strain of $10^{4.7}$PFU or PB1 strain of $10^{4.8}$PFU was inoculated to 1-day SPF chickens. On the 3rd, 7th, 10th, 14th, 17th, and 21st day after vaccination, autopsies on three chickens from each group were performed, and the viral isolation from liver, spleen, kidney, ovary/testis, lung, peripheral blood leukocyte, feather follicle, mouthwash, and cloaca swab was tried. If a sample was inoculated to a primary chick embryo cell and CPE was not found in 2-4 day incubation, and if subculture was conducted once and CPE was not found, negativity was determined.

**Result:**

In all groups, it was lymphatic tissues where the virus was detected for the first time on the 3rd day after inoculation (bursa Fabricii, thymus, and spleen). (The results are confidential and not open to the public.) A sample on 10th day after inoculation was not evaluated because of cell peeling. On 14th day after inoculation, virus was detected from almost all organs except mouthwash and cloaca swab. At any time, no virus was detected from mouthwash and cloaca swab.

**Considerations and Conclusion:**

HVT-NDV/F strain did not change from PB1 strains in the biodistribution of chicken after subcutaneous vaccination.

**Attachment 7**.....**Biodistribution of HVT-NDV/F and PB1 Strains at Intramuscular Inoculation (Overseas Test)**

**Purposes:**

Examined the biodistribution of HVT-NDV/F and PB1 strains used for producing them at intramuscular inoculation.

**Method:**

HVT-NDV/F strain of $10^{5.2}$PFU or PB1 strain of $10^{5.3}$PFU was inoculated to 1-day SPF chickens. On the 3rd, 7th, 10th, 14th, 17th, and 21st day after vaccination, autopsies three chickens from each group were performed on three chickens from each group, and the viral isolation from liver, spleen, kidney, ovary/testis, lung, peripheral blood leukocyte, If a sample was inoculated to a primary chick embryo cell and CPE was not found in 2-4 day incubation, and if subculture was conducted once and CPE was not found, negativity was determined.

**Result:**

**:**

In all groups, it was lymphatic tissues where the virus was detected for the first time on the 3rd day after inoculation (bursa Fabricii, thymus, and spleen) an liver (the results are confidential and not open to the public). On 7th day after inoculation, virus was detected from almost all organs except mouthwash and cloaca swab. At any time, no virus was detected from mouthwash and cloaca swab.

**Considerations and Conclusion:**

HVT-NDV/F strain did not change from PB1 strains in the biodistribution of chicken after subcutaneous vaccination.

## Attachment 8　Retrieval Results of Allergen Database on Amino Acid Coded to HVT

**Purposes:**

Retrieve the Allergen Database to study the possibility that Herpesvirus of turkey (HVT) produce toxic substances.

**Method:**

An amino acid sequence predicted from NVT whole base sequence (the Accession Number assigned by DNA Data Bank of Japan (DDBJ): AF291866) was retrieved in the Allergen Database for Food Safety (ADFS（http://allergen.nihs.go.jp/ADFS/index.jsp).

**Result:**

The allergen protein la g 1, which was deprived from cockroach, showed 25 % conformity to 269 residue units.　The amino acid is coded for UL36 Large tegument protein gene of HVT. Continuously matched amino acid was 3 residues or less.　Moreover, allergenicity suspected proteins were not retrieved.

**Considerations:**

In the FAO/WHO method, the allergen is positive if the protein is a 35 % or more match with 80 residue units of amino acid or matches the amino acid continuously for 6-8 residues. Therefore, HVT genes do not contain proteins similar to the previously known allergen protein.

## Attachment 9 Base Sequence of the Donor-Nucleic Acid

The DNA fragment sequence was inserted to the homologous recombination of HVT PB1 strain to produce HVT-NDV/F strain. The results were confidential and not open to the public. The sequence includes HVT (PB1 strain) homologous region, LTR promoter derived from RSV, the initiation and the termination codons of F protein gene derived from Newcastle disease, and the amino acid seqyebce if F protein.

A partial base sequence of PB1 strain is registered in DNA Data Bank of Japan (DDBJ) accession number M84473. The base sequences of PB1 and FC126 strains (AF291866) is 98.9% homology.

The sequences of LTR promoter derived from Rous sarcoma virus is registered in the accession number DQ075935.

The sequences of F protein gene derived from Newcastle disease virus is registered in the accession number Y18898.

The sequences of cloning vector pGEM-3Z is registered in the accession number X65304.

**Attachment 10　Documents concerning Donor-Nucleic Acid and Vector**

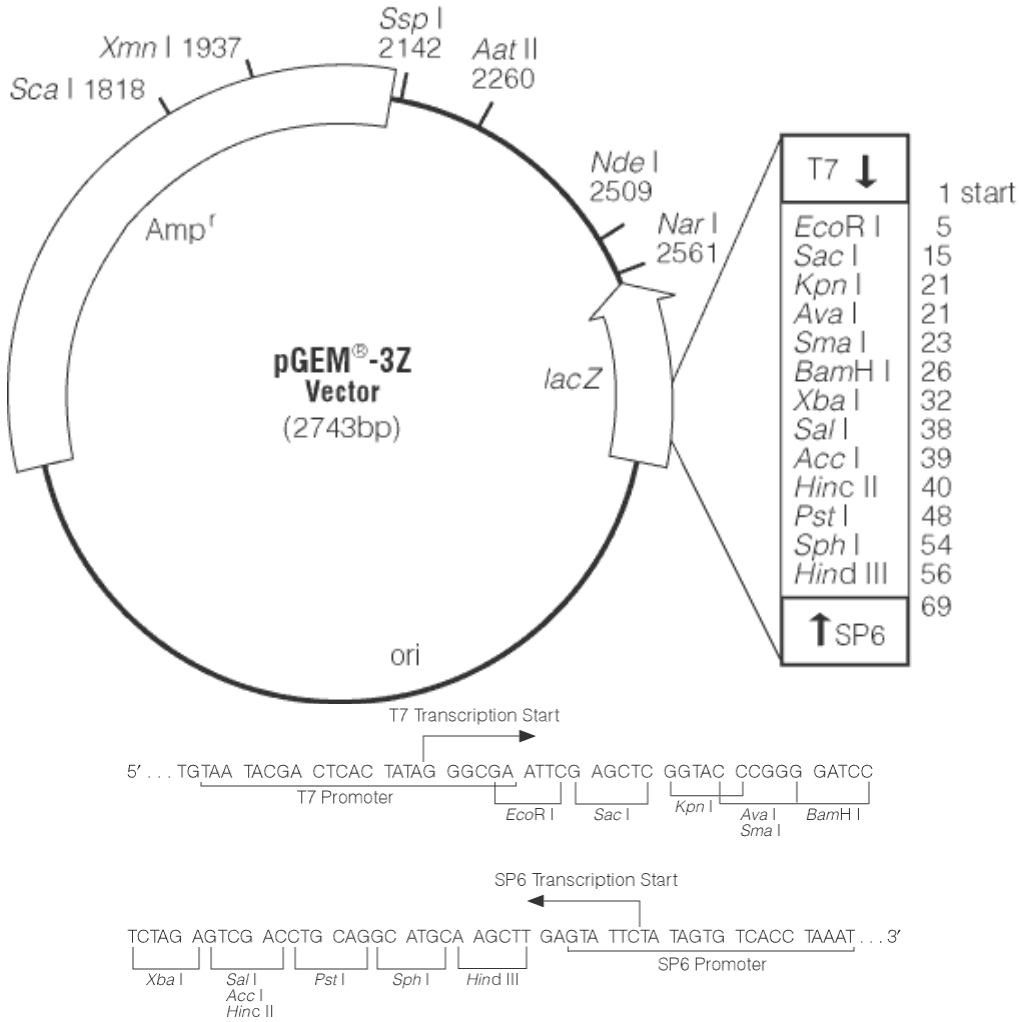
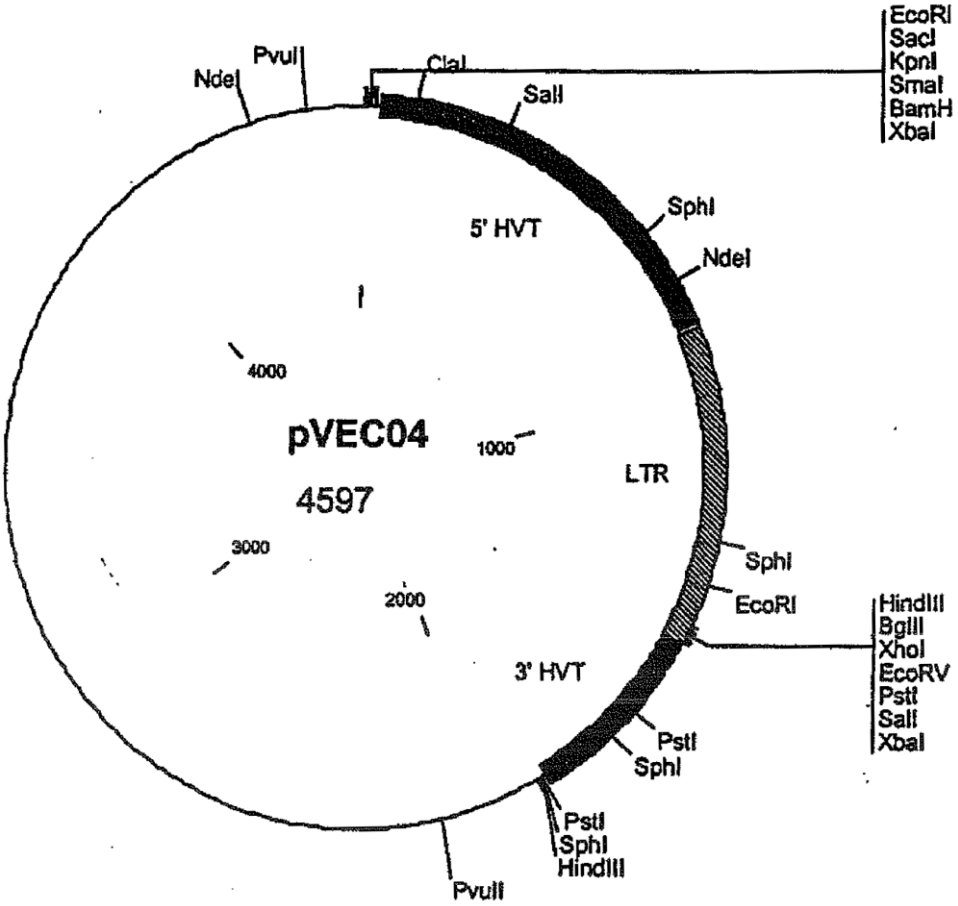



Fig.1 pGEM-3Z Map, Promoter, and Multi Cloning Sites

In constructing the vector pVEC04 (Fig. 2), the plasmid vector on market used as a backbone.

T7 RNA polymerase transcription initiation site 1
SP6 RNA polymerase transcription initiation site 69
T7 RNA polymerase promoter 2727-3
SP6 RNA polymerase promoter 67-86
Multicloning site 5-61
lacZ start codon 108
lac operon sequence 2561-2724; 94-323
lac operator 128-144
β-lactamase (Ampr)　code area 1265-2125
pUC/M13 Forward sequence primer-binding site (PES) 2686-2702
pUC/M13 Reverse sequence primer-binding site 112-128




**5'-AAGCTTAGATCTCTCGAGGATATCCTGCAGGTCGACTCTAGA-3'**

*Hin*dIII *Bgl*II *Xho*I *Eco*RV *Pst*I *Sal*I *Xba*I

Fig.2 Vector pVEC04 Map and Sequences in Cloning Site

The vector is constructed by using pGEM-3Z (Fi.1) as a backbone. It has LTR promoter sequence, cloning site on the downstream of LTR, and HVT-derived sequences on the both sides.

Three sites, that is, *Bgl*II, *Xho*I, and *Eco*RV can be used as unique restriction enzyme sites at the cloning site in the LTR downstream. The restriction enzyme site on 5' side is a sequence derived from pGEM-3Z.

*Bgl*II site is used for cloning F protein gene of Newcastle disease virus

HTV homologous sequence and LTR sequence are shown with sequences of F protein gene in Attachment 9.

What F protein gene was introduced to the vector became pNDV04 (Fig. 7)

Chicken embryo fibroblasts

↓

HVT (PB1 strain) inoculation

↓

Extraction of all DNAs

↓

Partial digestion by *Sau*3AI and fragment collection of 15-20 kbp

↓

Library by EMBL3 (phage vector)

↓

Hybridization by using HVT inoculation and DNA of non-inoculation cells
Screening

↓

Identification based on the restriction enzime map of known HVT (FC 126 strain)

↓

Phage clone λHTV04 including 17.5 kbp in $U_S$ region.

↓

Cloning of *Xho*I fragment including ORF2 (US10 homolog) in pGEM-3Z

↓

pMD07

Fig.3 HVT Homologous Sequence Cloning Procedure

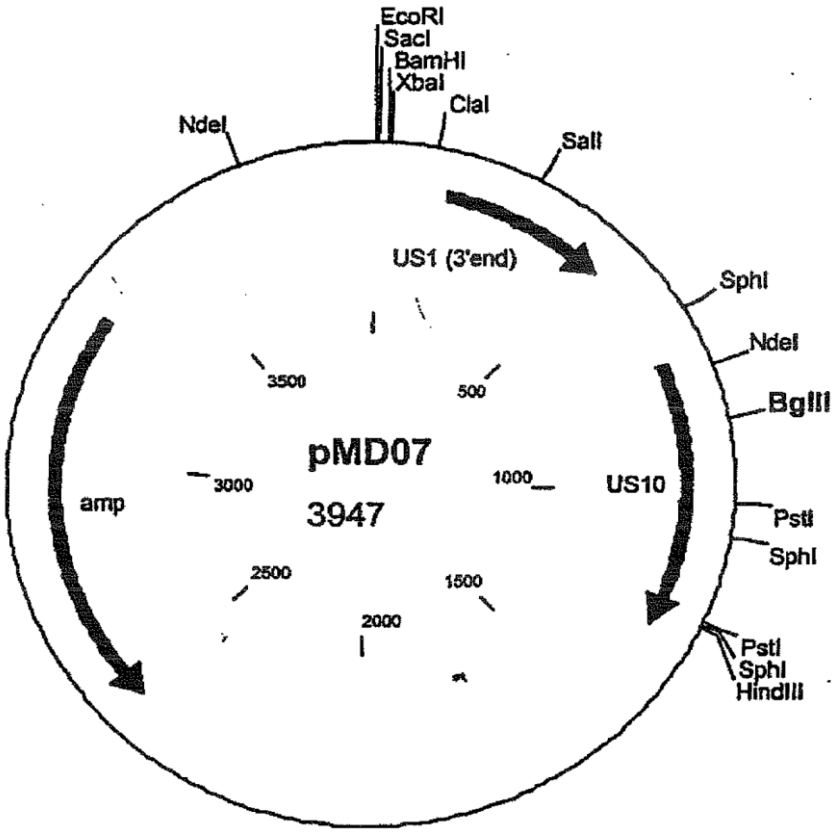
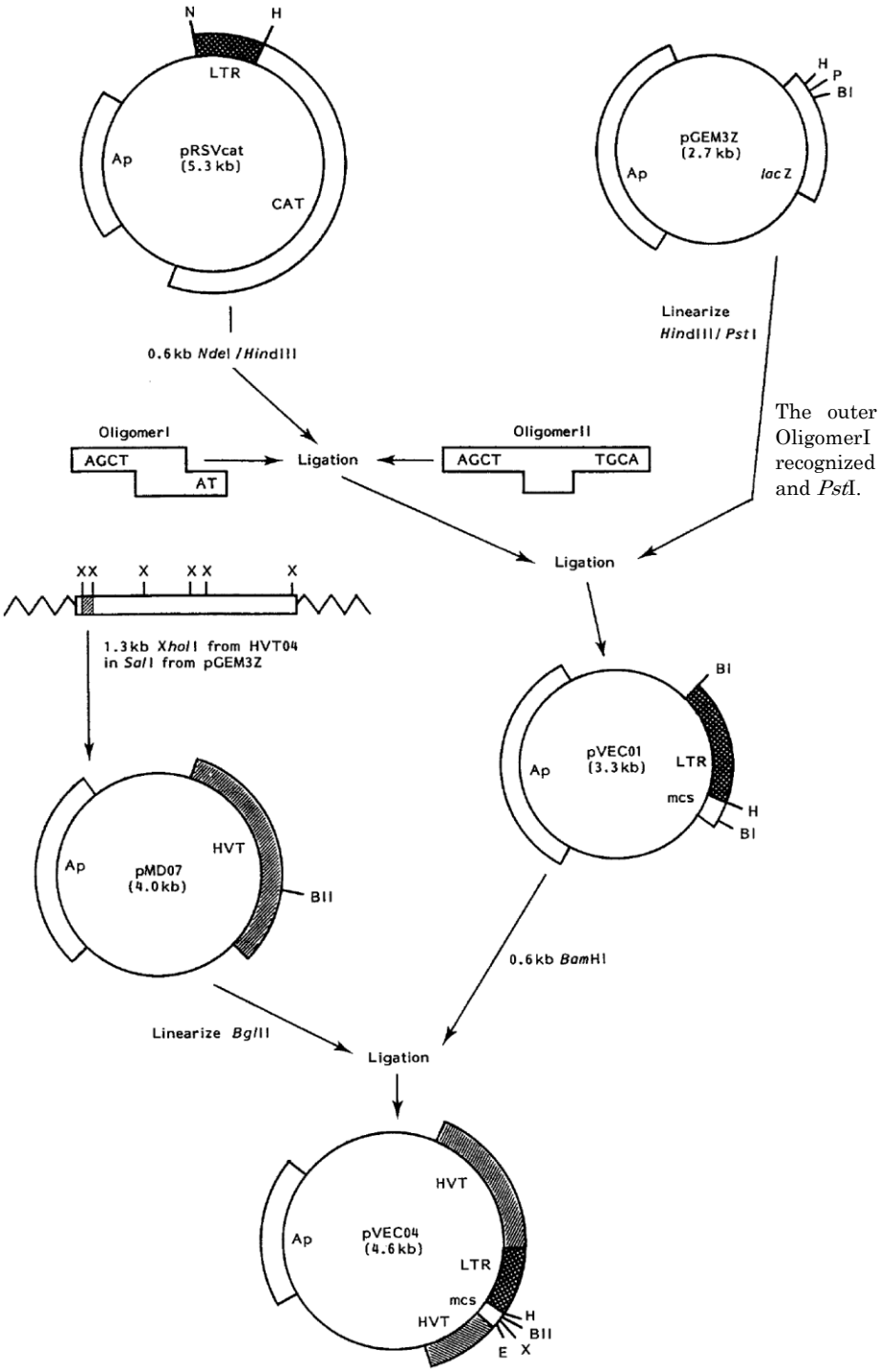



Fig 4 pMD07 Map

pM007 is a cloning of the fragment of HVT sequence (the region including ORF2 (US10 homolog) in pGEM-3Z. LTR sequence and a new cloning site were inserted to *Bgl*II site in US10 sequence to build pVEC04 (Fig.5).




