Molecular Epidemiology of

Newcastle Disease Virus in Japan

（日本におけるニューカッスル病ウイルスの分子疫学的研究）

The United Graduate School of Veterinary Science

Yamaguchi University

Dennis Villaseñor UMALI

March 2014

CONTENTS

GENERAL INTRODUCTION

Newcastle disease virus

Avian paramyxovirus (APMV) is classified in the genus *Avulavirus*, of the subfamily *Paramyxovirinae*, family *Paramyxoviridae*, of the Order *Mononegavirales*. APMV is comprise of twelve antigenically distinct serotypes (APMV-1 to APMV-12) capable of infecting most avian species [3, 11, 73, 103]. Among these, APMV-1 [also known as the Newcastle disease virus (NDV)], is identified as the most devastating and economically important serotype. It is the causative agent of Newcastle disease (ND), a highly contagious and fatal viral infection affecting poultry and most species of birds worldwide [3-6].

Newcastle disease

ND may be manifested as a velogenic viscerotropic (VVND), velogenic neurotropic (VNND), mesogenic, lentogenic or an asymptomatic enteric disease [2, 4-6, 51-55, 82, 107]. Chickens infected with VVND usually show signs of acute hemorrhagic gastrointestinal infection characterized by weakness, greenish diarrhea, facial edema, muscular tremors and death. Infection with VNND usually results to respiratory distress followed by neurologic disorders characterized by ataxia, incoordination and torticollis. When chickens are infected with mesogenic strains of NDV, the infection may lead to development of non-fatal respiratory disease. In layers and breeders, mesogenic ND may also result to drop in egg production with low mortalities. When chickens are infected with lentogenic NDV strains, mild respiratory and gastrointestinal signs are usually observed. In the absence of respiratory signs and when virus replication is limited to the gastrointestinal tract, ND maybe manifested as

an asymptomatic enteric infection [3-6]. Depending on several factors such as host species, host immune status and age, environmental stress, coinfection with other organisms, viral dose and route of exposure, clinical signs of ND may appear from 2 to 15 days after infection [3-6].

Molecular Epidemiology of NDV

Since the emergence of ND in 1926, at least four panzootics of NDV had been reported [3, 114]. The first panzootic spread very slowly and took almost over 20 years to spread to become a true panzootic [7]. It was reported to be caused by NDVs from genotypes II, III and IV [66]. Genotype II NDVs during the first panzootic were characterized by varying degree of virulence from lentogenic, mesogenic to velogenic and were consists mainly of North American isolates. Genotypes III and IV NDVs represent the early ND viruses which emerged from the Far East and Europe [1, 66, 76, 91]. In the 1960's, the second ND panzootic occurred. It spread rapidly from the Middle East reaching all continents and most countries by 1973 [2]. It has been suggested that this rapid spread was due to enormous trade and importation of captive caged birds as a result of technological advances in air transportation and commercialization and globalization of poultry production [2]. NDV strains isolated during the second pandemic were reported to belong to genotypes V, VIa and VIII [114]. Around 1970's, the third ND panzootic was reported. This was caused by the neurotropic form of NDV, which was suspected to have originated from the Middle East. It spread rapidly across Europe and into other continents through infected pigeons. NDV strains involved in the third panzootic were identified to belong to genotypes VIb, VIc and VId [114]. Recently, severe ND outbreaks in Indonesia and Taiwan in 1980's and South Africa, Middle East, Northern and Eastern Europe and

China in 1990's were reported [42, 61-63, 65, 114]. Phylogenetic analyses revealed that these outbreaks were caused by genotype VII NDVs. This genotype is currently the prevalent genotype circulating in Asia, Africa and Europe and at present constitutes the ongoing fourth ND panzootic [4, 42, 61-63, 65, 114]. Other genotypes of NDV have also been reported. Genotype IX has been reported in some regions in China whereas the novel genotype X has been recently described in Taiwan [106] and XI in Madagascar [66].

NDV Outbreaks in Japan

Since commercial vaccines became routinely available in the late 1960's, only a few sporadic ND outbreaks were observed in Japan. As a result, most Japanese NDVs were isolated mostly from unvaccinated backyard flocks, pet birds and wild migratory birds [68, 69]. It has been observed that NDVs from these hosts are genetically diverse and that these outbreaks may have been due to multiple etiologies [68, 69]. Recently, several cases of sporadic ND in vaccinated commercial poultry in Japan have been observed [79]. Several authors from other parts of the world have also reported the occurrence of velogenic ND in apparently healthy vaccinated chicken flocks [12, 50, 75, 85, 108].

At present, very limited data are available regarding the molecular epidemiology of NDV outbreaks especially in vaccinated commercial poultry farms in Japan. Molecular analyses at the subgenotype level and exhaustive investigation on the relationships of Japanese NDVs to other isolates from around the world are also lacking. The possible involvement of genomic factors in cases of breakthrough infection by NDV in vaccinated chickens has also never been thoroughly investigated. Because of these limitations, this study was therefore conducted.

Aim of the Thesis

This thesis was conducted to investigate the molecular epidemiology of NDV in Japan and to explore the possible roles of genomic factors on the dynamics of breakthrough infection of NDV in vaccinated commercial layer flocks.

Nine Japanese field strains of velogenic NDVs isolated from vaccinated commercial flocks from different prefectures from 1969 to 2002 were characterized. The epidemiological relationships of these isolates with other NDVs from different regions of the world were analyzed. Inferences on the possible origins, transmission mechanisms and dynamics of spread of these viruses were performed.

A case of atypical infection of velogenic ND in vaccinated commercial layer flock was reported. The clinical, serological and production profile of breakthrough infection of velogenic ND in vaccinated chickens were described.

The complete genome sequence of three velogenic NDV strains isolated from vaccinated commercial layer flocks in the span of three decades in Japan were determined. The complete sequence data of these strains were analyzed to investigate the possible role of genomic factors in the infection dynamics of NDV in vaccinated chickens.