The outer sequences of OligomerI and II are recognized by *Hind* Ⅲ and *Pst*I.

Fig.5 Cloning Vector pVEC04 Construct Procedure

pVEC04 is a vector for HVT recombinant with 3 cloning restriction enzymes sites. OligomerI includes *Bam*HI sequence and was used to create the *Bam*HI site on the LTR upstream of pVEC01. OligomerII includes *Bgl*II, *Xho*I and *Eco*RV sequences and each of them is the only sequence at the final plasmid pVEC04. LTR promoter is right-handed rotation, and *Bgl*II, *Xho*I and *Eco*RV sites, 5' and 3' sides are respectively adjacent to 800bp and 400bp HVT sequences.

Abbreviations Ap: ampicillin resistance marker, BI: *Bam*HI, BII: *Bgl*II, E: *Eco*RV, H: *Hind*III, N: *Nde*I, P: *Pst*I, X: *Xho*I, mcs, Multicloning Sites, CAT: chloramphenicol acetyltransferase

Newcastle disease virus incubated by embryonated eggs (Clone 30 strains).

↓

Virus particle puriferation

↓

Extract genome RNA (minus single strand RNA)

↓

cDNA synthesis

↓

Library by λgt10 (phage vector)

↓

Prepare DNA probe based on the sequence of previously known protein genes
Screening by hybridization

↓

Phage clone with F protein gene

↓

Subcloning in pGEM4Z

↓

Deleted redundant sequences before/after F protein genes

↓

pNDV01

↓

Subcloning in pVEC04

↓

pNDV04

Fig.6 Procedure of Cloning F Protein Genes of Newcastle Disease Virus
Insertion fragmentary of pNDV01 was created by cutting inserted fragmentary with *Bam*HI and subcloning in *Bgl*II site of pVEC04 (Fig.2) (*Bgl*II restriction enzyme site was lost by *Bam*HI and *Bgl*II ligation.)

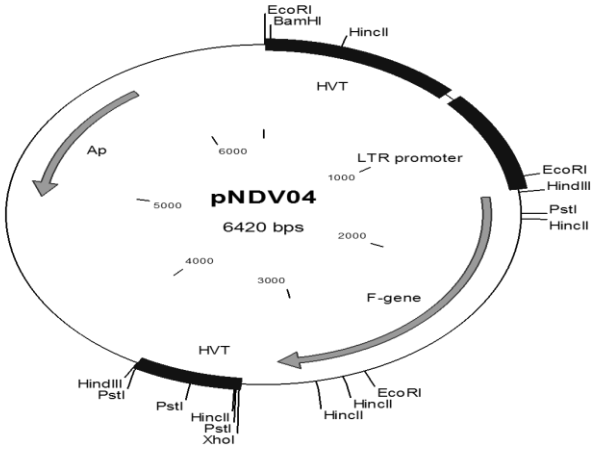



Fig. 7 Overall Structure of Plasmid pNDV04 including Donor-Nucleic Acid and the Restriction Enzyme Site

| | |
|---|---|
| F-gene | Newcastle disease virus F protein gene |
| LTRpromoter： | LTR promoter derived from Rous sarcoma virus (RSV) |
| HVT: | homologous sequence of Turkey Herpesvirus used for homologous recombination (ORF2 in $U_S$ region (US10 homorolog) |
| Ap: | Ampicillin resistance gene |

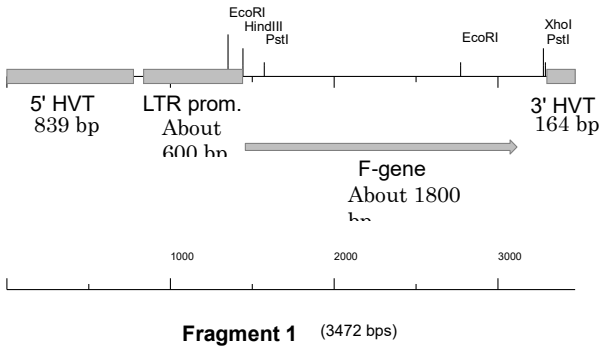



Fig 8　Donor-Nucleic Acid Used for Homologous Recombination

The plasmid in Fig. 7 is used in the straight-chain form for homologous recombination.

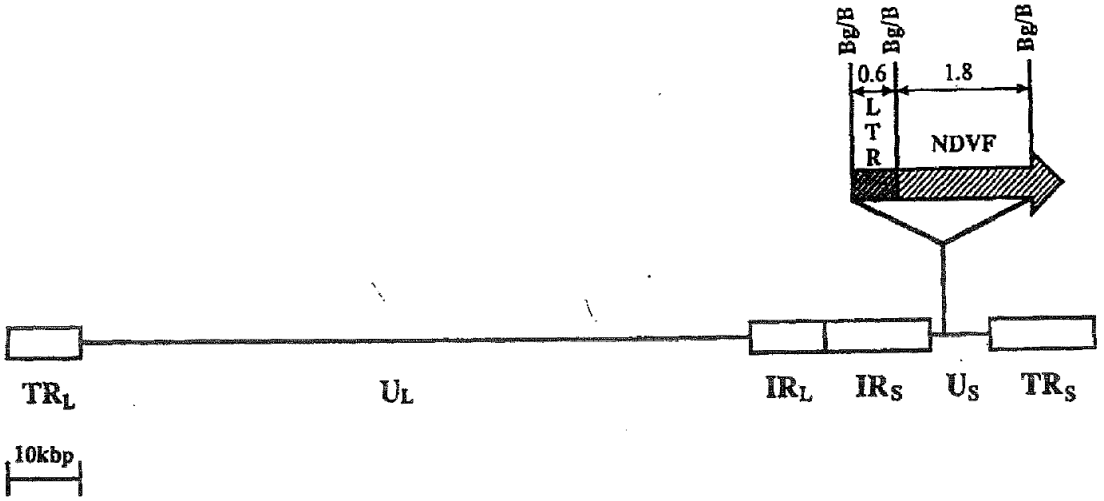



Fig. 9 Insertion Site of Donor-Nucleic Acid in Recombinant

The recombinant genomes are schematically shown.
LTR: LTR promoter derived from Rous sarcoma virus (RSV)
NDVF：Newcastle disease virus fusion (F) protein gene
$U_L$: $U_L$ region (unique long)
$U_S$: $U_S$ region (unique short)
$TR_L$: Terminal repeat　at the side of $U_L$
$TR_S$:　Terminal repeat　at the side of $U_S$
$IR_L$:　Inverted repeat at the side of $U_L$
$IR_S$:　Inverted repeat at the side of $U_S$

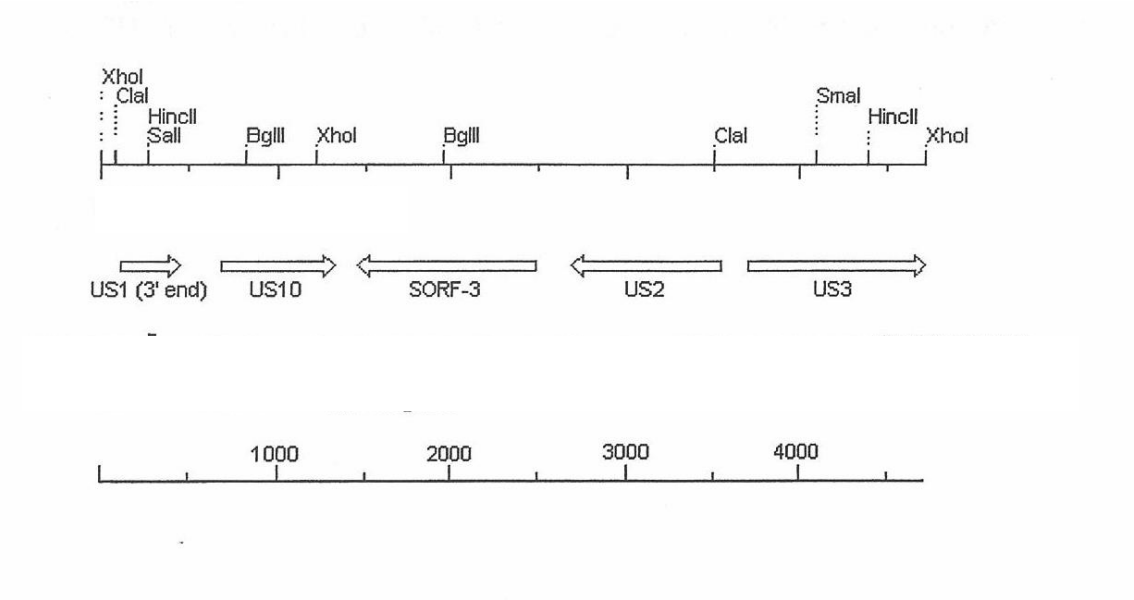



Fig 10　Restriction Enzyme Map around the Donor-Nucleic Acid Insertion Site
The restriction enzyme map of the vicinity of MVT's $U_S$ region (Fig.9 $U_S$).　Arrows show ORFs and their directions. The lower bar shows bp. The figure shows parent (PB1 strain) before insertion and the donor-nucleic acid was inserted into *Bgl*II site of US10 homolog.

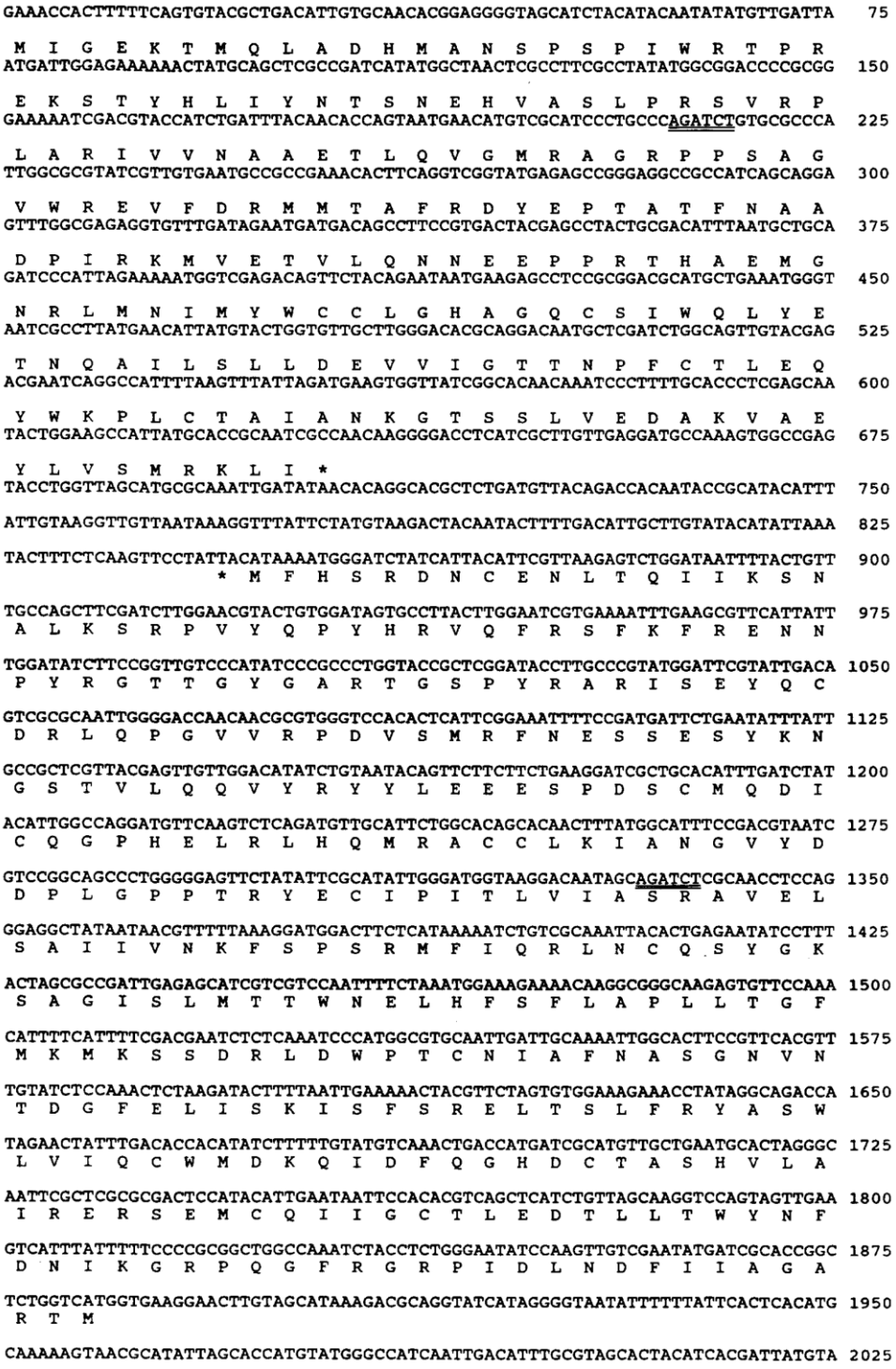



Fig 11 Base Sequences of the Donor-Nucleic Acid Insertion Site (the Part including HVT Unique Short Region ORF2 and ORF3)

ORF 2 (US10 homolog) is between base numbers 76 and 702. ORF3 runs in the reverse direction from the base numbers 1884 to 846. It is indicated that by the in vivo recombination using β galactosidase expression cassette, a foreign gene can be inserted into this region of HVT genome. It is where a marker gene is inserted into 2 *Bgl* restriction enzyme sites (double underlines). Finally, *Bgl*II restriction enzyme site in ORF2 is used as an insertion site for F protein derived from Newcastle disease virus.

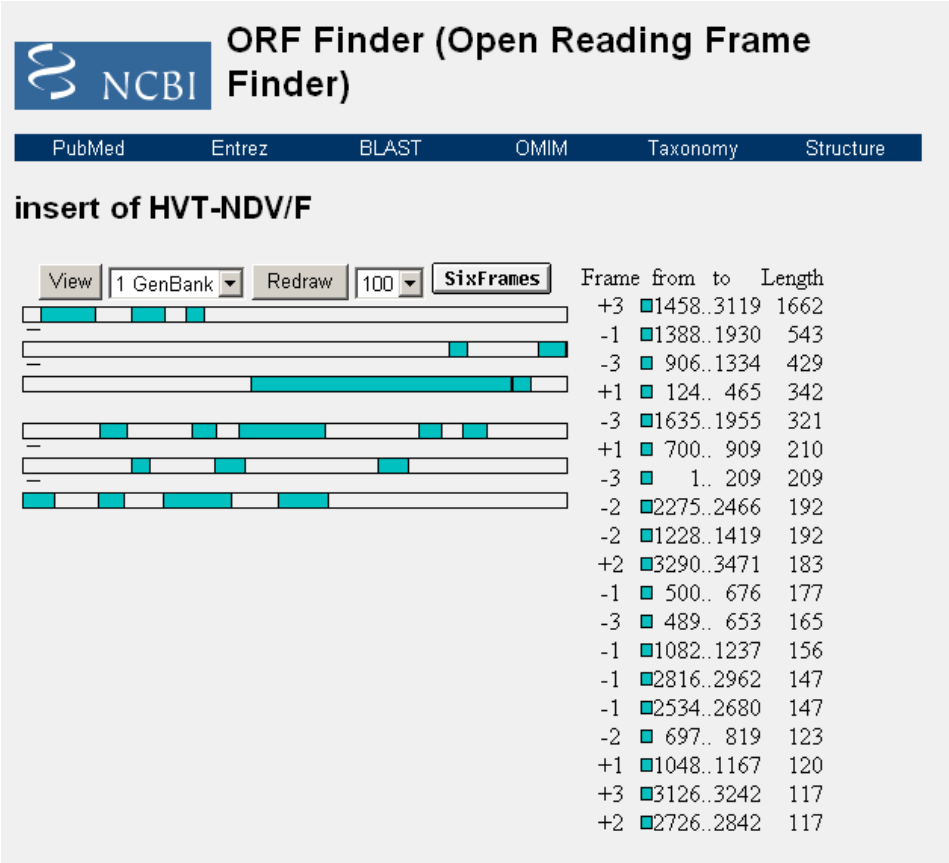



Fig.12 Retrieval Result of Open Reading Frame of Donor-Nucleic Acid Used for Recombinant

As for a donor-nucleic acid used for the recombination (3472 bp, Attachment 9), an analysis tool (ORF Finder) released by the National Center for Biotechnology Information (NCBI) was used to retrieve the Open Reading Frame (ORF).

Six reading frames including a complementary chain show the region of 100 or more amino acids (ORF) from the initiation codon to the termination codon in green. Numeric values on the right show reading frames, initiation position, termination position, and length in descending order of the longest of found ORFs.

The objective gene was retrieved as the longest ORF (1458-3119). Besides, short ORFs from 117 to 543 bp were found at 18 sites.

**Attachment 11Safety of Genotype and Phenotype of HVT-NDV/F Strain (Overseas Test)**

**Purposes:**

Confirm the safety of genotype and phenotype of HVT-NDV/F strain

For this purpose, the master seed virus(hereinafter called “MSV”) has been subcultured for 5 generations in chickens and the reversion of pathogenicity was confirmed. In addition, after the subculture for 11 generations by using cultured chick embryo cells, the stability of genotype by the Southern blotting technique and the stability of F protein expression were confirmed by fluorescent staining.

**Method:**

Subculture in chicken:

MSV was subcutaneously inoculated to 20 of 1-day-old SPF chickens, which were observed for 14 days. At the end of observation, blood drawing and necropsy were carried out, leukocyte was separated from blood and pooled, and it was inoculated into cultured primary chick embryo cell to check the existence of virus. Leukocyte was used for subculture and was subcultured for 2nd - 4th generations in the same way as the 1st generation. In the 5th generation, 55 chickens were used for the test. On the 14th day, blood was drawn from 20 chickens, and they were necropsied. The remaining chickens were raised for 8 weeks after inoculation and their Marek's disease symptoms were observed.

Subculture by cell:

MSV was subcultured for 11 generations by using primary chick embryo cell and was tested in Southern blotting technique and fluorescent staining.