A seven-year surveillance of NDV and other APMVs from overwintering migratory waterfowls in Japan were conducted. It has been demonstrated that wild birds were potentially capable of transmitting and spreading precursors of velogenic viral strains to domestic poultry [3, 27, 95]. In this chapter, several NDVs and APMVs were isolated, analyzed and compared to other field isolates from Japan.

CHAPTER I

Molecular epidemiology of Newcastle disease virus isolates from vaccinated commercial poultry farms in non-epidemic areas of Japan

Introduction

Newcastle Disease (ND) is a highly contagious and economically devastating disease of poultry. It is caused by the Newcastle disease virus (NDV) [also known as avian paramyxovirus type-1 (APMV-1)] of the genus *Avulavirus* of the family Paramyxoviridae. NDV infects a wide range of domestic and wild bird species worldwide. Among animal viruses, it is one of the biggest contributors of economic losses to the world's economy [4-5].

NDV is an enveloped, non-segmented, single-stranded, negative-sense RNA virus with a helical morphology. Its genome has six open reading frames (ORF) in the order of 3'-NP-P-M-F-HN-L-5'. These genes encode for the following proteins: nucleoprotein (NP), phosphoprotein (P), matrix protein (M), fusion protein (F), hemagglutinin-neuraminidase (HN) and the RNA dependent RNA polymerase (L) respectively. During P-gene transcription, two additional non-structural proteins, the V and the W proteins, are also generated through RNA editing [99]. Based on genomic size and the nucleotide sequences of the F and L genes, NDV strains can be categorized as class I or class II [4-5]. Class I NDVs, which have a genomic size of 15,198 nucleotides [64], are occasionally isolated from wild aquatic birds and domestic poultry and are mostly avirulent to chickens. Class II NDVs comprise the majority of virulent NDV strains and some avirulent NDV strains [4-5]. Class II NDVs are further subdivided into 11 genotypes (I-XI) [4, 14, 42, 63, 65, 66, 106]. Early sublineages of Class II NDVs that occurred before the 1960s (genotypes I to IV)

have a genomic size of 15,186 nucleotides, whereas late Class II NDV sublineages (genotypes VI to XI) have a genomic size of 15,192 nucleotides. Class II NDVs under genotype VI and VII are further subdivided into eight (a-h) subgenotypes [4, 14, 42, 63, 65, 66, 106]. Aldous *et al.* [1] proposed the creation of lineages and sublineages in classifying NDVs to make it possible to rapidly type future virus isolates on the basis of their nucleotide sequence and make inferences about their origins. They proposed that NDVs could be divided into six broadly distinct groups (lineages 1 to 6), where lineages 3 and 4 were further subdivided into four sublineages (a to d) and lineage 5 was further subdivided into 5 sublineages (a to e). Genotypes I and II correspond to lineage 1 and 2, while genotype III corresponds to sublineage 3a; genotype IV to sublineage 3b; genotype V to sublineage 3c; genotype VIII to sublineage 3d; genotype VIa and VIe to sublineage 4a; genotypes VIb, VIc and VId to sublineages 4b, 4c and 4d; genotypes VIIa, VIIb, VIIc and VIId to sublineages 5a, 5b, 5c and 5d; 5e to previously characterized genotype VII NDVs from Taiwan and a quarantine isolate in UK that formed a separate cluster from other lineage 5 NDVs; and lineage 6 represents a new NDV genogroup. Recently a novel lineage, provisionally named lineage 7 was reported in West and Central Africa [14].

Different NDV strains vary greatly in pathogenicity [3, 47, 51-55, 74, 107]. NDV isolates can be broadly grouped into five pathotypes on the basis of clinical signs in infected chickens. ND may manifest as viscerotropic velogenic, neurotropic velogenic, mesogenic, lentogenic and asymptomatic enteric [2, 4-6, 51-55, 82, 107]. Other factors, such as host species, host immune status and age, environmental stress, coinfection with other organisms, viral dose and route of exposure, may also influence the severity of the disease [3-4].

In Japan, ND was first reported during the first panzootic in the 1930s. This panzootic was caused by a genotype III NDV. After this time, large ND outbreaks were reported to occur until commercial vaccines became available in the late 1960s [68, 69]. Since then, sporadic outbreaks, mostly in small unvaccinated backyard flocks and pet birds, have been reported [68, 69]. In spite of vaccination, few sporadic outbreaks in vaccinated commercial poultry have also been observed [79].

At present, limited molecular epidemiological data are available regarding the causes of ND outbreaks in vaccinated poultry farms. Knowing the molecular characteristics of NDV strains affecting commercial poultry in spite of vaccination might give important insights on the possible origins and genetic nature of these viruses which may help in formulating more effective ND prevention and control strategies. In addition, no studies have been performed yet investigating the classification of Japanese NDVs at the subgenotype level and if recombination events occur in Japanese NDVs. Knowing the subgenotype classification of NDVs and occurrence of recombination events are essential since these may provide a more direct understanding on the epidemiological relationship of Japanese NDVs with other strains from different parts of the world, which may help further elucidate the mechanisms of global and transcontinental dynamics of transmission and spread of this disease. Therefore in the present study, field strains of NDVs with different geographical and temporal distribution patterns that were isolated from vaccinated commercial poultry flocks in non-epidemic areas of Japan were analyzed. Sequence data were extensively compared with 180 NDV strains from different parts of the world from different time periods.

Materials and Methods

NDV strains

Nine NDV strains isolated from commercial poultry farms with different spatial (Osaka, Ibaraki, Chiba, Fukushima and Miyagi Prefectures) and temporal distribution patterns (1969, 1987, 1999-2002) were used to investigate the molecular epidemiological relationships of ND outbreaks in vaccinated commercial poultry flocks in Japan. These strains were isolated by one to two passages of pooled infected tissues in 10-day-old embryonated specific pathogen-free (SPF) chicken eggs. Infective allantoic fluids were harvested and kept in lyophilized form or in serum tubes and stored at -80°C until further use. All isolates used in this study were provided by Poultry Products Quality Control Co. Ltd. (Fukushima, Japan).

Farm history and clinical profile

Records of management and farm history were obtained to characterize the clinical profile of nine suspected NDV strains (Table 1).