Southern blot:

Virai DNA was digested with the restriction enzyme *Xho*I, electrophoresis was conducted with 1.5 % agarose gel, and the DNA was transferred to the nitrocellulose film. Labelled 1.2kb fragmentary adjacent to the insertion site of PB1 strain with radioactive phosphoric acid ($^{32}$P) and used it as a probe. Detection was exposed to X-ray film. The expected band size of PB1 strain is 1.2bp and those of HVT-NDV/F strain are 0.4 and 3.3 kb. (Figures 1 and 2)

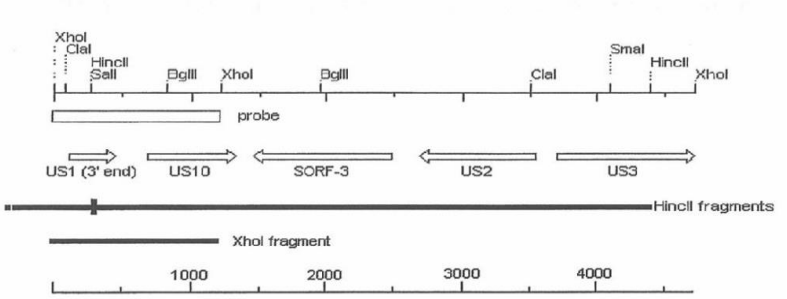



Fig.1 PB1 Strain Restriction Enzyme Map

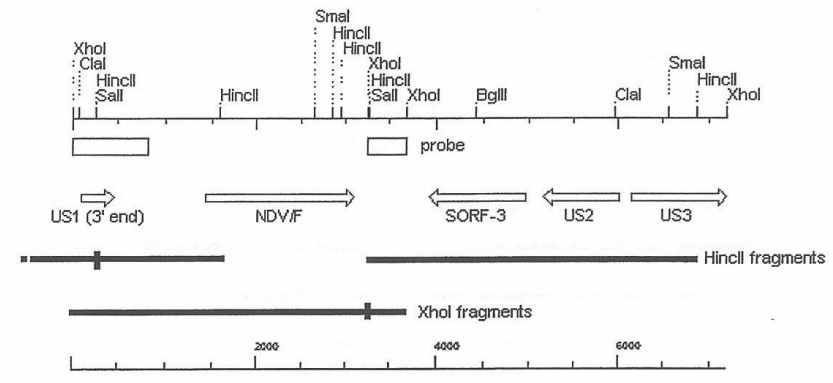


Fig.2 Restriction Enzyme Map of HVT-NDV/F Strain

Indirect fluorescent antibody method:

MSV of HVT-NDV/F strain, the virus subcultured in chickens for 5 generations, the virus whose cell was subcultured for 11 generations, and PB1 strain were inoculated to 2nd generation chick embryo cells in culture, incubated them for 5 days, and immobilize cells with 70 % aceton. Dyed viral infected cells with the indirect fluorescent antibody method using F protein specific monoclonal antibody (Mab-57) or HVT specific monoclonal antibody (L78.2) and measured the number of plaquesl

**Result:**

Subculture in chicken:

Two of test chickens in the third generation of subculture died. An autopsy revealed a cause of death is hip attack. From the blood collected from subcultures, 100 % virus was separated. An 8-week old chicken subcultured for 5 generations was necropsied, and no lesion of Marek's disease was found.

Southern blot:

As the virus in chicken for 5 geneations has small amount of virus, the one proliferated in cells subcultured for 4 generations was used. Bands of 3.3 kb and 0.4 kb were found in the virus subcultured for 11 generations and the virus subcultured for 5 generations in chickens, but not in PB1 strain. (Results were confidential and not open to the public.)

Indirect fluorescent antibody method:

In the virus in chicken subcultured for 5 generations and the one in cell for 11 generations, F protein expression was confirmed (Tables 1 and 2).

Table 1 F Protein Expression of HVT-NDV/F Stains Subcultured for 5 Generations in Chickens

| Virus | Average Pluque Number | |
|---|---|---|
| | F-57 (αF protein) | L78.2 (αHVT) |
| Virus subcultured for 5 generations in chickens | 138.6 | 142.2 |
| PB1 strain | 0 | 45.2 |

Table 2 F Protein Expression of HVT-NDV/F Stains Subcultured for 11 Generations in Primary Chick Embryo Cell

| Virus | Infection value (PFU/mL) | |
|---|---|---|
| | F-57 (αF protein) | L78.2（αHVT） |
| Master seed virus(MSV) | 699,300 | 649,450 |
| Virus subcultured for 11 generations | 5,483,400 | 6,060,600 |
| PB1 strain | 0 | 517200 |

**Considerations and Conclusion:**

It was confirmed that the virus subcultured for 11 generations and the virus subcultured in chickens for 5 generations expresses F protein of inserted Newcastle disease virus. In addition, it was confirmed that the virus subcultured for 11 generations and the virus subcultured in chickens for 5 generations did not lose genes or rearrange genomes and that F protein genes of inserted Newcastle disease virus are stable. MSV of HVT-NDV/F continues to hold the inserted genes of Newcastle disease and subculture for 5 generations in chickens did not reverse pathogenicity.

## Attachment 12 Stability of Genotype by the Sequence of HVT-NDV/F Strain (Overseas Test)

**Purposes:**

Use base sequences to study the ggenetic homeostasisof HVT-NDV/F strain.

**Method:**

As for manufacturing origin strains (hereinafter called “MSV”), virus subcultured for 11 generations (subculture with primary chick embryo cells), inserted genes and adjacent regions were proliferated with PCR and the base sequences of amplified products were determined in a dye-terminator cycle sequence method. The primer used for amplification and sequence are confidential and not open to the public.

**Result:**

Base sequences in the subculture levels were prepare by the alignment of overwrapped fragmented sequence. Then, base sequences were alligned in the subculture levels and compared to see whether the base was changed. As a result of comparison, analyzed regions were same in three subculture levels (results were confidential and not open to the public).

**Considerations and Conclusion:**

HVT-NDV/F strain was genetically stable during MSV through the subculture for 11 generations.

## Attachment 13 Stability of Genotype after Subculture of HVT-NDV/F Strain in Chickens (Overseas Test)

**Purposes:**

Study the stability of genotype when HVT-NDV/F strain is subcultured in chickens.

**Method:**

Virus:

Three strains of virus (Samples 2, 7, and 19), which were subcultured for one generation from the master seed virus (hereinafter "MSV+1"), subcultured 8 times in chickens, and re-separated with the primary chick embryo cells and whose plaque was purified, were offered for experiment. The virus subcultured for 2 generations from the master seed virus (hereinafter called MSV+2) was offered for experiment as a control.

Southern blot:

All DNAs were extracted from the primary chick embryo cells that infected the virus. The DNA was absorbed with *Hin*cII or *Xho*I, electrophoresis was conducted with 0.8% agarose gel, and the DNA was transferred to a nitrocellulose film. Labelled 1.2kb fragmentary adjacent to the insertion site of HVT genome with radioactive phosphoric acid （$^{32}$P） and used it as a probe. Detection was exposed to X-ray film. The expected band sizes are 0.8, 1/3, and 3.5 kb in case of *Hin*cII digestion and 0.4 and 3.3 kb in case of *Xho*I. See Fig. 2 of Attachment 11 for the Restriction Enzyme Map.

**Result:**

Samples 2 and 7 showed the same band pattern as MSV+2. The proliferation of virus was found in Sample 19, but the band was not observed.(Results were confidential and not open to the public.)

**Considerations and Conclusion:**

HVT-NDV/F strain genotype is stable after subculture in chickens.

## Attachment 14 Confirmation of the Expression of F Protein of HVT-NDV/F Strain (Overseas Test)

**Purposes:**

Confirm accurate molecular of F protein expressed by HVT-NDV/F strain. At the same time, confirm the stability of expression as protein.

**Method:**

The master seed virusof HVT-NDV/F strain (hereinafter called “MSV”) and the virus subcultured for 5 generations (hereinafter called “MSV+5”) were offered for experiment. As a positive control, chicken kidney cells infected with Newcastle disease virus (hereinafter called “NDV”) and as a negative control, HVT master seed virus (FC126 strain) were used.

The cell dissolution and immunoprecipitation were conducted according to Sieze X Immunoprecipitation Kit (Pierce). For immunoprecipitation, F protein specific monoclonal antibody (57NDV-INT) was used.

As for sedimented protein, electrophoresis was conducted with polyacrylamide and coomassie dyeing was applied for visualization.

**Result:**

A band was found around about 60kDa of the same sizes as a sedimented band from MDV infected cells in MSV and MSV+5 samples, but not in HVT samples. (Results were confidential and not open to the public.)

**Considerations and Conclusion:**

HVT-NDV/F strain expressed the same molecular mass of F protein as NDV. The moscular mass of F protein did not change after subculture for 5 generations from the master seed.

## Attachment 15 Immunogenicity of HVT-NDV/F Strain at Hypodermic Inoculation (Overseas Test)

**Purposes:**

Check the validity if HVT-NDV/F strain is subcutaneously inoculated to 1-day old chickens. For this purpose, a test was conducted with trial vaccines subcultured for the largest generations used for manufacturing (11 generations from the manufacturing origin strain).

**Method:**

Produced 2 types of trial vaccines with different virus contents and measured infection values by using 2nd generation chick embryo cells in culture and HVT and F monoclonal antibody. As shown in Table 1, 206 of 1-day old SPF chickens were groupd and the trial vaccine 0.2 mL was subcutaneously inoculated to the cervical region. Individual test chickens were identified with wing bands and bred in the isolator cage (Table 1).

Challenged IP of 5-day old chickens with virulent Marek's disease virus GA 5 strains and conducted the clinical observation until chickens get 49-day old. All chickens that died along the way were necropsied and lesions of the Marek's disease were observed. At the end of the test, all chickens were necropsied, and lesions of the Marek's disease were observed.

Intramuscularly challenged 28-day old chickens with a strong poison type of Marek's disease virus Texas BG strains and observed them for 2 weeks.

Table 1 Grouping

| Group | Treatment | Marek's disease The No. of challenged chickens | Newcastle disease The No. of challenged chickens |
|---|---|---|---|
| 1 | Trial vaccine 1 | 35 | 32 |
| 2 | Trial vaccine 2 | 35 | 32 |
| 3 | Attack control | 35 | 12 |
| 4 | Non-attack control | 25 | - |

**Result:**

The defense rate against the Marek's disease virus attack was 89 and 97%. The defense rate against the Newcastle disease virus attack was 97 and 100%.

**Considerations and Conclusion:**

The two types of trial vaccines satisfied the efficacy conditions stipulated in Article 9, the Code of Federal Regulations (at least 80 % defense rate for Marek's disease and at least 90 % defense rate for Newcastle disease). If one day old chickens are inoculated subcutaneously, the virus amount for at least trial vaccine 1 is effective for Marek's disease and Newcastle disease.

## Attachment 16 Innovax ND Marker Test

**Purposes:**

The live vaccine Innovax ND contains HVT-NDV/F strain created by introducing F protein genes derived from Newcastle disease vaccine into HVT as an active ingredient. Dye the master seed virusof HVT-NDV/F strain (hereinafter called “MSV”), PB1 strain used for the production, and 3 batches of vaccine product with the Newcastle disease virus F protein specific monoclonal antibody and study the specificity and the sensitivity of F protein detection.

**Method:**

Study the specificity:

Seed the 2nd generation chick embryo cells in culture on an 8-hole chamber slide, inoculate MSV, PB1 strain, or the vaccine to one hole (100 PFU per hole), and subculture the cells at 37 deg C for 2 - 5 days under the existence of $CO_2$ of 5 %. After the confirmation of CPE, immobilize cells with 70 % aceton, dye them with anti-F-protein monoclonal antibody (57NDV-INT) and Goat Anti-Mouse Ig G/FITC and observed with a fluorescence microscope.

Study the Sensitivity:

One ampule of vaccine was dissolved in one bag of dissolving liquid was used as liquid vaccine (0.2 mL for 1 bird). Liquid vaccine was diluted with medium for maintaining cells and used as a sample so that 0.1 mL can be for 1/25 bird. Additionally, performed a two fold dilution of a sample and prepare 1/25-1/1,600 diluted samples. Like a specificity test, inoculated each sample to a chamber slide for fluorescent staining.

The virus content of the liquid vaccine used at the test was measured in a plaque method using primary chick embryo cells.

**Result:**

Study the specificity:

Specific fluorescence was found in HVT-NDV/F strain and vaccinated cells 'Table 1, Photos 1 and 2). Whereas, specific fluorescence was not found in cells inoculated with PB1 stain and non-inoculated cells (Table 1, Photos 3, 4, 5, and 6).

Table 1 Results of Fluorescent Staining of Anti-F Protein Monoclonal Antibody

| Inoculum | Specific fluorescence |
|---|---|
| HVT-NDV/F strain (for manufacturing) | + |
| PB1 strains (parent strain) | － |
| Non-inoculated cell | － |
| Innovax ND　Batch 91790025 | + |
| Innovax ND　Batch 91790030 | + |
| Innovax ND　Batch 91790031 | + |

＋: positive　　－: negative

Study the Sensitivity:

Even if liquid vaccine was diluted by 400 times, specific fluorescence was found (Table 2). At that time, measured viral infection value was 11.3 PFU.

Table 2 Study the Sensitivity:

| Dilution Rate of Liquid Vaccine (Dose/ 0.1 mL) | 1/25 | 1/50 | 1/100 | 1/200 | 1/400 | 1/800 | 1/1600 | Non-inoculation |
|---|---|---|---|---|---|---|---|---|
| A calculated viral infection value (PFU/well) | 245 | 125 | 62.4 | 31.2 | 15.6 | 7.8 | 3.9 | 0 |
| Measured viral infection value (PFU/well) | 180 | 90.3 | 45.1 | 22.5 | 11.3 | 5.6 | 2.8 | |
| Specific fluorescence | + | + | + | + | + | － | － | － |

**Considerations and Conclusion:**

It was confirmed that the dying method performed in the test specifically dyed F protein in the test. It seems that the vaccine strain and other HVT stains could be identified in the dying method.

The detection sensitivity in the dying method was 11.3 PFU per inoculation dose 0.1 mL. It seemed that when at least 12 PFU vaccine virus was contained per analyte 0.1 mL, this could be detected in the dying method.

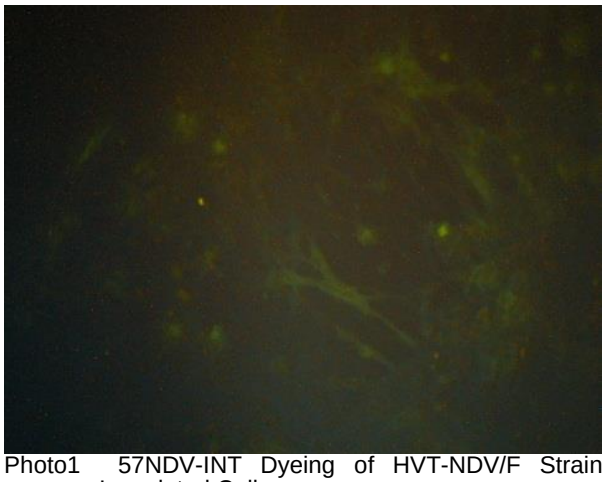


Photo1 57NDV-INT Dyeing of HVT-NDV/F Strain Inoculated Cell
Specific fluorescence was found.

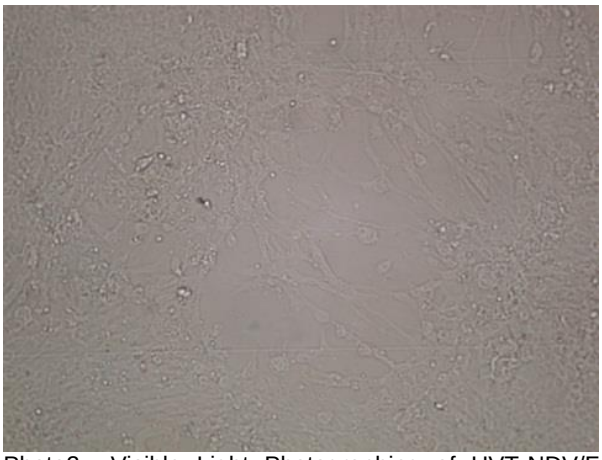


Photo2 Visible Light Photographing of HVT-NDV/F Strain Inoculated Cell
CPE of NVT was found.

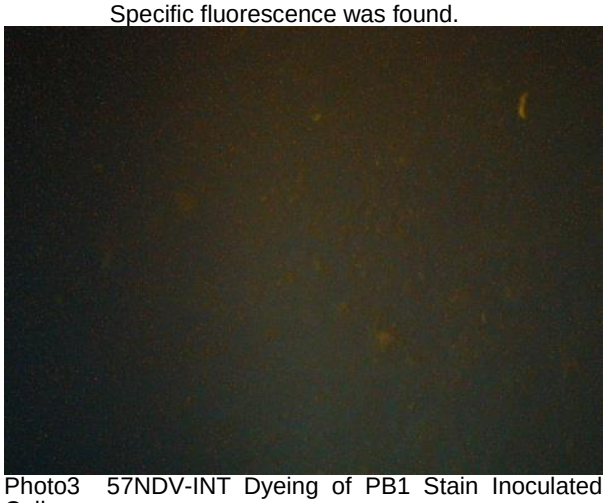


Photo3 57NDV-INT Dyeing of PB1 Stain Inoculated Cell
No specific fluorescence was found.

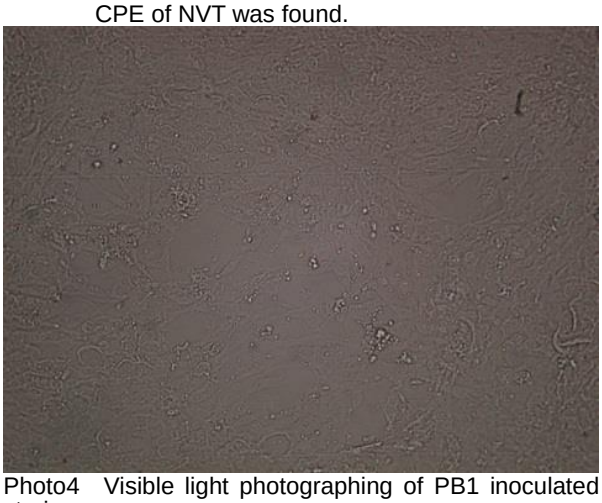


Photo4 Visible light photographing of PB1 inoculated strain
CPE of NVT was found.

Photo5 57NDV-INT Dyeing of Non-Inoculated Cell
No specific fluorescence was found.

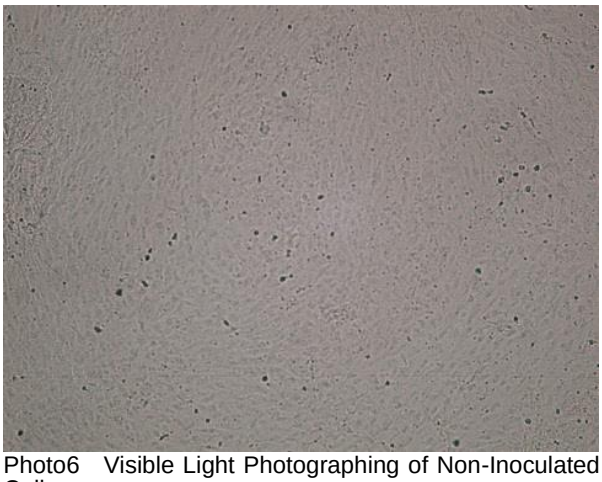


Photo6 Visible Light Photographing of Non-Inoculated Cell
CPE was not found.

## Attachment 17 Safety and Horizontal Infection of HVT-NDV/F strain (Overseas Test)

**Purposes:**

Confirm the safety of the HVT-NDV/F strain's master seed virus(hereinafter called “MSV”). At the same time, confirm the horizontal infection.

**Method:**

Grouped 18-day embryonated eggs per Table 1. For Group A, inoculated MSV of 10 birds/ 0.2 mL MSV, for Group D, dissoluving liquid 0.2 mL, and for other groups, non-inoculated. Observed their hatching rates.

After hatching, bred Group B with Group A cohabitationally and observed them up to 120-day old. Challenged Group G at 1-day old with super-virulent Marek's disease virus (PB1B stain) and observed them up to 50-day old.

At 6-week old, collected blood from 20 birds respectively from Groups A and B and separated the virus. Their leukocyte was separated from blood, inoculated to 2nd generation chick embryo cells in culture, incubated them for 5 days at 37 deg C under the existence of 5 % $CO_2$ to observe CPE.

At the end of the time period observed, measured weights and necropsied them, and Marek's disease lesions were found.

Table 1 Grouping

| Group | Treatment | No. of entered eggs | No. of breeding |
|---|---|---|---|
| A | MSV inoculation at 18-day old embryonated egg | 70 | 60 |
| B | Cohabitation breeding with Group A after hatching | 40 | 30 |
| C | Super-virulent MDV attack at the time of 1-day old | 66 | 56 |
| D | Control (pseudo inoculation) | 60 | 60 |

**Result:**

No abnormality was found in the hatching rate and the probability of survival of Group A inoculated with MAV, and the 120-day old necropsy showed no Marek's disease lesion. (Results were confidential and not open to the public.) All of Group C challenged with the virulent MDV showed Marek's disease lesion by 50-day old. The control Group D did not show any Marek's disease symptoms and lesions.

There are no significant difference between weights of Group A inoculated with MSV and those of the control Group D. In the analysis by sex, no significant difference between weights of Groups A and D was found. (Results were confidential and not open to the public.)

Virus was collected from all inspected individuals of Group A inoculated with MSV, and not from Group B kept cohabitationally (Table 2).

Table 2 Results of Viral Isolation of the Inoculation Group and the Cohabitationally Kept Group

| Group | Results of Viral Isolation |
|---|---|
| A (MSV inoculation) | 20/20 [1)] |
| B (cohabitation with Group A) | 0/20 |

1) Positive number /inspection number

**Considerations and Conclusion:**

Even if MSV 10 dosages of HVT-NDV/F strain was inoculated to embryonated eggs, neither the hatching rate nor the probability of survival was influenced and no lesion was found. In addition, HVT-NDV/F strain was not horizontally infected.

## Attachment 18 Retrieval Results of the Allergen Database on Amino Acid Coded to Donor-Nucleic Acid

**Purposes:**

Retrieve the Allergen Database to study the possibility that an amino acid coded to the donor-nucleic acid may produce harmful substances.

**Method:**

The amino acid sequence expected from 6 reading frames of donor-nucleic acid (it is confidential and not open to the public) is retrieved in the Allergen Database ADFS (http://allergen.nihs.go.jp/ADFS/index.jsp).

**Result:**

Among 6 reading frames, allergen tropomyosin derived from turban shell was retrieved in the third frame. In 44 residuals, 27 % match was recognized and continuously matched residues was 3 or less. Moreover, allergenicity suspected proteins were not retrieved.

**Considerations:**

In the FAO/WHO method, the allergen is positive if the protein is a 35 % or more match with 80 residue units of amino acid or matches the amino acid continuously for 6-8 residues. Therefore, the donor-nucleic acid does not contain proteins similar to the previously known allergen protein.

## Attachment 19 Immunity Continuity of HVT-NDV/F Strain

**Purposes:**

Study the infection persistence and antibody persistence of the vaccine virus until the 2nd year after inoculation of Innovax ND.

**Method:**

Inoculated vaccine for one bird into 30 18-day old embryonated eggs and subcutaneously inoculate 15 1-day old chickens to the cervical region and kept them until 104-week old (Table 1). Blood was collected on the 28th, 40th, 80th, and 104th weeks after inoculation, antibody tests and virus inspections were conducted. An antibody test inspects HVT's FA antibody and ELISA antibody of F protein. In a virus inspection, leukocyte was separated and inoculated into primary chick embryo cells, and at the same time, leukocyte was extracted and perform PCR of HVT. RCP refers to methods including Islam and proliferates Sorf1.

Table 1 Grouping

| Group | Inoculum | Inoculation dose | Virus dose | No. of test-pieces |
|---|---|---|---|---|
| Intraovular inoculation | Vaccine for one bird | 0.05mL | $10^3$PFU/ turkey | 30 |
| Hypodermic inoculation | Vaccine for one bird | 0.2mL | $10^3$PFU/ bird | 15 birds |

After hatching, 15 birds were bred in the intraovular inoculation group. Selected chickens at 7-week old and bred 5 birds per group.

**Result:**

Until 60 weeks after inoculation, all individuals are positive about PCR and viral isolation. In the 80th and 104th weeks after inoculation, three birds were PCR negative and one bird were viral isolation negative. Individually, either PCR or viral isolation was positive, and it was confirmed that the vaccine virus was collected from all individuals. F protein ELISA antibody and FA antibody are positive in all individuals until 28th - 104th weeks (Table 2).

Table 2 Inspection Results

| Group | Test Item | The number of post-inoculation weeks | | | | |
|---|---|---|---|---|---|---|
| | | 28 | 40 | 60 | 80 | 104 |
| Intraovular inoculation | PCR | 5/5 | 5/5 | 5/5 | 4/5 | 4/5 |
| | Viral isolation | 5/5 | 5/5 | 5/5 | 5/5 | 5/5 |
| | F protein ELISA antibody | 5/5 | 5/5 | 5/5 | 5/5 | 5/5 |
| | MD-FA antibody | 5/5 | 5/5 | 5/5 | 5/5 | 5/5 |
| Hypodermic inoculation | PCR | 5/5 | 5/5 | 5/5 | 5/5 | 4/5 |
| | Viral isolation | 5/5 | 5/5 | 5/5 | 4/5 | 5/5 |
| | F protein ELISA antibody | 5/5 | 5/5 | 5/5 | 5/5 | 5/5 |
| | MD-FA antibody | 5/5 | 5/5 | 5/5 | 5/5 | 5/5 |

1) In PCR, 2 reactions were tested per analyte and if one is positive, the analyte is determined as positive.
2) Viral isolation was determined after two backpassages.
3) F protein ELISA antibody shows the number/No. of test-pieces that indicates the extinction coefficient is less than 80 %.
4) FA antibody shows the number/No. of test-pieces is 40 times or more.

**Considerations and Conclusion:**

It was confirmed that the infection of HVT-NDV/F strain continues until 104th week after inoculation.

**Documents for Reference:**

Islam, A. *et al.* 2004. Differential amplification and quantification of Marek's disease virus using real-time polymerase chain reaction. J. Virol. Methods 119, 103-113

## Attachment 20 Study Excretion of HVT-NDV/F Strain under Stress

**Purposes:**

HVT-NDV/F strain horizontally infects a part of the cohabitationally kept group of chickens (Attachment 3). The strain seems to be excreted out of the body. Study whether infective virus is excreted from dander or feather follicle when imposing stress like forced molt through fasting.

**Method:**

Inoculate vaccine for one bird, which was produced from HVT-NDV/F strain to 18-day embryonated egg or 1-day old chicken subcutaneously to the cervical region and breed it until 10-week old. As a positive control that excretes, the group inoculated with CVI988 strain (Marek's disease virus serotype 1) is prepared (Table 1). Collected feather follicles and dander and conducted virus test before/after adding stress. For virus tests, materials were pooled by group, PCR using Islam method and the viral isolation with primary chick embryo cells are conducted. To impose stress, one-week fasting was conducted after 9-week old. However, to prevent death from starvation, about 1/3 of normal feed is given on the 4th day.

Table 1 Grouping

| Group | Injection materials | Inoculation dose | Virus dose | No. of test-pieces |
|---|---|---|---|---|
| Intraovular inoculation | Vaccine for one bird | 0.05mL | 6,244PFU/ turkey | 30 |
| Hypodermic inoculation | Vaccine for one bird | 0.2mL | 6,244PFU/ bird | 10 birds |
| Positive control | MD live vaccine (CVI) for one bird | 0.2mL | At least 1,000 PFU/ bird | 10 birds |

After hatching, 10 birds were bred in the intraovular inoculation group.

**Result:**

If no stress is imposed, feather follicles and danders in all groups are positive, and the virus was isolated from the positive control group, but not from the group inoculated with HVT-NDV/F strain. After imposing stress, no virus was isolated from the group inoculated with HVT-NDV/F strain (Table 2).

Table 2 Results of Virus Inspection before/after Imposing the Stress

| Straight | Group | Feather follicle epithelium | | Dander | |
|---|---|---|---|---|---|
| | | PCR | Viral isolation | PCR | Viral isolation |
| No addition (5-week old) | Intraovular inoculation | + | — | + | — |
| | Hypodermic inoculation | + | — | + | — |
| | Positive control | + | + | + | + |
| No addition (9-week old) | Intraovular inoculation | + | — | + | — |
| | Hypodermic inoculation | + | — | + | — |
| | Positive control | + | + | + | + |
| After addition (10-week old) | Intraovular inoculation | + | — | — | — |
| | Hypodermic inoculation | + | — | + | — |
| | Positive control | + | — | + | + |

If CPE is not accepted, the viral isolation was judged by blind passage for 3 generations.

**Considerations and Conclusion:**

The result is positive in PCR and negative in the vital isolation because the virus associated with cell exists but the particle has not matured. If the virus is isolated from dander, it is judged that excretion was done. Chickens inoculated with HVT-NDV/F strain did not excrete virus at 5-week and 9-week old, and when the stress was imposed, they do not excrete vvirus.

**Documents for Reference:**

Islam, A., *et al.* 2004. Differential amplification and quantification of Marek's disease virus using real-time polymerase chain reaction. J. Virol. Methods 119, 103-113

## Attachment 21 Study Excretion Period when HVT-NDV/F Strain Is Inoculated into Eggs

**Purposes:**

Collect dander and feather follicles from chickens that had intraovular inoculation and study the period when infective virus is detected.

**Method:**

The vaccine for one bird was inoculated into an 18-day old embryonated egg. Bred hatched and control poults for 6 weeks, collected feather follicles and danders from 5 birds of the group every week for virus inspection. For virus tests, materials were pooled by group, PCR using Islam method and the viral isolation with primary chick embryo cells were quantitatively conducted.

**Result:**

The excretion of virus from dander was found in the 2nd, 3rd, and 5th weeks. The two-week old chicken gave the largest excretion amount and then decreased the amount. The viral DNA amount in dander and feather follicles is the highest in the 1st week, and decrease after that (results were confidential and not open to the public).

**Considerations and Conclusion:**

A large amount of viral DNA was detected from 1-week old chicken's feather follicles or dander. The excretion to dander was not recognized because particles matured in feather follicles had not been included in dander at this time.

The virus excretion period is 2-5 week old when an intraovular inoculation of HVT-NDV/F strain was performed to 18-day embryonated egg.

**Documents for Reference:**

Islam, A., *et al.* 2004. Differential amplification and quantification of Marek's disease virus using real-time polymerase chain reaction. J. Virol. Methods 119, 103-113

## Attachment 22 Study the Excretion Period in Subcutaneous Inoculation of HVT-NDV/F Strain

**Purposes:**

Collect dander and feather follicles from chickens that had intraovular inoculation and study the period when infective virus is detected.

**Method:**

Inoculated 1-day chicken subcutaneously to the cervical region with vaccine for one bird or 100 birds. Bred hatched and control poults for 6 weeks, collected feather follicles and danders from 5 birds of the group every week for virus inspection. For virus tests, materials were pooled by group, PCR using Islam method and the viral isolation with primary chick embryo cells were quantitatively conducted.

**Result:**

The excretion of virus from dander was found the 2, 3, and 4 weeks later in case of the hypodermic inoculation group, and 2, 3, and 5 weeks later in case of the hypodermic inoculation. The two-week old chicken gave the largest excretion amount and then decreased the amount. The viral DNA amount in feather follicles is the highest at 2-week old, that in dander at 1-week old and decreases after that (results were confidential and not open to the public).

**Conclusions and Considerations:**

The virus produced in feather follicles is considered to be excreted in dander over time. At 1 week old, the viral DNA amount in dander is larger than that in feather follicles. This is because the feather follicle weight does not only reflect not only the feather follicle epithelium (FFE) but also the wing shaft.

Virus DNA is detected from the feather follicles and dander at 1-week old. Live virus was not collected because at this time, virus associated with cell proliferates and particles have not matured.

In case of hypodermic inoculation to a 1-day old chicken, the virus excretion period seems 2-4 week old.

**Documents for Reference:**

Islam, A., *et al.* 2004. Differential amplification and quantification of Marek's disease virus using real-time polymerase chain reaction. J. Virol. Methods 119, 103-113

## Attachment23 Vertical Infection of HVT-NDV/F Strain

**Purposes:**

Collect sperm eggs from breeding hens that were inoculated intraocularly or hypodermically with HVT-NDV/F strain, and the virus was detected from eggs, embryonated egg, and poults.

**Method:**

Raising breeding hens

Vaccine for one bird was inoculated into an egg or hypodermicly inoculated into 1-day chicken, and raised poults for 28 weeks (Table 1). Eggs collected during 19-28 weeks were tested. In addition, all test chickens were checked to see whether they were infected by HVT-NDV/F strain before selecting an intraovular inoculation group and after the completion of test.

Table 1 Breeding Hen

| Group | Inoculum | Infection value | Inoculation dose | No. of test-pieces |
|---|---|---|---|---|
| Intraovular inoculation | Vaccine for one bird | 6244PFU | 0.05mL | 30 |
| Hypodermic inoculation | Vaccine for one bird | 6244PFU | 0.2mL | 15 birds |

Select chickens at 42-day old and breed 1 male and 4 females per group.

Egg test:

After 5 adding salmon sperm DNA to egg yolk and egg albumen of 5 eggs of each group as carriers, extract DNA, and perform PCR to detect SORF1 of HVT by using Islam and other methods.

Embryonated Egg Test:

Collect embryos from 5 embryonated eggs from each group, remove the head, create emulsifiable concentrate, inoculate primary chick embryo cells, and isolate virus. In addition, extract DNA from emulsifiable concentrate and perform PCR to detect HVT.

Poult Test:

Breed 5 poults derived from breeding hens until 10-day old, collect blood, kidney, and spleen. Separate leukocyte from blood and perform PCR to isolate virus and detect HVT. Pool kidneys and spleens by the group, create emulsifiable concentrate, and perform PCR to isolate virus and detect HVT.

**Result:**

Egg test:

Perform PCR so that the detected virus is negative from an egg derived from a breeding hen either intraocularly or hypodermically inoculated (Table 2).

Table 2 Virus Detected from Eggs

| Egg origin | RCR results[1)] |
|---|---|
| Intraocularly inoculated breeding hen | 0/5 |
| Hypodermically inoculated breeding hen | 0/5 |

1) Positive number /inspection number

Embryonated Egg Test:

HVT was not detected from either group of embryonated egg(Table 3).

Table 3 Virus Detected from Embryonated Egg (11-day Old)

| Derived fromchick embryo | RCR results[1)] | Results of Viral Isolation[1)] |
|---|---|---|
| Intraocularly inoculated breeding hen | 0/5 | 0/5 |
| Hypodermically inoculated breeding hen | 0/5 | 0/5 |

1) Positive number /inspection number

Poult Test:

HVT was not detected from either group of poults (Table 4). In addition, no clinical abnormality was found during breeding poults, and no abnormality was found during the breeding of poults.

Table 4 Virus Detected from Poults (10-day Old)

| Origin of Poult | Materials | PCR | Viral isolation |
|---|---|---|---|
| Intraocularly inoculated breeding hen | Blood | 0/5 | 0/5 |
| | Spleen | 0/1 | 0/1 |
| | Kidney | 0/1 | 0/1 |
| Hypodermically inoculated breeding hen | Blood | 0/5 | 0/5 |
| | Spleen | 0/1 | 0/1 |
| | Kidney | 0/1 | 0/1 |

1) Positive number /inspection number

**Considerations and Conclusion:**

It was confirmed that eggs used at the test were born from breeding hens infected with HVT-NDV/F strain. The detected from egg in PCR was negative. At the prior examination, 100 PFU per egg could be detected. Poults are inspected at 10-day old. It is reported that if HVT 1-5 PFU is inoculated to day old chicks, more than half individuals suffer from viremia 1 week later (Churchill, et al.). In case of vertical infection, the timing is detectable.

From the above, there is very little possibility for a test virus to vertically infected.

**Documents for Reference:**

Islam, A., *et al.* 2004. Differential amplification and quantification of Marek's disease virus using real-time polymerase chain reaction. J. Virol. Methods 119, 103-113

Churchill, A. E., *et al.* 1973. Viremia and antibody development in chicks following the administration of Turkey Herpesvirus. Vet. Rec. 92, 327-334

**Attachment 24 Study DNA Extraction Procedure from Chicken Egg**

**Purposes:**

To study the vertical infection of HVT-NDV/F strain, it is necessary to extract virai DNA from eggs and inspection should be conducted according to PCR. The test adds a various amount of vaccine virus to chicken eggs, extract NDA, and solve for DNA detected limit from an egg.

**Method:**

Results were confidential and not open to the public.

**Result:**

Results were confidential and not open to the public.