The oldest strain was from Osaka Prefecture in 1969 (JP/Osaka/2440/69). This isolate was recovered from six dead layer birds that were submitted to the Osaka Veterinary Municipal Office for diagnosis. The flock was vaccinated with the live B1 vaccine in drinking water at 5-7 days of age and killed ND vaccine (Sato strain) in aluminum adjuvant at 25 days. The affected flock was around 80 days of age when the disease occurred. Mortality was reported to be around 60-70% but no data were given regarding observed clinical signs and production performance.

The second strain was from a layer farm in Ibaraki Prefecture isolated in 1987 (JP/Ibaraki/SM87/87). Total population of affected farm was 33,360 birds in 12 open-type houses of 2,780 birds each. The disease was reported in one of the houses.

The flock was vaccinated with the live B1 spray at 10days of age, killed Ishii/B1 in aluminum adjuvant at 45 days, live B1 spray again at 60 days and killed Ishii/B1 in aluminum adjuvant again at 120 days. The disease occurred two months after the last vaccination at 180days of age. The disease was characterized by gasping and 25% drop in egg production. Mortality was less than three percent.

The third strain was from Ibaraki Prefecture in 1999 that was isolated from dead spent hens sent for diagnosis (JP/Ibaraki/SG106/99). The isolate was from an unknown farm raising spent hens for liquid egg production. No detailed farm information was obtained. Chickens submitted for diagnosis were approximately more than 700 days old. Necropsy findings were proventriculitis, hemorrhagic lesions in duodenum and petechiae in lymphocytic tissues.

The fourth strain was from a replacement pullet farm in Chiba Prefecture isolated in 2001 (JP/Chiba/BY103/01). The disease occurred in a flock of 21,000 birds. The flock was vaccinated with the live B1 strain in drinking water at 4 and 10 days of age, live B1 spray at 28 days and killed Ishii strain in aluminum adjuvant at 45 and 90 days of age. It was reported that the killed vaccines were injected by hired professional vaccination staff that travel from farm to farms. Six days after last vaccination, the disease occurred characterized by gasping, nervous symptoms, leg weakness, twisting of neck and greenish diarrhea. Mortality was around 10%.

The fifth strain was from a layer farm in Ibaraki prefecture isolated in 2002 (JP/Chiba/BY7/02). The affected farm had a population of 125,000 birds (five houses of 25,000 birds) and affected flock was around 25,000 birds (one house). The flock was vaccinated with the live B1 strain in drinking water at 10 days of age, live B1 spray at 24 days, killed Ishii strain in aluminum adjuvant at 45 days, live B1 spray again at 60 days and killed Ishii strain in aluminum adjuvant again at 95 days. The

disease occurred at 110 days of age characterized by gasping and infectious bronchitis (IB)-like respiratory signs and 20% drop in egg production without significant mortalities. Necropsy findings were necrotic ovarian follicles and necrotic catarrhal inflammation of the intestines.

The sixth strain was from a layer farm located in Ibaraki prefecture in 2002 (JP/Ibaraki/IS5/02). The affected farm had a population of approximately 200,000 birds and the affected flock was around 41,000 birds. The flock was vaccinated with the live B1 spray at 10 and 28 days old, killed Ishii strain in aluminum adjuvant at 45 days, live B1 spray again at 60 days and killed Ishii strain in aluminum adjuvant again at 90 days. The disease occurred at 336 days of age characterized by mild respiratory signs such as gasping, seven percent decrease in egg production with no marked mortalities.

The seventh strain was recovered from dead birds from a replacement pullet farm in Ibaraki prefecture in 2002 (JP/Ibaraki/IS2/02) that were submitted for diagnosis. Total farm population was around 10,000 birds. The flock was vaccinated with the live B1 spray at 10 days of age. The disease occurred four days after vaccination characterized by severe depression.

The eight strain was isolated from a layer farm in Fukushima prefecture (JP/Fukushima/NYF-3/02). The farm had a population of around 120,000 birds and affected flock was approximately 16,000 layers. The flock was vaccinated with the live B1 spray at 10 and 28 days of age, killed Ishii strain in aluminum adjuvant at 45 days, live B1 spray again at 60 days and killed Ishii strain in aluminum adjuvant again at 90 days. The disease occurred around 532 days of age characterized by mild gasping, increase in soft shelled eggs and eight percent decrease in egg production. Mortalities were minimal and within production standards.

The ninth strain was isolated from a layer farm in Miyagi prefecture in 2002 (JP/Miyagi/AGT/02). The flock was vaccinated with the live B1 in drinking water at 10 days of age, live B1 spray at 24 days, killed Ishii strain in oil adjuvant at 45 days, live B1 spray again at 60 days and killed Ishii strain in oil adjuvant again at 95 days. It was informed that the flock was kept in multiple age houses (six different flocks in one house). Disease occurred at 250 days of age characterized by gasping, greenish white diarrhea and 70% decrease in egg production. No marked mortalities were observed.

Biological and pathotypical characterizations

Biological and pathotypical characterization of isolates were performed using mean death time (MDT) in 10-days-old embryonated SPF chicken eggs and intracerebral pathogenicity index (ICPI) in 1-day old chicks according to the protocols described previously [82]. Confirmation of pathotypes was performed by nucleotide sequence analysis of the F0 proteolytic cleavage site (residues 112-117).

Reverse Transcription Polymerase Chain Reaction (RT-PCR)

Nested RT-PCR was performed to confirm the identities of suspected NDV strains. In brief, isolates were propagated once in 10-day-old embryonated SPF eggs. Viral RNA from infected allantoic fluids was extracted directly by using a QIAamp® Viral RNA Mini Kit (Qiagen, West Sussex, UK). Viral RNA was transcribed to cDNA by using random hexamers and Primescript® Reverse Transcriptase (Takara Bio-Inc, Shiga, Japan). cDNA was amplified by PCR as described previously [68-69]. A two-step nested PCR was performed to amplify the region comprising the 3' end of the M-gene and the 5' end of the F-gene using KOD dash® (Toyobo, Osaka, Japan),

5uM of external and internal primers as described by Mase *et al.* [68-69] (Table 5). Thermocycling conditions for the first and second PCR steps were as follows: prewarming at 94^{0}C for 2 min (1 cycle), denaturation at 94°C for 30 sec, annealing at 50°C for 30 sec and extension at 72°C for 1 min. Amplification steps were performed for 35 cycles. The final extension was performed at 72°C for 30 sec (1 cycle).

Nucleotide sequence analysis

Confirmed NDV strains were subjected to additional RT-PCR amplification to characterize the open reading frame of the F-gene sequences (4504-6295 nt of whole NDV genome), 3-prime end of the NP-gene sequences (16-783 nt of whole NDV genome) and 5-prime end of the L-gene sequences (13995-14719 nt of whole NDV genome). In brief, RT-PCR was performed by using SapphireAmp® Fast PCR Master Mix (Takara Bio), 5uM of forward and reverse primers (Table 5) and cDNAs that were transcribed previously. Thermocycling conditions consisted of initial denaturation at 95^{0}C for 2 min followed by 35 cycles of 98^{0}C for 10 sec (denaturation), 55^{0}C for 10sec (annealing), 72^{0}C for 10sec (extensions) and final extension at 72^{0}C for 2min. PCR products were analyzed by electrophoresis with 1.2% agarose gel and purified by using QIAquick® Gel Extraction Kit (Qiagen, Valencia, CA). The nucleotide sequences of PCR products were determined by Big Dye terminator cycle-sequencing kit version 3.1 (Applied Biosystems Inc., Foster City, CA) and an ABI Prism 3130 Genetic Analyzer (Applied Biosystems). DNA products were sequenced from both directions.

Phylogenetic studies

Sequence assembly and editing were performed using CodonCode Aligner® (version 3.7.1, CodonCode Corporation, MA) and ClustalX® (version 2.1, Conway Institute UCD Dublin, Ireland). Deduced amino acid sequences were determined using Bioedit® software package version 7.1.3.0 [40]. Confirmation of identity and homology were performed using BLAST http://www.ncbi.nlm.nih.gov.

To determine the molecular epidemiological relationships of field strains, 180 NDV strains isolated from different regions of the world at different time periods were obtained from GenBank. These reference strains were representatives of all the different NDV genotypes and subgenotypes. Phylogenetic and molecular evolutionary analyses were conducted using MEGA version 4 [101]. Phylogenetic trees of the variable region of the F-gene sequences (47-421 nt), complete coding region of the F-gene sequences (1-1662 nt), coding region of the 3-prime end of NP-gene sequences (1-622 nt) and coding region of the 5-prime end of L-gene sequences (5629-6333 nt) were constructed by the neighbor-joining method with the maximum composite likelihood substitution model at 1000 bootstrap replicates.

Determination of recombination events, evolutionary distances and selection profile

Intragenic recombination events in the NDV nucleotide sequences were determined using RDP v3.44 program [67]. Seven different algorithms integrated in the program namely RDP, GeneConv, Bootscan, MaxChi, Chimaera, SiScan and 3Seq were applied to detect any putative recombination breakpoints and to estimate the occurrence of any recombination events within all the analyzed genes. Sequences with recombination events identified by at least two detection methods ($p < 0.01$) were considered as true recombinants. Intragenic recombination events within the F-gene

were also determined by comparing the tree topology of phylogenetic analyses using the variable region (47-421 nt) and complete coding region (1-1662 nt) of the F-gene. Intergenic recombination events were determined by comparison of topologies of the generated F, NP and L-gene phylogenetic trees.

Evolutionary distances were calculated using MEGA version 4 using the Maximum Composite Likelihood method. Codon positions included were the 1st+2nd+3rd+noncoding. All positions containing gaps and missing data were eliminated from the dataset (complete deletion option). Estimates of s.e. were obtained by a bootstrap procedure of 1000 replicates. Analysis of evolutionary selection profile was performed using Datamonkey http://www.datamonkey.org/ following the Fixed- Effect Likelihood (FEL) method [Hasegawa, Kishino and Yano (HKY) model, p-value less than 0.05] [31, 86].

Results

Biological and pathotypical characterizations

All suspected NDV isolates yielded a 766-bp product in the nested PCR amplification step. This confirmed that all isolates belong to Avian Paramyxovirus type-1 viruses. All strains exhibited mean death time (MDT) of 48 to 56 hours in embryonated chicken eggs. Intracerebral pathogenicity index (ICPI) values ranged from 1.7 to 1.9 (Table 1). Nucleotide sequence analyses of the variable region of the F-gene (47-421 nt) showed that all isolates had multiple basic amino acids at its F0 proteolytic cleavage site (residues 112-117). Predicted amino acid sequence of the F0 cleavage site was ^{112}RRQKR^{116} at the F2 protein and phenylalanine (^{116}F^{117}) at the N-terminus of the F1 protein for all strains. These results indicated that all isolates were velogenic.

Genetic and phylogenetic characterizations

A total of 1662 nucleotides encoding for 553 amino acid residues were identified in the complete coding region of the F-gene of all field strains. Six potential N-glycosylation sites (Asn-X-Ser/Thr where X is any amino acid except proline or aspartic acid) located at positions 85 to 87, 191 to 193, 366 to 368, 447 to 449, 471 to 473 and 541 to 543 were recognized. Twelve cysteine residues located at positions 25, 76, 199, 338, 347, 362, 370, 394, 399, 401, 424 and 523 were identified. Comparison of glycosylation sites and cysteine residues showed no changes in the amino acid sequence in all field strains, which may indicate that these sites were highly conserved.

Analysis of seven neutralizing epitopes located at positions 72, 74, 75, 78, 79, 157 to 171 and 343 of the F-protein showed a K to R amino acid substitution at position 78 in seven of the nine strains (Table 2). These strains were JP/Ibaraki/SG106/99, JP/Ibaraki/IS5/02, JP/Chiba/BY103/01, JP/Ibaraki/IS2/02, JP/Miyagi/AGT/02, JP/Chiba/BY7/02 and JP/Fukushima/NYF-3/02. Furthermore, analysis of amino acid substitutions showed 13 point mutations in the variable region of the F-gene of the Japanese field strains (Table 2). Genotypic and subgenotypic-specific amino acid substitutions were also observed, which were consistent with the proposed theory of NDV evolution as reported previously [114].