**Considerations and Conclusion:**

The detection limit of vaccine virus in this method is 100 PFU per egg.

## Attachment25 HVT-NDV/F Strain Detected from Fece of Meat-type Chickens Inoculated with Vaccine (Overseas Test)

**Purposes:**

Study whether HVT-NDV/F strain is excreted in fece of a chicken inoculated with HVT-NDV/F strain.

**Method:**

Inoculated a dose of HVT-NDV/F strain to 18-day old meat-type chickens. Provided eye-drop vaccination of Newcastle disease to newly hatched, 1-day old chickens. Test chickens were kept inan isolater. Collected fece on the 4th, 5th, 6th, and 7th week after inoculation, and isolated virus by using primary chick embryo cells from supernatant water and precipitate after removing feces.

**Result:**

No infectious virus was detected from feces sample at all inspection times (Table 1).

Table 1 Results of Viral Isolation

| Sample | The number of post-inoculation weeks | | | | |
|---|---|---|---|---|---|
| | 3 | 4 | 5 | 6 | 7 |
| Supernatant | － | － | － | × | － |
| Precipitation | － | － | － | － | － |

－: Negative ×: cannot evaluate due to contamination

**Considerations and Conclusion:**

No infectivity virus was detected from fece samples of meat-type chickens inoculated with vaccine.

## Attachment 26 Survival Test of HVT-NDV/F Strain in the Environmental Conditions for the Natural World

**Purposes:**

Studied the survival capability of HVT-NDV/F strain in mud and rain water, blood clot, and serum,

**Method:**

Mix about $2\times10^6$PFU of the vaccine virus of HVT-NDV/F strain with sterilized mud water (±10%), rainwater, chicken blood or chicken that has just been collected, and stand it for 4 hours to 7 days at the room temperature. Removed supernatant by centrifugal separation (2,500rpm and 5-minute) and floated it to the medium for cell culture (MEM), inoculated it to primary chick embryo cells, cultured them under the existence of 5%$CO_2$ at 37 deg C for 10 days and observed CPE. As a control, inoculated the virus associated with cell standing in PBS and newly fused virus associated with cell and observed CPEs.

**Result:**

After HVT-NDV/F strain stands for 1 day in serum (24 hours), 4 days in clotted blood, and 7 days in mud/rain water, and PBS, it has lost the infectivity. For the virus control, observed CPEs during all inoculation (Table 1).

Table 1 Results of Survival Tests of HVT-NDV/F Strain (Virus Associated with Cell) in the Environment

| Test condition | Treatment times | | | | |
|---|---|---|---|---|---|
| | 4 hours | For 1 days | For 2 days | For 4 days | For 7 days |
| Muddy water | + | + | + | + | — |
| Rainwater | + | + | + | + | — |
| Clotted blood | + | + | + | — | — |
| Serum | + | — | — | — | — |
| Positive control (PBS) | + | + | + | + | — |
| Non-inoculation control | — | — | — | — | — |
| Viral control | | + | + | + | + |

+: CPE positive —: CPE negative

**Considerations and Conclusion:**

The recombinant virus HVT-NDV/F strain cannot survive 1 week or more in rain water, mud water, clotted blood, and serum.

**Attachment27 Stability of HVT-NDV/F Strain in the Vaccination Environment (Overseas Test)**

**Purposes:**

Study the survival capability of HVT-NDV/F strain vaccine in the extracorporeal environment.

**Method:**

Melted 1 ample of vaccine, dissolve it into 200mL dissolving liquid and dispensed it, left to stand at the room temperature, avoiding direct sunlight. After standing still, a virus was inoculated into 2nd generation chick embryo cells in culture to observe plaques. As a control, measured the vaccine infection values of the vaccine immediately after dissolution and those of HVT (FC 126 stains)

**Result:**

On the 2nd day after standing, the number of plaque remarkably decreased and the plaque could not be detected 7 days later (Table 1).

Table 1 The Number of Vaccine Plaque Standing Still at Room Temperature

| Vaccine | The number of days after dissolution | | | |
|---|---|---|---|---|
| | At the time the employee starts working | One day later | Two days later | Seven days later |
| HVT-NDV/F strain | 126 | 29 | 7 | 0 |
| HVT（FC126） | 123 | 46 | 18 | 0 |
| Soon after dissolution | — | 65 | 89 | 87 |

**Considerations and Conclusion:**

HVT-NDV/F strain cannot live more than 1 week in the vaccination environment.

## Article 28 Study of the Viability of HVT-NDV/F Strain Excreted in Dander

**Purposes:**

Collect dander from a poult inoculated with HVT-NDV/F strain. On the date of collection, and 1st, 7th, and 28th day after collection, determine the quantity of infectious virus and study the survival period of the virus excreted in dander.

**Method:**

Inoculated 50 1-day old chicken subcutaneously to the cervical region with vaccine for 100 chickens, and collected their dander when they became 2-week old. Measured the weight of dander and dispensed it to test tubes, preserved it at 4 deg C and at room temperature, extracted cell free virus 1, 7, and 28 days later, measured infection values by using 2nd generation chick embryo cells in culture, and determined the quantity of DNA of HVT through the realtime PCR by using the method of Islams'.

**Result:**

If dander is preserved at 4 deg C, infection value per 10 mg dander decreased until the 7th day and the infectivity remained on the 28th day.

In case of normal temperature preservation, little change was observed on 1st day, but the value decreased on 7th day and was undetectable on 28th day.

DNA amount per dander 10 mg changed among samples, but infection values per DNA amount decreased with time under any condition.

**Considerations and Conclusion:**

If HVT-NDV/F strain eliminated from dander is preserved at 4 deg C, its infectivity remains on the 28th day. In addition, it lost effect by the 28th day at room temperature.

HVT-NDV/F strain seemed to keep infectivity for extended periods under the environment where a low-temperature state was kept. In addition, the strain lost effect at room temperature within about 4 weeks from excretion.

**Documents for Reference:**

Islam, A., *et al.* 2004. Differential amplification and quantification of Marek’s disease virus using real-time polymerase chain reaction. J. Virol. Methods 119, 103-113

**Attachment 29 Inactivate H-VTNDV/F Strains by Disinfectant**

**Purposes:**

Study the inactivation by HVT-NDV/F strain disinfectant (alcohol, chlorine, invert soap, and lime hydrate).

**Method:**

HVT-NDV/F strain vaccine was dissolved, and solutions of alcohol (70 % ethanol), chloride (sodium hypochlorite 100 ppm), invert soap (benzalkonium chloride 200 ppm), or lime hydrate (0.1 % calcium chloride) were added to 2×106 PFU virus. After standing at room temperature for 10 minutes to 4 hours, collected virus-infected cell by centrifugal, inoculated to primary chick embryo cells to isolate virus.

**Result:**

CPE was not found 10 minutes later in case of invert soap and 30 minutes later in case of alcohol and lime hydrate. In case of chlorine, CPE was found 30 minutes later but not 4 hours later. In case of PBS, or control, CPE was found both after 30 minutes later and 4 hours later (Table 1).

Table 1 Results of Survival Tests of HVT-NDV/F strain (Virus Associated with Cell) in Disinfectant

| Disinfectant | Treatment times | | |
|---|---|---|---|
| | 10 minutes later | 30 minutes later | 4 hours later |
| Alcohol | | － | － |
| Chloride | | + | － |
| Invert soap | － | | － |
| Lime hydrate | | － | － |
| Control (PBS) | | + | + |

+: CPE positive －: CPE negative

**Considerations and Conclusion:**

HVT-NDV/F strain was inactivated by any disinfectant. The time necessary for inactivate is 10 minutes for invert soap, 30 minutes for alcohol and lime hydrate, and 4 hours for chloride.

## Attachment 30 Inactivate HVT-NDV/F Strain by Artificial Gastric Juice

**Purposes:**

Confirm if a virus gets inactive by gastric juice when you eat vaccine virus remained in chickens.

**Method:**

The artificial gastric juice is aqueous solution of 0.2 % sodium chloride, 0.7 % hydrochloric acid, and 0.6 % pepsin derived from pig. Added artificial gastric juice, 10-time diluted artificial gastric juice or PBS to about $3.5\times10^6$PFU vaccination virus of HVT-NDV/F strain and shake it at 37 deg C. After shaking, collected virus associated with cell from the solution, inoculated the virus to cultured primary chick embryo cells for viral isolation.

**Result:**

The vaccine virus treated in 10-time diluted artificial gastric juice lost the activity after 30 minutes. In case of the vaccine virus treated with PBS, CPB was observed in the chick embryo second generation cells inoculated with cells infected with HVT-NDV/F strain.

Table 1 Results of Survival Test of HVT-NDV/F Strain (Virus Associated with Cell) in Artificial Gastric Juice

| Test condition | Treatment times | |
|---|---|---|
| | 0.5 hours | 4 hours |
| Artificial gastric juice (pH1.2) | — | — |
| 1/10 artificial gastric juice (pH2.2) | — | — |
| Control (PBS and pH7.3) | + | + |

**Considerations and Conclusion:**

HVT-NDV/F strain exposed to the artificial gastric juice was inactivated at least 30 minutes later. It seems that if the vaccine strains remained in chicken meat are eaten, HVT-NDV/F strain is inactivated. In addition, if an inoculated chicken was preyed by a wild animal, the vaccine virus was thought to be inactivated.

**Attachment 31 The Information on the Product**

| Scheduled Trade Designation (Provisional) | |
|---|---|
| Nobilis　Inno-Fusion ND | |
| Name of the marketing business | Intervet K.K. |
| Formulation classification | Biological preparations |
| Dosage forms | Frozen　Raw |

| Principal ingredient |
|---|
| Turkey Herpesvirus HVT-NDV/F strain with F protein genes derived from Newcastle disease transferred　$10^{7.1}$PFU or more/ ample (1.8mL for 4,000 birds) |

| Route of administration | |
|---|---|
| 1 | Hypodermic needle |
| 2 | Egg injection |

| Target animals |
|---|
| Chickens |
| Indication |
| Prevention of chickens' Marek's and Newcastle diseases |
| Scheduled usage and dosage |
| 1. Hypodermic inoculation to cervical region<br>Readily melt frozen vaccine, dissolve it with the solvent for dissolving frozen vaccine “Nobilis Diluent CA (pending formation)” so that is can be 0.2 mL per bird and inoculate a day old chick subcutaneously to the cervical region with 0.2 mL.<br><br>2. Inoculation into embryonated egg<br>Readily melt the frozen vaccine, dissolve it with the solvent for dissolving frozen vaccine “Nobilis Diluent CA” so that is can be 0.05 mL per bird and inoculate an 18-day egg with 0.05 mL by using an automatic intraovular inoculation machine. |

| Scheduled Trade Designation (Provisional) | |
|---|---|
| Nobilis　Inno-Fusion ND-SB | |
| Name of the marketing business | Intervet K.K. |
| Formulation classification | Biological preparations |
| Dosage forms | Frozen　Raw |

| Principal ingredient |
|---|
| Turkey Herpesvirus HVT-NDV/F strain with F protein genes derived from Newcastle disease transferred　$10^{6.8}$PFU or more/ ample (1.8mL for 4,000 birds) |
| Non-tumor primary Marek's disease virus　SB-1 strains $10^{6.8}$PFU or more/ample (1.8mL for 4000 birds) |

| Route of administration | |
|---|---|
| 1 | Hypodermic needle |
| 2 | Egg injection |

| Target animals |
|---|
| Chickens |
| Indication |
| Prevention of chickens' Marek's and Newcastle diseases |
| Scheduled usage and dosage |
| 1. Hypodermic inoculation to cervical region<br>Readily melt frozen vaccine, dissolve it with the solvent for dissolving frozen vaccine “Nobilis Diluent CA (pending formation)” so that is can be 0.2 mL per bird and inoculate a day old chick subcutaneously to the cervical region with 0.2 mL.<br><br>2. Inoculation into embryonated egg<br>Readily melt the frozen vaccine, dissolve it with the solvent for dissolving frozen vaccine “Nobilis Diluent CA” so that is can be 0.05 mL per bird and inoculate an 18-day egg with 0.05 mL by using an automatic intraovular inoculation machine. |

**Attachment 32 Horizontal Infection of HVT-NDV/F Strain to Birds (Overseas Test)**

**Purposes:**

Study excretion from chickens inoculated with HVT-NDV/F strain, the existence or absence of horizontal infection with chickens, pigeons, ducks, and turkeys cohabitationally kept with inoculated chickens.

**Method:**

Inoculated $10^{4.1}$PFU of HVT-NDV/F strain to twenty of 1-day SPF chickens. Bred 10 noninoculated SPF chickens, 10 pigeons, 15 turkeys, and 10 ducks in separate cages in the same room. Collected blood from all animals, isolated peripheral blood leukocyte, and inoculated to primary chick embryo cells, and isolated virus 4, 5, 6, 7, and 8 weeks after inoculation.

**Result:**

All chickens directly inoculated with HVT-NDV/F strain suffered from viremia, but no viremia was found in cohabiting chickens, pigeons, and ducks at any time of investigation. For the duration of the test, 11 out of 15 turkeys suffered from viremia caused by HVT-NDV/F strain (Table 1).

Table 1 Results in Virus Collection from Leukocyte

| Group | The number of post-inoculation weeks | | | | |
|---|---|---|---|---|---|
| | 4 | 5 | 6 | 7 | 8 |
| Inoculated chickens | 19/19 | 20/20 | 19/19 | 20/20 | 19/19 |
| Cohabiting chickens | 0/10 | 0/10 | 0/10 | 0/10 | 0/10 |
| Cohabiting pigeons | 0/10 | 0/10 | 0/10 | 0/10 | 0/9 |
| Cohabiting ducks | 0/10 | 0/10 | 0/10 | 0/10 | 0/10 |
| Cohabiting turkey | 0/14 | 4/11 | 4/13 | 2/13 | 11/13 |

Positive number /inspection number

**Considerations and Conclusion:**

Inoculated chickens and chickens, pigeons, or ducks in cohabitation breeding are not horizontally infected with HVT-NDV/F strain. However, cohabiting turkeys are not horizontally infected.

## Attachment 33 Risk Evaluation Statement of HVT-NDV/F Strain in the U.S. (Department of Agriculture (USDA) Authorization Code: 17H1.R2）

### 1. Background

Newcastle disease virus (NDV) is classified into Family Paramyxoviridae, Subfamily Paramyxovirinae, and Genus Rubulavirus. Many viruses in the genus infected birds through inhalation and feeding (Alexander, 1997). Newcastle disease is a poultry disease caused by NDV, highly pathogenic and economically significant. Many known NDV strains have very different pathogenicities: 1) asymptomatic-enteric, 2) lentogenic (nonvirulent), 3) imesogenic (intermediate virulence), and 4) velogenic (highly virulent). Lentogenic (nonvirulent) or imesogenic (intermediate virulence) are used as NDV live vaccines

In the U.S., several types of nonvirulent live vaccines can be used to prevent Newcastle disease (ND). Nonvirulent La Sota strain and B1 strain are often used for vaccines. Still, nonvirulent live vaccines may have side effects depending on vaccine strains, environmental conditions, and the presence of complex infections. In addition, some ND live vaccines have problems: some NDV maternal antibodies may prevent vaccine-associated protection.

The herpesvirus of turkey (HVT) is categorized as Herpesviridae, Alphaherpesvirinae and used as Hetero type vaccine outdoors. Nonpathogenic HTV is categorized as Marek's disease virus serotype 3 (MDV3), the most common vaccine. HVT is not pathogenic to chickens and the risk of spreading in the environment is lower than Mark's disease virs types 1 and 2. Therefore, HVT is useful as live virus vector to exogenous antigen into vaccinated animals (Sondermiejer et al., 1993).

To protect against Marek's disease, a large-scale vaccination for day old chicks was conducted by using HVT live vaccine. The wide use of vaccine remarkably reduced the outbreak of Marek's disease, but sometimes it occurs in spite of the use of vaccine.

Intervet Inc. developed a recombinant live vaccine by integrating NDV nonvirulent genes into HVT so that one vaccination may help young chickens produce vaccine to protect MDV and NDV. It is essential to induce systemic immunization to prevent highly virulent ND. As existing vaccines find it difficult to achieve this, Intervet looked for the possible of the recombinant live vaccine, considering it may be most suitable. MDV type 2 (SB-1 stain) was added to prevent highly virulent Marek's disease. The USDA code number of Marek's Disease-Newcastle Disease Vaccine, Serotypes 2, Live Virus, Live Marek's Disease Vector is17H1.R2.

### 2. Concern Area

Animal and Plant Health Inspection Service (APHIS) of USDA has a concern over 3 areas: 1) the animal safety, 2) public health safety, and 3) environmental safety. Intervet Inc. evaluated risks in connection with a notification to conduct a clinical trial of the vaccine under development. The following conclusions are based on risk assessments of areas.

### 2.1 Safety for Animals

We evaluated safety characteristics of the live vaccine including HVT-NDV/F strain (USDA code: 17H1.R2). HVT-NDV/F strain has low risk to animal safety. As soon as receiving the risk evaluation, we asked a permission to conduct a product's clinical safety test.

To confirm the safety of the vaccine, the master seed viruswas inoculated to 60 18-day old SPF embryonated eggs. The inoculation dose is for 10 or more birds. No abnormality was found in the hatching rate and the probability of survival. All poults survived for 5 days from hatching. Then, they were bred until 120-day old, and none of them exhibit Marek's disease and ND lesions. Noninoculated cohabiting chickens did not exhibit lesion of Marek's disease. Challenging nonvaccinated chickens with highly virulent Marek's disease virus RB1B stain, we found all of them exhibited Marek's disease lesions. For this reason, tested chickens are confirmed to be susceptible to Marek's disease. The non-challenged control group did not exhibit Marek's disease lesions. There were no significant difference between weights of the vaccinated group and the control group. The analysis of weights by sex resulted in the same.

The safety of this vaccine was studied in the effectiveness test. The group of intraovular inoculation (inoculated to 18-day embryonated egg), the group of hypodermical inoculation with HVT plain vaccine, the noninoculated group , and the group challenged with MD and ND

were respectively challenged with RB1B at 5-day old and with ND virus Texas GB stain at 28-day old. As a result, no abnormality was found in the hatching rate and the probability of survival. It was revealed that the vaccination of 18-day old embryonated egg protected chickens from highly virulent Marek's disease virus and highly virulent Newcastle disease.