Phylogenetic analyses of nine field strains and 180 NDV strains from GenBank were performed by using contiguous nucleotide sequences of the F-gene, NP-gene and L-gene. Reference strains from GenBank were selected as representatives of nine of the 11 ND genotypes (genotypes I to XI) representing isolates from different regions of the world (Figures 1-3).

Field NDV strains were observed to belong to two distinct genotypic groups (genotype VI and VII) using phylogenetic analysis of the complete coding sequence of the F-gene. Early isolates such as JP/Osaka/2440/69 and JP/Ibaraki/SM87/87 were genotype VI, while field strains isolated from 1999 onwards were from genotype VII (Figures 1 and 4). Phylogenetic analysis using the 3-prime portion of NP-gene and 5-prime portion of L-gene yielded the same tree topology and phylogenetic groupings (Figures 2-3). Phylogenetic analyses at the subgenotype level using the variable region of the F-gene sequences revealed that JP/Osaka/2440/69 belongs to subgenotype VIa, JP/Ibaraki/SM87/87 to subgenotype VId and all the recent field isolates to subgenotype VIId (Figures 5 and 6).

F-gene nucleotide sequence of JP/Osaka/2440/69 was found to be closely related (98.5-99.2% nucleotide sequence identity) to isolates from the Middle East. The F-gene sequence of JP/Ibaraki/SM87/87 was closely related (96.3-100.0%) with isolates from Japan and China. All the other VIId isolates were highly similar (97.2-100.0%) to isolates from Japan, China and Taiwan and interestingly to a goose isolate from China (98.4-98.6%) (Table 3).

No intragenic nor intergenic recombination events (Unique events = 0; Recombination signals = 0) were observed involving the field isolates using all the described methods. Analysis of the over-all mean evolutionary distance among the Japanese field isolates showed rates of $3.9x10^{-2}$ [standard error (s.e) 0.01], $3.1x10^{-2}$ (s.e. 0.01) and $2.0x10^{-2}$ (s.e. 0.01) base substitution per site in the full F-gene, partial NP-gene and partial L-genes, respectively. In contrast, over-all mean evolutionary distance among the recent field strains (1999-2002) were $1.0x10^{-3}$ (s.e. 0.001) base substitution per site for the F and NP-genes and zero base substitution for the L-gene. In addition, 25 sites in the F-gene of all field strains were observed to be under

negative selection (p-value < 0.05) (Table 4).

Discussion

ND remains a serious threat to commercial poultry even though intensive vaccination programs are being applied. In Japan, occasional outbreaks have been reported in commercial poultry mostly due to improper vaccination, immunosuppression due to infectious and non-infectious causes, and challenge by more velogenic viruses [79]. However, limited data are available regarding the genomic characteristics of NDVs occurring in vaccinated commercial poultry flocks. Knowing the genetic characteristics of wild strains of NDV affecting vaccinated poultry might give important insights on the possible origins, transmission mechanisms and infection routes of these viruses. Molecular and phylogenetic studies like this are important since these might lead to better understanding on how to prevent, control and manage future ND cases.

Molecular characterization of NDV strains mostly considered the F-gene with particular emphasis given on the variable region (47-421 nt) because it codes for a number of functionally important structures such as signal peptide [amino acid (aa) 1-31], cleavage activation sequence (aa 112-116), portion of the fusion inducing hydrophobic region (aa 117-142) and it is characterized by both variable and conserved regions [70, 105]. Nucleotide sequence of the F-gene fragment (nt 47-420) is regarded as standard criterion for genotyping [88]. A molecular basis of pathogenicity has also been well established through sequence analysis of F-protein cleavage site. It was reported that the motiff ^{112}R/K-R-Q-K/R-R^{116} at the C-terminus of the F2 protein and F (phenylalanine) at the N-terminus of the F1 protein (residue 117) are major determinants of viral virulence [3-4, 36, 69, 78, 83, 114]. A huge

database of sequence data especially on F-gene sequences of NDVs isolated throughout the world has also been published and available for sequence comparison and phylogenetic studies [49].

Records of management and farm history showed that NDV strains used in this study originated from farms with diverse geographical, temporal and disease profiles. It is noteworthy to emphasize that these farms were thoroughly vaccinated against NDV but they were still infected with the disease. Moreover, deduced amino acid sequence of cleavage site of the F-gene of all field isolates revealed the motif ^{112}R-R-Q-K-R-F^{117} indicating that all strains were velogenic. This was further confirmed by MDT and ICPI tests. These indicate that in spite of the regular use of inactivated and live vaccines, velogenic ND may still occur in vaccinated flocks. However, some affected birds showed only mild respiratory symptoms without significant mortalities and severe pathological lesions. In some flocks, only mild to moderate decrease in egg production was observed.

Seven major epitopes have been identified involving the fusion inhibition and neutralization of F-protein [80, 105, 115]. Individual amino acids at 72, 74, 75, 78, 79 and 343 and a stretch of amino acids from residues 157-171 were identified to be critical for both structures and functions of the F-gene. In this study, nucleotide substitution in one of the fusion inhibition and neutralizing epitope (p.K78R) was identified in all of the seven VIId strains. 13 point mutations were also identified in the variable region of the F-gene. Comparison with sequence data from reference strains (n= 180) showed that among these mutations, p.K4I were conserved only in NDV strains originating from Japan while p.L21P, p.I52V, p.K78R and p.R101K were conserved in strains originating from the Far East Asia (Japan, China and Taiwan). These substitutions maybe used as crude molecular markers of geographic origins of

NDVs.

Analysis of genotypic and subgenotypic substitutions in the hypervariable region of the F-gene showed findings that were in conformity with the proposed theory of NDV genetic evolution [114]. It was proposed that subgenotype VIa from the second pandemic probably evolved to VIc by production of a crucial p.S107T substitution and VId by production of p.S93T substitution; VIIb evolved from VIb via a VII-specific p.V121I substitution; VIIb evolved to become VIIa and VIIc through p.K101R substitution; and VIIc evolved to become VIId by the production of additional p.I52V and p.F314Y substitution [114].

A point mutation in the F-gene resulted to neutralizing epitope variant. However whether this mutation was part of adaptive mechanism of NDVs to evade the immune response to be able to infect vaccinated chickens is not clear or whether this mutation was actually the effect of selective immune pressure exerted on ND viral particles as a consequence of vaccination is also unknown. To understand how wild NDVs infect vaccinated chickens, this identified mutation may be useful for future site-directed mutagenesis studies.

Phylogenetic analyses on the field strains using the variable region of the F-gene (47-421 nt) revealed that JP/Osaka/2440/69 belongs to genotype VIa (Figure 5). As reported previously [2], genotype VIa was responsible for the second ND panzootic that started in the Middle East during the 1960s and then spread to most countries around the world as a result of enormous trade and importation of captive caged birds and technological advances in air transportation. Interestingly, this isolate shared 100% sequence identity with JP/Narashino/68, which was isolated from a Japanese Blue Magpie. It is interesting to note that nucleotide sequence identity of Iraq/AG-68 was 98.5% similar while strains Kuwait/256/68, Lebanon/70 and

Israel/70 were 98.7-99.2% similar with these Japanese strains. It is possible that JP/Narashino/68 was a foreign strain that was introduced to Japan from wild birds. JP/Narashino/68 and/or its progenitor might have been then spread to domestic chickens in Japan, leading to the isolation of JP/Osaka/2440/69 (100% similar) (Table 2).

Phylogenetic analyses on JP/SM87/87 showed that this strain belongs to VId ND viruses (Figure 5). VId viruses together with VIb and VIc were responsible for the third panzootic, which were reported to be spread by pigeons. This strain shared 100% sequence identity with JP/Tochigi/85 and JP/Ibaraki/85, which may indicate that JP/SM87/87 was a product of the ongoing outbreak.

The seven remaining field isolates belong to genotype VIId (Figure 6). Genotype VII is the most predominant NDV genotype that is responsible for most outbreaks in East Asian countries including Taiwan, Korea and China since the 1980s, constituting the fourth pandemic [4, 42, 61-63, 65, 114]. Also in Japan, the isolation of genotype VII viruses was reported previously [68-69]. Therefore, this genotype has been the most predominant NDV in recent outbreaks in Japan.

The earliest VIId viruses on record infected chickens from South Korea in 1995 (Figure 6). These strains include Kr-279/95, Kr-146/95 and Kr-077/95. On the other hand, the earliest VIId NDVs that were reported from Japan were JP/Tokyo/96 from chickens and JP/Ibaraki-ph/97 from a pheasant (99.0% similar to one another), which might indicate that the two strains were part of an ongoing outbreak. Remarkably, JP/Tokyo/96 shared 99.0% sequence identity with GX-1/97, which was isolated from a chicken flock in Western China, FJ-2/99 from a fowl from China and GD/1/98/Go from a goose from China (Table 2). Remarkably, GD/1/98/Go was 98.4-98.6% similar with JP/Ibaraki/SG106/99 and all the other VIId field strains in

this study. These findings may indicate that wild birds have played a role in the circulation of VIId viruses across the Far East Asian countries (Korea, Japan and China). A comparison of homologies with contemporary isolates also showed that JP/Ibaraki/SG106/99 and all the other VIId field strains were highly similar (99-100%) with JP/Ibaraki/00. Interestingly, a p.K78R amino acid substitution in the F-protein of this strain was also reported previously [69]. Moreover, it was shown that chickens that were challenged with JP/Ibaraki/00 survived a cross-protection test after vaccination with B1 strain, however it was noted that vaccination did not prevent infection and excretion of the virus [69]. This result was partially correlated with the clinical profile of the infected flocks seen in this study. Although the infected flocks survived the infection in this study, problems with production performance were observed.

Comparison among the recent Japanese field strains showed that these strains have high F-gene homologies (99.7-100%), which may indicate that these strains may have been epidemiologically related. Computation of the over-all mean evolutionary distance of the F-gene of these recent strains showed a substitution rate of $1.0x10^{-3}$ (s.e. 0.001) base substitution per site in the span of 3 years (1999-2002). In contrast, over-all F-gene mean evolutionary distance in all field strains (1969-2002) was $3.9x10^{-2}$ (s.e. 0.010) base substitution per site. Interestingly, the partial NP gene showed an almost same substitution rate ($1.0x10^{-3}$ and $3.1x10^{-2}$) while partial L-gene had the lowest rate of substitution (zero and $2.0x10^{-2}$). However, because of incomplete sequence data in NP and L-genes, direct comparison among these substitution rates is not feasible in this study. In other studies, it was reported that among the NDV proteins, the F and P-protein have the highest rate of change ($0.78\text{-}1.98x10^{-3}$ and $0.78\text{-}2.32x10^{-3}$ substitution/site/year, respectively) while L and

NP-proteins have the lowest rate of change ($0.59\text{-}1.44\times10^{-3}$ and $0.45\text{-}1.50\times10^{-3}$ substitution/site/year, respectively) [23, 76]. In addition, analysis of evolutionary selection profiles of the Japanese field strains revealed 25 sites in the F-gene sequence that were under negative selection (p-value < 0.05) (Table 4). No positive selection sites were identified. This is in agreement with the findings of other authors that over-all NDV proteins are under strong purifying and negative selection pressures [23, 76].

Phylogenetic analyses using the nucleotide sequences of NP gene, L-gene, complete F-gene coding sequence and variable region of the F-gene resulted to almost similar tree topologies. Surprisingly phylogenetic analysis using NP gene resulted to a clearer differentiation among field strains. These may indicate that NP and L-genes may be alternative methods to characterize NDVs given that like the F-gene, these genes are also involved in the dynamics of viral virulence, play important functional roles in the NDV replication cycle and are also characterized by regions with high conservation necessary to identify homologies among strains but also characterized by regions with high variations necessary to identify specific variations between strains. Phylogenetic analysis of NP and L-genes in conjunction with the F-gene may also help detect possible natural or artificial recombination events.