HVT is nonpathogenic to chickens. The viruses existing on the feather follicle epithelium are limited. Therefore, the infection through excretion and between chickens are limited. (Cho, 1975; Zygraich and Huygelen, 1972). Unlike Marek's disease virus serotypes 1 and 2, serotype 3 (HVT) is replicated on the feather follicle epithelium only for a while (Zygraich and Huygelen, 1972). The safety on the diffusion from vaccinated chickens to the nonvaccinated was evaluated. 18-day SPF embryonated eggs were inoculated with HVT-NDV/F strains for 10 chickens, and after hatching, they cohabited with the noninoculated. In the 6th week after inoculation, blood was withdrawn from 20 inoculated chickens and 20 noninoculated, and leukocyte was isolated for inspection. All inoculated chickens suffered from HVT viremia, but the disease did not diffuse to the noninoculated. No lesion was found in them. From the above, the inoculation of HVT-NDV/F strains to chickens did not cause any safety problem.

The diffusion of vaccine virus was studied for the case where 10-bird vaccine was inoculated to chickens. The vaccinated chickens cohabited with nonvaccinated chickens, pigeons, turkeys, and ducks in the same room. Blood was drawn, leukocyte was separated, and virus was isolated 4, 5, 6, 7, and 8 weeks later. the virus was isolated only from inoculated chickens and turkeys cohabiting with them. That means recombinant virus does not diffuse to cohabiting chickens, pigeons, and ducks. However, if inoculated chickens cohabit with turkeys, the diffusion occurs.

As there is a concern: the insertion of NDV genes into HVT may change the affinity of HVT, the tissue affinity of HVT-NDT/F stain was evaluated.

A high dose of recombinant virus or HVT to be used to produce recombinant immunized chickens and virus was collected from varous organs. On the 3rd, 7th, 10th, 14th, 17th, and 21st day after inoculation, three chickens from each group were euthanized, and the virus inspection was conducted on liver, spleen, kidney, ovary/testis, lung, thymus, bursa Fabricii, peripheral blood leukocyte, feather follicle, mouthwash, and cloaca swab. In all groups, it was lymphatic tissues where the virus was detected for the first time on the 3rd day after inoculation (bursa Fabricii, thymus, and spleen). On 14th day after inoculation, virus was separated from almost all organs except mouthwash and cloaca swab. At any time, no virus was detected from mouthwash and cloaca swab. From the above, no difference between the recombinant and HVT in the biodistribution of target animals.

Other birds, namely, turkeys, pigeons, and ducks were inoculated with 10 birds of manufacturing origin strains and studied the safety of non-target animals A high dose of PB1 strains of HVT was inoculated to another group: turkeys, pigeons, and ducks. Based on bibliographic information, it is expected that HVT, which is a virus separated from turkey, is replicated in turkeys (Witter et al., 1970).

Thje result was the recombinant HVT-NDV/F strain was not replicated in pigeons and ducks. Similarly, PB1 strain used for production was not replicated in pigeons and ducks. Based on these results, it was confirmed that the range of animals susceptible to the master seed virusof HVT-NDV/F strain was similar to PB1 strain used for the production. Therefore, it is not a safety risk for other birds to use HVT－NDV/F strain for chickens.

We studied the replication in the recombinant rabbit. The recombinant virus for one chicken was inoculated to eyes of two rabbits. At the same time, other two rabbits were inoculated with PB1 strain. AS a result, neither virus caused clinical manifestations and conjunctivitis to rabbits. Furthermore, viremia was not caused. Neither recombinant nor PB1 strain are replicated in the rabbit, a non-target animal.

The genotype and the phenotype of HVT-NDV/F strain were confirmed to be stable during the backpassage for 5 generation in chickens to confirm the reversion of pathogenicity. It was confirmed through the fluorescent antibody method that NDV genes express manufacturing origin strain, strains subcultured for 11 generations, and backpassage strains for 5 generations. Similarly, it was confirmed through the Western blotting technique that during the time, genes are stable without deletion and rearrangement.

**2.1 Public Health Safety**

It is considered that there is no risk on the public health safety. There is no report on HVT infection on species other than chickens and turkeys. The virus is replicated in chickens and turkeys but does not cause clinical symptoms. The infection of human beings has not been reported. The strain used for producing a vaccine under development has been used as a vaccine for chickens since 1972 and there is no risk of side effects and public health. The exposure to human beings is limited to workers engaging in vaccine inoculation and observers on the post-inoculation. There is no report that HVT vaccine causes the infection on human beings.

**2.2 Safety for Environment**

The risk of safety for the environment is low. The ability of recombinant vaccine to be excreted and diffused is limited. The range of animals susceptible to vaccine under development has not changed from HVT used for production and is limited to birds (chickens and turkeys). HVT exists mainly in the place where chickens and turkeys are used. It seems the safety aspects of vaccine under development towards non-target animals have not changed since the commercially available vaccine strains, which were used for production. The distribution of HVT vaccine strains has not been reported, and there is no side effects caused by the outside use of this vaccine. It seems that the implementation of clinical tests of the vaccine under development has no adverse effect on the environment.

To confirm the safety toward the environment, we examined whether HVT-NDV/F strain would survive in the susceptible animal's extracorporeal environment. One vial of vaccine was melted and dissolved in the solvent, dispense it, and stand it still for 1, 2, and 7 days. As a result, HVT-NDV/F strain cannot survive 1 week or more in the environment. Therefore, the viability of HVT-NDV/F strain is low, there is little chance of diffusion, and there is no safety risk.

The horizontal transmission of nuclear acid has no connection with HVT. The possibility of recombination is reported on Aujeszky virus, vaccine strains of other herpes virus (Henderson et al., 1990, 1991; Katz et al., 1990). However, these researches are experimental: 2 types of stock viruses with high infection values are mixed and injected into animals by a syringe. Under such circumstances, vaccine strains lead to various gene deletions. It is reported that in a cell culture, MDV1 infects latent effected established cells with MDV2, so that a recombinant virus was produced (Hrai et al., 1990). But as this phenomenon has not been reproduced, the ability to induce recombinant is quite low.

When a recombinant occurs between 2 vaccine strains, two viruses exit : the original and recombinant strains. No alteration was made on HVT, the backbone of the recombinant, for gene deletion. It seems that the ability of recombinant with wild HVT and MDC has not been changed since the conventional HVT live vaccine. Polyvalent vaccines including Marek(s virus senotypes 2 and 3 have been used for 10 years outside, and there is no report that any recombination has occurred between the viruses.

The recombinant ability is not likely to be enhanced by inserting MDV F gene. It seems that Neither NDV nor HVT can transmit genes.

The ability to horizontally transmit or recombine genes of vaccine that uses HVT as vector is extraordinally low.

**Table 3 Risk Examination**

The risk examination identifies risks, integrates the evaluation, and consists of 4 items: 1) the probability of adverse events, 2) results of occurrence of adverse events, 3) risk evaluation, and 4) discussion on evaluation. The evaluation of probability and results is modified by the reliability of evaluation including evaluation validity. The evaluation validity consists of the corresponding section identifying hazards, and calculation and evaluation of risks.

Probability evaluation
Low: It is unlikely that any adverse event occurs.
Intermediate: An adverse event may occur.
High: An adverse event is very likely to occur.

Evaluate the result

Low: Results of the occurrence of adverse event are serious. For example, adverse events are self-limited and effects may be negligible.
Intermediate: The occurrence of adverse events are intermediately serious. For example, effects of adverse events are irreversible but there is a coping process.
High: The occurrence of adverse events are serious. For example, effects of adverse events are irreversible and there is no coping process.

Evaluation certainty
Certain: The evaluation is supported by a direct scientific evidence.
Intermediately certain: The evaluation is supported by an indirect scientific evidence.
Uncertain: The evaluation is not supported by any scientific evidence.

Numeric values are assigned to an incidence, evidence, and evaluation certainty (Table 1). Every evaluation by numeric value is based on the importance of evaluation in the region. Assigned numeric values show the significance of potential risk seriousness. To determine the scale of potential risk, numeric value shoul be multiplied.

Table 1 Risk Calculation Method

| |
|---|
| Incidence<br>Low (L) LL = 1.00<br>Middle (M) LM = 0.50<br>High (H) LH = 0.10 |
| Result<br>Low (L) CL = 1.00<br>Middle (M) CM = 0.10<br>High (H) CH = 0.01<br><br>If the incidence is intermediate or high and results are intermediate or high, use the certainty evaluation. In other combination, use the certainty evaluation II. |
| Certainty evaluation I<br>Certain (C) C = 0.50<br>Intermediately certain (MC) MC = 0.75<br>Uncertain (U) U = 1.00 |
| Certainty evaluation II<br>Certainty (C) C = 1.00<br>Intermediately certain (MC) MC = 0.75<br>Uncertain (U) U = 0.50 |
| Expected risk<br>Expected risk = [incidence x certainty] x [result x certainty] |

Risk assessment
Low: Acceptable risk (Expected risks are 0.5000 - 1.0000)
There are few concerns on application. (There is no validity to reject the application.)
Intermediate: Acceptable risk (Expected risks are 0.0025 - 0.3750)
Concerns on application is intermediate. (Mitigation measures are taken or the application is rejected)
High: Acceptable risk (Expected risks are 0.0003 - 0.0019)
Concerns on application are significant. (The application will be rejected.)

**3.1 Safety for Animals**

Evaluation on the incidence of adverse events Low
Evaluation certainty: Certain
Evaluation on results of occurrence of adverse events: Low
Evaluation certainty: Certain
Risk examination: LL.C.CL.C （[1.00×1.00]×[1.00×1.00]）

Expected risk: 1.0000
Risk assessment: Low
Validity of evaluation 2.1 Safety for Animals

**3.2 Public Health Safety**

Evaluation on the incidence of adverse events Low
Evaluation certainty: Certain
Evaluation on results of occurrence of adverse events: Low
Evaluation certainty: Intermediately certain
Risk examination: LL.C.CL.MC ([1.00×1.00]×[1.00×0.75])
Expected risk: 0.7500
Risk assessment: Low
Validity of evaluation 2.2 Public Health Safety

**3.3 Safety for Environment**

Evaluation on the incidence of adverse events Low
Evaluation certainty: Certain
Evaluation on results of occurrence of adverse events: Low
Evaluation certainty: Intermediately certain
Risk examination: LL.C.CL.MC （[1.00×1.00]×[1.00×0.75]）
Expected risk: 0.7500
Risk assessment: Low
Validity of evaluation 3.2 Safety for Environment

**4. Conclusion**

Based on test results to analyze risks, Intervet Inc. concludes the vaccine combining HVT-NDV/F strain, a recombinant virus, and SB-1 has lower risks on the animal safety, public health safety, and the environmental safety. Marek's/Newcastle disease vaccine (code No.: 17H1.R2) was confirmed to be safe in using chickens. We ask the permission to conduct a clinical safety test for this vaccine by applying this risk assessment to Animal and Plant Health Inspection Service (APHIS).

Documents for Reference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**Attachment34 Risk Evaluation Statement of HVT-NDV/F Strain in Canada**
**(USDA Authorization Code: 17H1.R2)**

**Abstract**

Newcastle /Marek's disease (Marek's disease virus Type 2, HVT vector) live vaccine is a HVT live vaccine where Marek's disease virus Type 2 and genes derived from Newcastle disease virus are introduced. The vaccine is used for intraovular inoculation (inoculated to 18-day embryonated egg) to prevent chickens from suffering from super-virulent Marek's disease and Newcastle disease.

The vaccine was evaluated by the Canadian Centre for Veterinary Biologics (CCVB) of the Canadian Food Inspection Agency (CFIA). The environmental impact assessment was conducted as a part of requirement for sales approval of the product in Canada. The Environmental Impact Assessment is an official document including the molecular and the biological characterization of live recombinant virus, the safety of application items and non-target animals, human safety, concerns on the environment, and risk mitigation methods.

The Environmental Impact Assessment is based on not only information provided from the manufacturer (Intervet Inc, Millsboro, DE, U.S. Veterinary License No. 165A) but also the information independently obtained by CCBV examiner.

## 1. Prolusion

### 1.1 Application for Examination

The Canadian Centre for Veterinary Biologics (CCVB) of the Canadian Food Inspection Agency (CFIA) has a legal authority to be responsible for regulating the biological preparations for animal use under the Health of Animals Act and other regulations.

In Canada, the biological preparations for animal to be sold or used must comply with the standards on product safety, purity, effects and efficacy. Intervet Inc. (Millsboro, DE, US) applied for the approval on the following vaccine through Intervet Canada Corp (Kirkland, Quebec).

- Newcastle /Marek's disease (Marek's disease virus Type 2, HVT vector) live vaccine (trade designation “INNOVAX-ND-SB, ” USDA product code number 17H1.R2, CCVB registration code: 800VV/M10.0/I6.2)

The Environmental Impact Assessment was prepared by CCVB as a part of the permission examination on the vaccine above.

### 1.2. Background

Newcastle /Marek's disease (Marek's disease virus Type 2, HVT vector) live vaccine is a HVT live vaccine is manufactured by Intervet Inc. and sold in the U.S. This recombinant vaccine is a HVT where Marek's disease virus Type 2 and genes derived from Newcastle disease virus are introduced.

Newcastle disease (ND) is a very quickly spreading avian virus disease found in many regions of the world. The virus pathogenicity depends on strains: nonvirulent strain, which causes asymptomatic infection, through velogenic (highly virulent) whose death rate is 100 % (King 2006).

Marek's disease (MD) is a viral neoplastic disease in poultry and found all over the world. Although the disease is localized, it is assumed that all chickens have the virus unless SPF management is strict. For some reasons, it is very difficult to eradicate MD virus from the chicken group. Whether chickens are vaccinated or not, the virus very quickly transmits among individuals, the infection continues for a long time, and excreted virus survives for months in the environment (Fadly, 2006).

## 2. Purposes and the Necessity for Examination

### 2.1 Significance

A label of INNOVAX-ND-SB Newcastle/Marek's diseases says “To prevent super-virulent Marek's and Newcastle diseases, inoculate the vaccine to 18-day old embryonated eggs.”

### 2.2. Background

CCVB examines applications for permission of biological preparations for animals under the Health of Animals Act and other regulations. The general standard for permission stipulates the

following items: 1) The product is pure, safe, and effective, 2) the product has received permission in the country of manufacture, 3) vaccine components of the product are suitable for the status of the outbreak of diseases in Canada, and 4) the product is manufactured and its quality is controlled in accordance with the generally accepted GMP standards. As the U.S. vaccine meets these general standards and an application was filed without any unacceptable import risk, CCVB examined the product for approval.

As this product is a biotechnology vaccine categorized into Class 2, CCVB found it necessary to conduct environmental impact assessments before this product can be relieved into the environment. The environmental impact assessment is made to determine whether the product release carries the safety risk to animals, public health, or environment.

**3. Options**

There are two options. The first is if all approval requirements are satisfied, a permission for import the biological preparations for animals will be issued so that Intervet Canada Corp can import INNOVAX-ND-SB. The second is unless all approval requirements are satisfied, a permission for import the biological preparations for animals will not be issued.

**4. Molecular and the Biological Characteristics of the Host and Recombinant Microorganism**

**4.1 Categorization, Origin, and Strains**

The applied vaccine falls into Class 2 biologics (live expression with at least one foreign gene vector coding immunogen or immunological stimulus). The gene construction of recombinant vaccine is prepared by inserting genes expressing the protein derived from Newcastle disease virus (NDV) into nonpathogenic HVT vector. The vaccine includes Non-tumor primary Marek's disease virus Type 2 (MDV2).

**4.2 Origin and Functions of Foreign Gene**

NDV gene sequence was selected from those likely to stimulate protective immunity of chickens. Details of actual sequences are kept at CCVB.

**4.3 Method of Achievement of Genetic Modification**

Details of the method used to construct the recombinant microorganism is kept at CCVB. The master seed virusis examined for the denial of adventitious virus, purity, and safety in accordance with the method stipulated in Article 9, the Code of Federal Regulations (CFR).

CCVB examines and keeps these test results.

**4.4 Safety of Vaccine Microorganism Gene and Phenotype**

The Southern blot analysis found the recombinant microorganism genes are stable after subculture for 5 generations in cell. The fluorescent antibody method and the Southern blot analysis found even when the living organism is subcultured, the phenotype and the genotype are stable. In case of the subculture for 5 generations in chickens, a target animal species, any change in genes and phenotype were recognized.

**4.5 The Possibility of Horizontal Transmission and Recombination of Genes**

The vaccine is a mixture of recombinant microorganisms and other herpesvirus and their genes have obvious homology (Alfonso et al., 2000; Fukuchi et al., 1984; Gibbs et al., 1984). Guessing from the literature that show cells and living organism were co-infected with herpesvirus and that experimental recombination was performed, there is a logical risk of transmission and recombination between 2 viruses. However, comparing other polyvalent combination vaccines, this product has particularly higher risk of recombination.

The pathogenicity is a characteristic of virus that may change at the time of recombination. Either herpesvirus strain seems to be non-pathogenic. There is a logical risk that a new recombinant may increase pathogenicity as recognized in other herpesvirus research (Henderson et al., 1990; Kats et al., 1990). This MdV2 strain and a different HVT strain have been used as approved polyvalent combination vaccines for yeard, but there is no sign of pathogenicity. It seems as for this products, either virus is little likely to get pathogenic through recombination.

**4.6 The Range, specificity, Tissue Affinity, Excretion, and Diffusion Ability of Susceptible Animal**

The manufacturer performed a living-body test to see the range of recombinant susceptible animals. The replication of the recombinant was detected only from chickens and turkeys, not from pigeons and ducks. The biodistribution of the recombinant showed that it has not been

changed from the host HVT. The transmission of recombinant microorganisms from vaccinated chickens to unvaccinated ones and ducks was not found. However, they were transmitted to turkeys. As the turkey is an animal naturally holding the virus used to produce the recombinant, this is an expected result.

As NDV has been reported to cause mild conjunctivitis to mammals, a recombinant virus was inoculated to rabbits, but no replication occurred and no symptom was found.

**4.7 Comparison of Characteristics of Recombinant and Host**

It seems that the range, tissue affinity, excretion, and diffusion ability of the recombinant susceptible animals are same as those of the host HVT vaccine strain.

**4.8 Portal of Entry and Propagation**

Avian herpesvirus are mainly extracted from dander and diffused. However, when HVT is inoculated to day old chicks, it is not frequently diffused (Cho and Kenzy, 1975). Like the host, recombinant microorganisms did not diffuse from vaccinated chickens to cohabiting nonvaccined ones. It is known that a wild-type of HVT survives in the environment. The manufacturer found it survives for 7 days or less in the environment of virus associated with cell.

**5 Safety for Human Beings**

**5.1 Past Safety Use**

The recombinant has no history of usage in Canada while in the U.S. it has been used since 2007. The HVT used for production has been safely used for dozens of years as the one to control Marek's disease. The NDV gene insertion is collecting only a part where one protein is expressed from complete virus and we cannot imagine any concern over safety. The solvent and preserving agent used for the product is also used in other products and there is no safety concern.

**5.2 Possibility of Exposure to Human Beings**

The possibility of direct vaccine (virus associated with cell) exposure to human beings is likely to be limited to the inoculation works by veterinarians, farmers, and animal technicians. Like other recombinant HVT vaccine observations, inoculated animals may excrete virus in a limited frequency. Therefore, matured virus excreted from feather follicle epithelium may be exposed to human beings.

According to the bibliographic information, herpesvirus has continuous infection ability, and HVT seems to be replicated in birds' bodies for extended periods or for an indefinite time (Calnek and Witter, 1991; Cho 1974), workers at the poultry slaughterhouse have chances to be exposed to the recombinant virus.

The recombinant virus locally exists in lymphocyte of internal organs and the feather follicle, not in the meat, a main part offered for human consumption. The possibility for the virus to be exposed to human being through edible meat derived from vaccinated chickens decreases after meat dressing. Furthermore, if the recombinant virus remain in chicken meat, most taken nucleic acid will be degradated in the human alimentary canal (Jonas et al., 2001).

As mentioned above, there are chances of exposure to human beings, but it is unlikely to exert adverse effects.

**5.3 Results from Exposure to Human Beings**

There is no report on HVT infection on species other than chickens and turkeys. In these species, virus is replicated but does not cause clinical symptoms. Newcastle disease is a zoonotic infection and causes a transient conjunctivitis, but NDV components used for gene insertion in this vaccine are not likely to cause adverse effects to the health of exposed human being. The exposure of this vaccine to human being including misinjection is not likely to cause serious adverse effects.

**5.4 Pathogenicity of Microorganism Used for Production toward Human Beings**

As mentioned above, HVT used for production shows no pathogenicity towards human beings, but NDV is a mild pathogen of zoonotic infection. This vaccine includes one NDV protein gene, and is not pathogenic to human beings.

**5.5 Influence of Gene Manipulation on Pahogenicity to Human Beings**

It seems that NDV gene insertion does not change HVT pathogenicity.

**5.6 Risk of Wide Use of Vaccine**

The wide use of this vaccine is not likely to cause any serious public health problem.

## 6 Safety for Animals

### 6.1 Past Use

It was experimentally confirmed that the use of recombinant microorganisms for 1 or 10 birds for the target animal species, chicken, is safe. Similarly, it is confirmed that the use for non-target animals, that is, birds (turkey, pigeon, and duck) and mammals (rabbit) is safe. INNOVAX-ND-SB was inoculated to 3 million or more birds in an outdoor safety test implemented by the manufacturer and was approved in the U.S. in 2007. The HVT used for production has been used for dozens of years as the vaccine for chickens to control Marek's disease.

### 6.2 Destiny of Vaccine Virus in Target Animal Species and Non-target Animal Species

A wild-typeHVT has not been reported to cause any clinical disease toward any animal species. In the backpassage in chickens did not show any sign that the recombinant gets pathogenic.

The test using the target and non-target animals show the range and affinityof animals susceptible to the recombinant vaccine have not been changed since HVT strains. In addition, this experiment shows the infection continued for 21 days (until the completion of the experiment period) in turkeys. Based on bibliographic information, the inspection would continue through life (Calnek and Witter, 1991; Cho 1974).

No long-term test on safety of this product in target species chicken and non-target species turkey has not been conducted.

### 6.3 Diffusion Ability of Vaccinated Animals to Target and Non-target Animals

According to the literature, HVT is excreted from dander of vaccinated chicken on a sporadic basis.

The manufacturer's experiment showed no recombinant was diffused to non-turkey species (Zygraich and Huygelan, 1972; Cho 1974). The turkey is an animal naturally holding the virus used to produce the recombinant.

### 6.4 Obtain Pathogenicity through Backpassage

Manufacturing origin strains were stable in subculture for 5 generations in genotype and phenotype. No exogenous factor stipulated in CFR 9.133.300 existed. The backpassage in chickens, target animal species, showed the recombinant virus did not get pathogenic.

### 6.5 Effect of Excessive Inoculation to the Target and Non-target Animals

The manufacturer reported that 10 times inoculation to embryonated eggs did not exert any detrimental effect on the hatching rate and the rate of maturity and no lesion was found.

### 6.6 The Range of Susceptible Animals and the Vector Mobility

The range of susceptible animals is very narrow and limited to turkeys and chickens. The recombinant virus is excreted like the virus used for production, and was not horizontally infected among chickens, but was among cohabiting turkeys.

## 7. Effect on the Environment

### 7.1 Frequency of Release to the Environment

Recombinant viruses were excreted from feather follicles of vaccinated chickens. Most of the vaccinated chickens were bred inside with biosecuriity and the direct exposure to the environment is small. However, when the ventilation is conducted or the poultry house is cleaned, the recombinant virus may be released. HVT pollutant wastes and air (dusts) are likely to infect turkeys (Witter and Solomon, 1971).

In the daily use of vaccine, accidental discharge and unintended emission of aerosol from injection cylinder or pollution in the inoculation site into the environment may occur.

### 7.2 Effects of Persistence of Vector and the Accumulation in the Environment

A research shows HVT may be collected from the environment of vaccinated chicken groups in the 8th week after inoculation and may persist longer (Islam and Walkden-Brown, 2007). Another research shows MD virus excreted from feather follicle persists for 12 months in the environment.

The manufacturer shows that the recombinant virus (virus associated with cell) cannot persist for 7 or more days in the environment. The survival period of the matured virus excreted from the feather follicle and the persistence of the virus in the natural environment (what those excreted from feather follicle epitheliums and exist in wastes and dusts at the poultry house will

become after cleaning the house and how they change depending on barnyard manure and the sunlight irradiation) have not been tested.

Typically, herpesvirus is deactivated by the UV of the sun (Lytle and Sagripanti, 2005). However, MD virus included in dried feather and dusts at the poultry house retain the infectivity for at least 1 year (urajda and Klimes, 1970; Schat, 1985).

**7.3 Range of Exposure to Species Other than Target Animals**

The range of animals susceptible to herpesvirus is very narrow. The risk of diffusing the virus to mammals, or non-target animals. Turkeys bred near vaccinated chickens or turkeys have a risk to contact bedding and wastes from the chickens' breeding area or directly contact the chickens.

The manufacturer tested the recombinant vaccine virus and concluded that the virus was not pathogenic to turkeys, but preventive measures should be taken to decrease the possibility of diffusing virus to the population of turkeys. Therefore, the manufacturer agreed with INNOVAX-ND-SB label indicates to call a special attention: “This product should not be used for a chicken group that may directly or indirectly be exposed to turkeys.”

**7.4 Behavior of Virus Used as Vector or for Production in Non-target Animals**

The test carried out by the manufacturer syggests the recombinant virus should be directly inoculated to non-target animals including turkeys for safety. There is no realistic way to investigate how this virus behaves and settles in natural turkeys for a long time.

**8. Conclusion on the Environment**

**8.1 Risks and Advantages**

We think this vaccine is effective in inducing immunity even when it is inoculated to maternal antibody-positive chickens. The immunity induction to win the maternal antibody is a challenge for many currently available ND vaccines. Furthermore, this recombinant vaccine has no side effects recognized in ND live vaccine, which has very high immunogenicity but remains pathogenic.

The first risk recognized in INNOVAX-ND-SB is the possibility of side effects as recognized in any vaccine. The general safety of the product is confirmed by a clinical test for chickens conducted by the manufacturer.

Morevover, there is a logical risk: the virus may be diffused to the turkey population (or other birds of the Phasianidae family). The more important thing, however, is if the recombinant virus should be diffused to turkeys, the virus will not show pathogenicity to turkeys. In the test conducted by the manufacturer, turkeys were infected with the recombinant virus (through direct and horizontal infections) remain healthy during the test until about 4 weeks old like turkeys infected with wild-type HVT. To further mitigate the risk of diffusing to turkeys, the label of INNOVAX-ND-SB for Canada tells a preventive statement, saying measures should be taken to avoid contacts between vaccinated chickens and turkeys.

In conclusion, there is a logical risk that turkeys are unintentionally infected with the vaccine virus. Still, there is no way for confirmation when such phenomena occurs among turkeys. To promote the health of chickens, the vaccine is used, and this is more beneficial and reduces risks of diffusing vaccine virus to turkeys.

**8.2 Relative Safety by Comparison with Other Vaccines**

This vaccine consists of 2 types of non-pathogenic live MD and ND virus genes, and all of them are not pathogenic. Like the conventional MD vaccines, this vaccine is safe for target and non-target animals. This vaccine is safe because there is no possibility of obtaining pathogenicity, and adjuvant, which is typically used for inactive vaccines, is not used.

**9. Reducing Measures**

**9.1 Safety of Workers**

A veterinarian, workers of poultry industry, and animal technicians may be exposed to live recombinant virus during vaccination. As shown above in 5, there is no safety concern on such exposure. The intraovular inoculation, which is a recommended way to vaccinate NNOVAX-ND-SB, causes less misinjection than inoculation to poults. Moreover, as the vaccine does not contain adjuvant, no clinical problem of adjuvant occurs at the time of misinjection. Still, measures should be taken to protect workers from exposure in accordance

with the attached sentences of the product.

**9.2 Handling of Vaccination or Exposed Animals**

Chickens are bred at facilities with biosecurity, and rarely handled by human hands. Preventive biosafety measures are taken to workers for the poultry industry. The workers are seldom exposed to vaccinated poults. However, the workers for poultry industry may be exposed to the vaccine virus through the air and dust at poultry house polluted by the virus excreted with dander. Still, the vaccine virus is unlikely to be pathogenic to human beings.

**10. Monitoring**

**10.1 General Examples**

According to Canadian legal requirement for the vaccine permits, the manufacturer must submit the information on a serious and obvious side effect to CFIA within 15 days when they obtain such information from an owner or a veterinarian. A veterinarian may directly report to CIFA adverse events, which looked side effects. Receiving a report on side effects, CCIV requires the manufacturer to conduct an investigation and report to owners' veterinarians and CIFA. If the problem is solved to satisfy owners, CCVB will not require any more. Yet if investigation results are not satisfactory, CCVB will, depending on the situation, exercise control such as further safety tests, temporary suspension of shipment, or rescission of permission.

**10.2 Human Being**

This product is monitored for human beings.

**10.3 Animals**

As shown above, a veterinarian must report any suspect of side effect to CCVB. Use the format CFIA/ACIA2205 “Notification of Suspicious Side Effect of Biologics for Animals” to report a suspicious side effect.

**11. Reference and Contact**

**Importer** Intervet Canada Corp.
16750, route Transcanadienne
Kirkland, QC H9H 4H7

**Manufacturers:** Intervet Inc.
29160 Intervet Lane, Box 318
Millsboro, Delaware USA 19966-0318

**12. Conclusion**

According to the effect evaluation based on available information, CCVB concludes the import and use of Newcastle /Marek's disease (Marek's disease virus Type 2, HVT vector) live vaccine to Canada will not exert bad influence on the environment as far as the products are produced according to the approved manufacturing process and are used according to the manual.

We believe this effect evaluation and the completion of Canada's permission examination on biological preparations for animals will modify the permission for import of biological preparations for animals to permit the import and sales of the following product to/in Canada.

- Newcastle /Marek's disease (Marek's disease virus Type 2, HVT vector) live vaccine (trade designation “INNOVAX-ND-SB, ” USDA product code number 17H1.R2, CCVB registration code: 800VV/M10.0/I6.2)

All batches of this product were certificated by the USDA before export to Canada. In accordance with all conditions described in an import license for biologics for animals, this product is imported and sold.

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**Attachment 35 Infectious Virus Residual of HVT-NDV/F Strain in the Edible Part of Chicken after Poultry Dressing (Overseas Test)**

**Purposes:**

Evaluate the stability in muscles and edible organs after poultry dressing of chickens inoculated with HVT-NDV/F strain.

**Method:**

Inoculated a high dose of HVT-NDV/F strain to 5-day old chickens. All 4-week old test chickens were slaughtered, collect musculus (pectoralis muscle and thigh), liver, cardiac, muscular stomach and preserved them at 4 deg C and -20 deg C. Tried to collect virus from the organ samples 24, 48, and 72 hours after slaughter.

**Result:**

HVT-NDV/F strain with infectivity was defected from cardiac and liver preserved for 24 hours at 4 deg C.
At any one time, no virus was collected from muscle of thigh, pectoral muscle, and muscular stomach. No virus was collected from any organ samples 48 and 72 hours after slaughter. At any time, no virus was collected from organs preserved at - 20 deg C (Table 1).

Table 1 Existence of infectious virus in muscle and edible organs after preservation at 4 deg C and -20 deg C.

| Inspection portion | 24 hours | | 48 hours | | 72 hours | |
|---|---|---|---|---|---|---|
| | 4 deg C | -20 deg C | 4 deg C | -20 deg C | 4 deg C | -20 deg C |
| Pectoral muscle (breast meat) | — | — | — | — | — | — |
| Thigh mus | — | — | — | — | — | — |
| Liver | + | — | — | — | — | — |
| Cardiac | + | — | — | — | — | — |
| Muscular stomach (gizzard) | — | — | — | — | — | — |

**Conclusion:**

HVT-NDV/F strain is stable in the liver and cardiac for 24 - 48 hours after slaughter. No infectivity virus was detected in muscles and muscular stomach 24 hours after slaughter.
The virus sensitivity was deactivated by freezing at - 20 deg C.

## Attachment 36 Inspection on Death Examples of Wild Birds of the Phasianidae Family in Japan

If mass morality or abnormal death of wild beasts and birds is found in Japan, a prefecture in jurisdiction receives a report, and as required, press reports and inspections at a livestock hygiene service center are conducted. The prime concern about mass morality of birds is avian influenza. The Ministry of Environment is in charge of the inspection results of the influenza. Since 2010, types of tested wild birds were released to the public. Table 1 shows the summary of tested birds of the Phasianidae family.

The number of birds of the Phasianidae family tested in the investigation of wild bird that died from the avian influenza was 6 in total. The death situations are unknown, but these birds of the Phasianidae family were found to be negative for high pathogenicity and low virulence influenza virus

We retrieved death of wild birds other than the avian influenza by using information materials and academic reports. We examined 64 death examples and the number of death examples of birds of the Phasianidae family is one. The death cause of copper pheasant in Case 23 is a traumatic wound.

No other case concerning the death or pathogenesis of wild birds of the Phasianidae family was found. Some information may not be collected, but the frequency of the death or pathogenesis of wild birds of the Phasianidae family seems low. In addition, no case could be found that show a relation between a poultry house and wild bird of the Phasianidae family.

Table 1 The Number of Birds of the Phasianidae Family Tested in the Investigation of Wild Birds that Died from the Avian Influenza

| Dead Wild Bird Investigation | Investigation Season | | | Total |
|---|---|---|---|---|
| | 2010-11[1)] | 2011-12[2)] | 2012-2013[3)] | |
| The total number of inspections | 5649 | 444 | 370 | 6463 |
| Birds of the Phasianidae family | Pheasant4* Bamboo partridge 1 | None | Copper pheasant 1 | 6 |

*The number shows the one of analytes and the number of examples is unknown.

1) *Saku Season (22-23 Nendo) no Yacho niokeru Kogensei Tori Influenza no Hassei ni kansuru Kosatsu* (Considerations on the Occurrence of High Pathogenicity Avian Influenza in the Last Season (2010-2011)) (September 9, 2011, Nature Conservation Bureau, Ministry of Environment)

2) *Heisei 23-24 Nen Season no Tori Influenza Virus Hoyu Jokyo no Kekka ni Tsuite* (Results of the Investigation on the Conditions of Birds Keeping Avian Influenza in the season of 2011-12) (August 22, 2012, Office of Wildlife Managemen, Nature Conservation Bureau, Ministry of Environment)

3) *Heisei 24-25 Nen Season no Tori Influenza Virus Hoyu Jokyo no Kekka ni Tsuite* (Results of the Investigation on the Conditions of Birds Keeping Avian Influenza in the season of 2012-13) (September 6, 2013, Office of Wildlife Managemen, Nature Conservation Bureau, Ministry of Environment)

## Attachment 37 Inspection on Death Examples of Wild Birds of the Phasianidae Family

In the U.S., the National Wildlife Health Center collects the information on the death examples of wild animals in the U.S., and compiles sites of occurrence of death examples, animal species, and death causes. The collected information is released to the public, aiming at the use by natural resource managers, researchers, public health agencies, establishing laws on the prevention and decrease of diseases, measures against common diseases among human beings, domestic animals, and wild animals, and identifications of normal diseases and biosecurity. The death example of wild birds of the Phasianidae family other than turkeys are selected from this report (Table 1).

Six death examples of wild birds of Phasianidae family in the U.S. were confirmed. Among the examples, the causes of 5 deaths were clear, but the example of greater sage grouse was not. The site of occurrence of the example of greater sage grouse in 2012 is Clear Lake National Wildlife Refuge in the northern part of California, and the geographical point is shown in Fig. 1. Figs. 2, 3., and 4 show the locations of feeding regions for turkeys, meat type chickens, and hens. The turkey farm nearest to Clear Lake is in the middle of California, 200 or more km away. The nearest chicken farm is a chicken egg farm in the south of Oregon, about 100 km away. The geological locations suggest the death cause of the greater sage grouse has nothing to do with breeding of turkeys and chickens.

No other example of death or symptom of birds of the Phasianidae family could be found around feeding regions for turkeys.

Table 1 Inspection on Death Examples of Wild Birds of the Phasianidae Family in the U.S.

| Date of occurrence | Place | Species | Cause |
|---|---|---|---|
| 9/3/2012 -11/30 | California (Clearlake) | Greater sage grouse: about 40 birds | Unknown (to be deleted) |
| 8/7/2006 -9/16 | Oregon | Sage grouse: about 69 birds | Viral infection: West Nile virus |
| 8/1/2003 -8/31 | Wyoming and Montana | Sage grouse About 22 | Viral infection: West Nile virus |
| 8/14/2001 -8/31 | North Dakota | Ring-necked pheasants, American white pelicans, unidentified ducks, and unidentified sea gulls: 136 birds | Type C botulin toxin |
| 4/16/1992 -4/17 | Texas | Ring-necked pheasant, recurvirostra avosetta, Anas discors, and Bartramia longicauda: about 100 birds | Traumatic wound (hail) |
| 8/2/1986 -8/11 | Idaho | Sage grouse: about 50 birds | Poisoning (organophosphorus pesticide) |

Source: Quarterly Mortality Reports, National Wildlife Health Center

*Death examples of turkeys are not included in the table.