This investigation showed that all field isolates from vaccinated commercial poultry were part of much bigger outbreaks affecting not only provinces or regions but even entire continents. To determine how commercial farms are being infected with NDV, the epidemiology of NDV in the whole of Japan and parts of Far East Asia was analyzed. This study showed that Japanese poultry was affected by at least four panzootics and that outbreaks were mostly characterized by co-circulation of genetically distinct virus lineages that were consistent with the predominant virus

genotype circulating in a particular time period. Moreover, no distinct transition was observed from each panzootics. Aside from involvement of local strains, ND outbreaks in Japan were mostly due to virus transmission from infected wild birds either by international bird trade or migration patterns. It is possible that these strains were then introduced to the affected farms through direct or indirect contact with these wild birds or through the movement of farm workers, fomites and contaminated equipments.

A point mutation in one of the neutralizing epitopes of the F-protein resulting to occurrence of neutralizing epitope variants was also identified. This identified mutation may be useful for future site-directed mutagenesis to understand the dynamics of NDV infection in vaccinated chickens.

Table 1. Clinical profile and biological characterization of the Japanese field strains

NDV isolate	Host (Approximate age)	Region of origin	Year isolated	Clinical profile of affected flocks	ICPI	MDT	Genotype
JP/Osaka/2440/69	layer chickens (80d)	Osaka	1969	mortality of 60-70%; no detailed history; sample received for diagnosis;	1.8	56h	VIa
JP/Ibaraki/SM87/87	layer chicken (180d)	Ibaraki	1987	mortality less than 3%; 25% drop in egg production; gasping and mild respiratory signs	1.7	56h	VId
JP/Ibaraki/SG106/99	layer chicken (700d)	Ibaraki	1999	no detailed history; sample received for diagnosis; necropsy lesions were proventriculitis, petechiae in duodenum and lymphocytic tissues	1.7	48h	VIId
JP/Chiba/BY103/01	layer chicken (96d)	Chiba	2001	mortality around 10%, nervous signs, gasping, leg weakness; twisted neck	1.8	48h	VIId
JP/Chiba/BY7/02	layer chicken (110d)	Chiba	2002	mild respiratory signs; gasping, swollen face, 20% drop in egg production; no significant mortality	1.7	56h	VIId
JP/Ibaraki/IS5/02	layer chicken (336d)	Ibaraki	2002	mild respiratory signs; gasping, seven percent decrease in egg production; no significant mortality	1.8	48h	VIId
JP/Ibaraki/IS2/02	layer chicken (14d)	Ibaraki	2002	Severe depression	1.9	48h	VIId
JP/Fukushima/NYF3/02	layer chicken (532d)	Fukushima	2002	mild respiratory signs; gasping, soft-shelled eggs; eight percent decrease in egg production; no significant mortality	1.8	48h	VIId
JP/Miyagi/AGT/02	layer chicken (250d)	Miyagi	2002	mild respiratory signs, gasping; greenish diarrhea; 70% decrease in egg production; no significant mortalities	1.8	48h	VIId

Table 2. Amino acid substitution in the variable region and neutralizing epitopes of the F-gene sequences of the Japanese field strains

	Hypervariable region													Neutralizing epitopes						
Virus	4	10	11	13	20	21	27	52	63	78	93	101	121	72	74	75	78	79	157-171	343
Consensus[a]	K	P	A	L	M	L	C	I	V	K	T	R	V	D	E	A	K	A	SIAATNEAVHEVT	L
JP/ Osaka/2440/69	-[b]	-	V	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
JP/Ibaraki/SM87/87	-	L	V	P	T	-	-	-	I	-	S	-	I	-	-	-	-	-	-	-
JP/Ibaraki/SG106/99	I	-	-	-	-	P	R	V	-	R	-	K	-	-	-	-	R	-	-	-
JP/Chiba/BY103/01	I	-	-	-	-	P	R	V	-	R	-	K	-	-	-	-	R	-	-	-
JP/Ibaraki/IS5/02	I	-	-	-	-	P	R	V	-	R	-	K	-	-	-	-	R	-	-	-
JP/Ibaraki/IS2/02	I	-	-	-	-	P	R	V	-	R	-	K	-	-	-	-	R	-	-	-
JP/Miyagi/AGT/02	I	-	-	-	-	P	R	V	-	R	-	K	-	-	-	-	R	-	-	-
JP/Chiba/BY7/02	I	-	-	-	-	P	R	V	-	R	-	K	-	-	-	-	R	-	-	-
JP/Fukushima/NYF3/02	I	-	-	-	-	P	R	V	-	R	-	K	-	-	-	-	R	-	-	-
JP/Sato/30	R	-	-	-	A	-	H	-	-	-	-	-	I	-	-	-	-	-	n.d.	n.d.
US/B1/47	R	-	-	M	A	-	-	-	-	-	-	-	I	-	-	-	-	-	-	-
LaSota/46	R	-	-	M	A	-	-	-	-	-	-	-	I	-	-	-	-	-	-	-
US/JP/Ishii/62	R	-	V	-	V	-	-	-	-	-	-	-	I	-	-	-	-	-	-	-

[a] The consensus amino acid sequence was derived from 100-180 velogenic, mesogenic and lentogenic NDV strains from GenBank; [b] Same as consensus sequence.