* As the North American quali belongs to Odontophoridae, not Phasianidae, it is not included in the table.

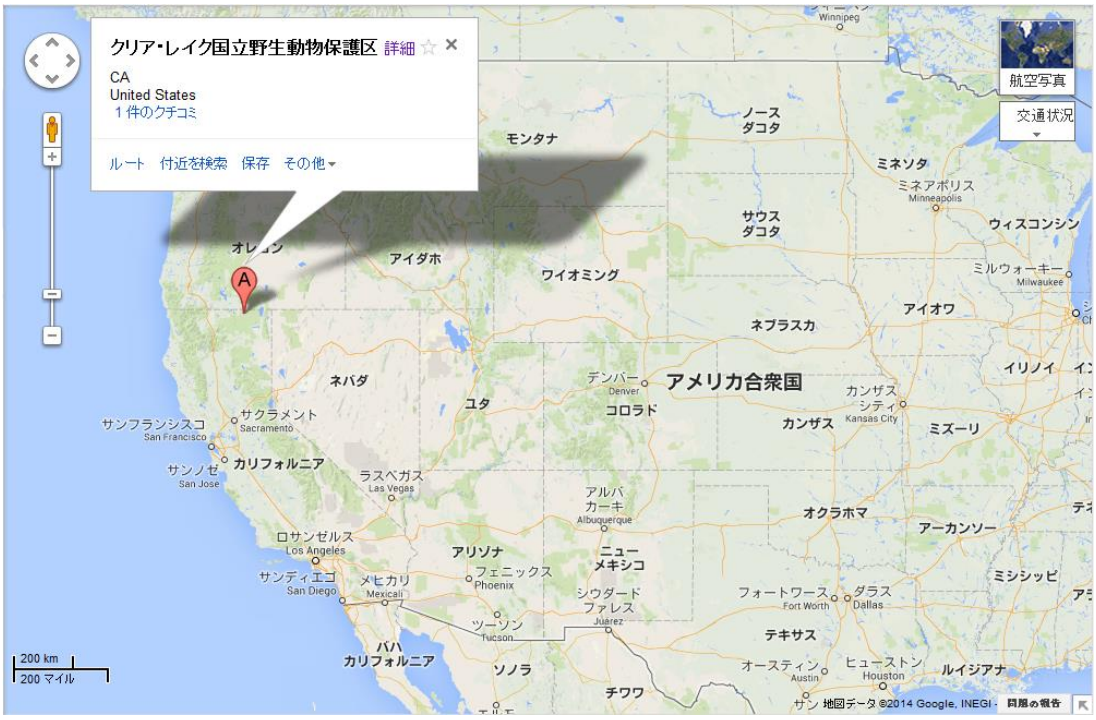


Fig.1 Geological Location of Clear Lake National Wildlife Refuge

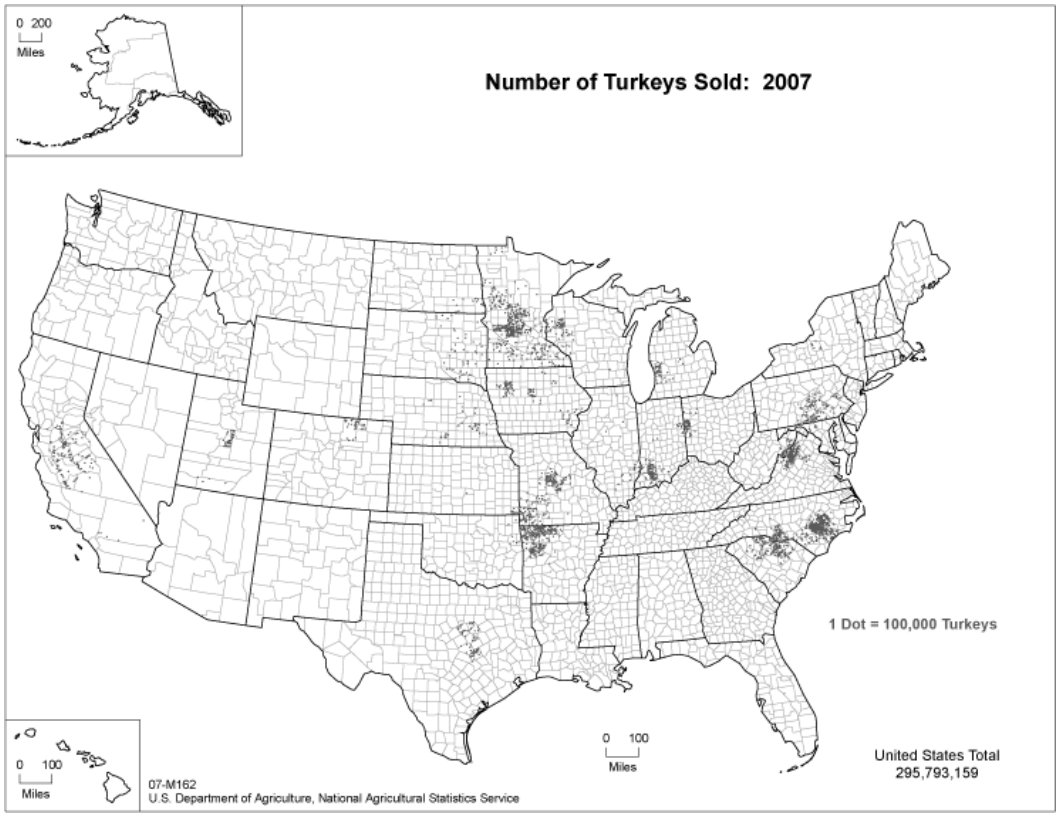


Fig.2 Geological Locations of Feeding Regions for Turkeys

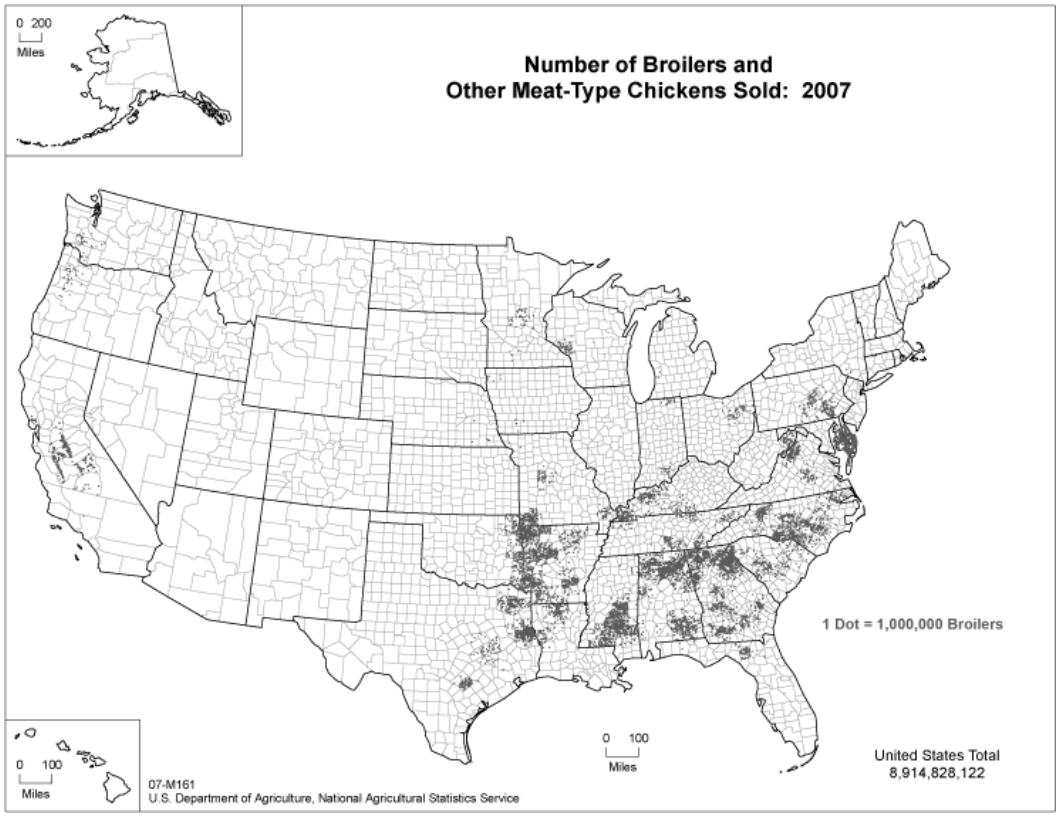


Fig.3 Geological Locations of Feeding Regions for Meat-Type Chickens

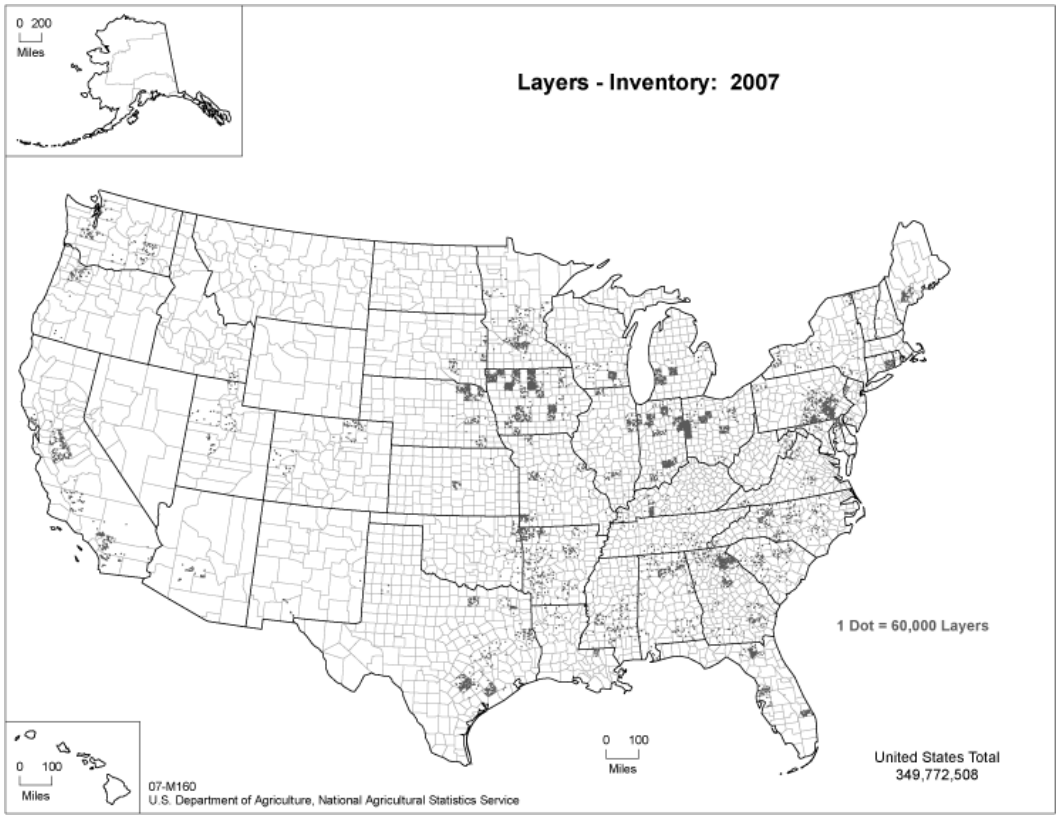


Fig.4 Geological Locations of Feeding Regions for Hens

## Attachment 38　HVT in Susceptible Birds and Maintenance in the Nature

Marek's disease is a disease in chickens characterized by lymphoid tumor formation and neuropathy caused by pathogenic Marek's disease virus serotype 1 (hereinafter called "MDV1") infection, and is one of the most important diseases in the chicken industry. Live vaccine is a means effective for prevention of Marek's disease. Nonvirulent MDV1, non-pathogenic Marek's disease virus Type 2 (hereinafter called "MDV2"), and Herpesvirus of turkey (hereinafter called "HVT") are used as live vaccines.

HVT is derived from healthy turkeys and widely distributed among their group.　There is an example of separating from wild turkeys. HVT's different name is the Marek's disease virus type 3 (hereinafter called "MDV3") and antigenically similar to MDV1 and MDV2. While MDV1 shows pathogenic, MDV2 and the virus are nonpathogenic.

PB1 strain used by a host of recombinant was separated from healthy turkey's blood in Norfolk, the UK, around 1969 [1)].

An animal holding HVT in the natural world is a turkey. Birds other than the original holding animal, which are experimentally confirmed the infection of HVT are the chicken[1)], quali [2)]and ring-necked pheasant[9)], and those that are not be infected are the duck [10)] pigeon [10)], and bobwhite ([9)]. The chicken, quali, and ring-necked pheasant, which are infected with HVT, are in the family Phasianidae, and the pheasant, copper pheasant, Lagopus muta, which are also in the family Phasianidae, may display susceptibility to HVT. But as they are not close to turkeys in systematology, the possibility of infection seems lower.

The turkey, the original animal holding HVT, is naturally infected when it is farmed in a region where HVT exists, and by 10-week-old, all individuals in a group become carriers of the virus and antibody [3)]. If the turkey is inoculated with HVT, all inoculated individuals are affected and all of those living with them are affected, too [4)]. There is a small amount of data on the HVT susceptibility of other birds of the Phasianidae family. All inoculated individual chickens are affected, but those living with them are not affected or only some are if any [5, 6, 7, 8, 11)]. There is no data on the rate of infection of quali. When inoculated, quali produces antibodies and infection is realized. In addition, HVT is used as Marek's disease vaccine in a quail farming as well as in a chicken farming[2)]. Only HVT inoculated individuals among ring-necked pheasants carry the virus. It is suggested that the susceptibility of HVT is low [9)]. Based on the knowledge above, the turkey is originally an animal holding HTV and the susceptibility of other birds of the Phasianidae family is low. Even if birds of the Phasianidae family such as the pheasant, copper pheasant, and Lagopus muta have high susceptibility to HVT, their susceptibility is thought to be lower than the turkey. Therefore, even if a part of individuals are infected, it is not likely that the infection spreads and is kept.

The differences of HVT infection in turkeys and chickens is whether the horizontal infection easily occurs.

HVT, which is proliferated in a feather follicle and excreted to outside of the body, is thought to be an infectious agent. If it is infected to chickens, the proliferation in the feather follicle shows transient lapse a few weeks after infection[7, 8] and the short-time completion of excretion is thought to be a reason why the horizontal infection of HVT in chickens is limited to a part of individuals.

If HVT is inoculated to chickens, it proliferates in lung, thymus, bursa of Fabricius, spleen, and other lymphatic tissues and spread to other internal organs by infecting leukocytes 1-4 days after inoculation. Early individuals develop viremia 6 days later and most 3-4 weeks later[7].

As HVT is cell associated, the living cell is essential for its viability and it can nether be proliferated nor viable in the extracellular environment. Still, because HVT-infected turkey's emulsion on the skin is infectious, HVT is matured in the feather follicle epithelium, and substances excreted as cell-free virus seems to be an infectious agent[7, 8]. Cell-free virus is detected from feather follicle a few weeks after inoculation, in case of chickens, and it is difficult to detect it when the time is over. Even after the completion of excretion, chickens gets a continuous infection and maintain immunity for extended periods[7].

As for the infectious route of HVT excreted from the body, it is confirmed that HVT is airborne infection (dust infection) to chickens and turkeys though cases for chickens are limited[4, 5, 6]. Literatures say [7, 8]HVT is transmitted by contact. This is based on an observation that the infection is caused by cohabitation . The data that proves whether the infection is caused by contacting skins and mucosa or whether HVT is orally infected through excreted virus-infected feed and water is not included, and the infection by contact is not fully verified. In addition, virus excretion from oral cavity or feces was not recognized [12, 13], droplet and droplet nuclei infections may be a cause. In addition, it was confirmed that there is no vertical transmission [7, 8].

From the above, HVT's main infection route is thought to be airborne infection (dust infection), which causes infection by breathing virus excreted outside.

As for recombination among strains in homologous viruses, there is a possibility of homologous recombination in HVT if there is a chance of coinfection with different strains, but there is little chance of coinfection in the outdoor infection because there is no example of HVT separation from unvaccinated chickens[7] and there is no wild animal holding HTV in Japan.

Reference Materials

1) “Viraemia and antibody development in chicks following the administration of turkey herpesvirus.” Churchill A.E. *et al.*, 1973, Veterinary Record 92(13):327-334 (Literature 1)
2) “The effect of HVT vaccine in Japanese Quail I.” Sugiura R. *et al.*, 1982, Research Bulletin of Aichi Agricultural Research Station 14:450-455 (Literature 2)

3) “Epidemiology of a herpesvirus of turkeys: Possible sources and spread of infection in turkey flocks” Witter R. L. and Solomon J. J., 1971, Infection and Immunity 4(4):356-361 (Literature 3)
4) “Experimental infection of turkeys and chickens with a herpesvirus of turkeys (HVT)” Witter R. L. and Solomon J. J., 1972, Avian Diseases 16(1):34-44 (Literature 4)
5) “Horizontal transmission of turkey herpesvirus to chickens. 1. Preliminary observation” Cho B. R. *et al.*, 1971, Poultry Science 50(3):881-887 (Literature 5)
6) “Horizontal transmission of turkey herpesvirus to chickens 5. Airbone transmission between chickens” Cho B. R., 1976, Poultry Science 55:1830-1833 (Literature 6)
7) “Turkey herpesvirus and Marek’s disease virus. A comparative appraisal” Prasad L. B. M., 1979, Comparative Immunology, Microbiology & Infectious Disease 2(2-3):335-358. (Literature 7)
8) “Nononcogenic turkey and chicken herpesvirus (HVT)” Schat K. A. and Nair V., 2008, in “Disease of Poultry 12th Edition” (Sarif Y. M. ed.) p491-492, Backwell Publishing Professional. Iowa.(source material of Attachment 8)
9) The effect of HVT/ILT-138 on non-target species (source material of Attachment 1)
10) *In-vivo* growth after intramuscular inoculation of the recombinant herpesvirus of turkey HVT-NDV/F and its parent strain PB1 in various avian species (source material of Attachment 2)
11) Spread of the recombinant herpesvirus of turkey HVT-NDV/F and of the parent HVT Strain PB1 to in-contact chickens (source material of Attachment 3)
12) Dissemination of the recombinant herpesviruses of turkey HVT-NDV/F and HVT-NDV/HN in SPF chickens after inoculation by the subcutaneous route (source material of Attachment 6)
13) Dissemination of the recombinant herpesviruses of turkey HVT-NDV/F and HVT-NDV/HN in SPF chickens after inoculation by the intramuscular route (source material of Attachment 7)