Table 3. Nucleotide sequence identity of the field strains using the complete coding region of the F-gene sequences (1-1662 nt)

Strain name (genotype)	Nucleotide sequence identity (%)								
	JP/Osaka/ 2440/69	JP/Ibaraki/ SM87/87	JP/Ibaraki/ SG106/99	JP/Chiba/ BY103/01	JP/Chiba/ BY7/02	JP/Ibaraki/ IS-5/02	JP/Ibaraki/ IS2/02	JP/Fukushima/ NYF-3/02	JP/Miyagi/ AGT/02
JP/Osaka/2440/69 (VIa)	100.0	96.1	92.2	92.2	92.3	92.2	92.2	92.1	92.3
JP/Narashino/68 (VIa)	100.0	95.5	91.4	91.4	91.4	91.4	91.4	91.2	91.4
JP/Chiba/69 (VIa)	99.5	94.9	90.9	90.9	90.9	90.9	90.9	90.6	90.9
Lebanon/70 (VIa)	99.2	95.9	90.8	90.8	90.8	90.8	90.8	90.5	90.8
Kuwait/256/68 (VIa)	99.0	96.1	90.5	90.5	90.5	90.5	90.5	90.2	90.5
Israel/70 (VIa)	98.7	95.9	90.8	90.8	90.8	90.8	90.8	90.5	90.8
Iraq/AG-68 (VIa)	98.5	95.6	90.0	90.0	90.0	90.0	90.0	89.7	90.0
ASTR/74 (VIa)	97.3	94.1	89.3	89.3	89.3	89.3	89.3	89.0	89.3
California/1083(Fontana)/71 (VI)	96.8	94.8	91.9	91.9	91.9	91.9	91.9	91.8	91.9
NewYork/44407/84 (VIb)	95.8	93.9	90.1	90.1	90.2	90.1	90.1	90.1	90.2
JP/Ibaraki/SM87/87 (VId)	96.1	100.0	90.1	90.1	90.2	90.1	90.1	90.0	90.2
JP/Tochigi/85 (VId)	95.5	100.0	88.0	88.0	88.0	88.0	88.0	87.7	88.0
JP/Ibaraki/85 (VId)	96.2	99.9	90.1	90.1	90.2	90.1	90.1	90.0	90.2
Sweden/95 (VId)	95.1	97.6	89.5	89.5	89.6	89.5	89.5	89.4	89.6
DK-1/95 (VId)	94.6	97.3	87.4	87.4	87.4	87.4	87.4	87.1	87.4
CH-1/95 (VId)	94.7	96.8	87.4	87.4	87.4	87.4	87.4	87.2	87.4
TW/99-154 (VId)	94.9	96.3	88.0	88.0	88.0	88.0	88.0	88.2	88.0
Kr-102/89 (VIf)	95.7	95.3	90.3	90.3	90.4	90.3	90.3	90.2	90.4
Zhj-2/86 (VIg)	96.3	98.7	90.2	90.2	90.3	90.2	90.2	90.1	90.3
Sh-1/97 (VIg)	95.8	98.3	89.8	89.8	89.9	89.8	89.8	89.7	89.9
JP/Ibaraki/SG106/99 (VIId)	92.2	90.1	100.0	100.0	99.9	100.0	100.0	99.8	99.9
JP/Chiba/BY103/01 (VIId)	92.2	90.1	100.0	100.0	99.9	100.0	100.0	99.8	99.9
JP/Chiba/BY7/02 (VIId)	92.3	90.2	99.9	99.9	100.0	99.9	99.9	99.8	100.0
JP/Ibaraki/IS-5/02 (VIId)	92.2	90.1	100.0	100.0	99.9	100.0	100.0	99.8	99.9
JP/Ibaraki/IS2/02 (VIId)	92.2	90.1	100.0	100.0	99.9	100.0	100.0	99.8	99.9
JP/Fukushima/NYF-3/02 (VIId)	92.1	90.0	99.8	99.8	99.8	99.8	99.8	100.0	99.8
JP/Miyagi/AGT/02 (VIId)	92.3	90.2	99.9	99.9	100.0	99.9	99.9	99.8	100.0
JP/Ibaraki/00 (VIId)	91.4	88.0	100.0	100.0	100.0	100.0	100.0	99.7	100.0
JP/Gunma/01 (VIId)	91.4	88.0	100.0	100.0	100.0	100.0	100.0	99.7	100.0
FJ-2/99 (VIId)	91.7	88.2	98.7	98.7	98.7	98.7	98.7	98.4	98.7
GD/1/98/Go (VIId)	93.1	91.0	98.5	98.5	98.6	98.5	98.5	98.4	98.6
JP/Tokyo/96 (VIId)	92.0	88.5	98.4	98.4	98.4	98.4	98.4	98.1	98.4
JP/Ibaraki-ph/97 (VIId)	92.0	88.5	98.4	98.4	98.4	98.4	98.4	98.1	98.4
GX-3/98 (VIId)	92.9	90.0	98.3	98.3	98.3	98.3	98.3	97.9	98.3
TW/98-1 (VIId)	91.8	88.4	98.2	98.2	98.2	98.2	98.2	97.9	98.2
TW/98-2 (VIId)	91.5	88.2	98.2	98.2	98.2	98.2	98.2	97.9	98.2
XJ-2/97 (VIId)	93.6	91.2	97.8	97.8	97.9	97.8	97.8	97.7	97.9

Table 4. Nucleotide positions in the F-gene sequence of the Japanese field strains that were under negative selection[a]

Nucleotide position	dS[b]	dN[c]	dN/dS	Normalized dN-dS	p-value
29	70.9	0.0	0.0	-599.4	0.01
80	10.4	0.0	0.0	-88.1	0.04
94	64.8	0.0	0.0	-547.6	0.01
102	32.4	0.0	0.0	-273.7	0.01
106	175.4	0.0	0.0	-1482.8	0.04
109	31.2	0.0	0.0	-263.8	0.03
139	10.5	0.0	0.0	-88.1	0.03
151	196.7	0.0	0.0	-1662.4	0.02
164	10.2	0.0	0.0	-85.9	0.03
173	10.4	0.0	0.0	-88.1	0.04
198	17.6	0.0	0.0	-148.7	0.03
229	33.4	0.0	0.0	-282.5	0.03
230	58.2	0.0	0.0	-491.6	0.02
278	10.4	0.0	0.0	-88.1	0.04
294	17.6	0.0	0.0	-148.7	0.03
295	36.7	0.0	0.0	-310.1	0.03
354	33.4	0.0	0.0	-282.5	0.03
378	86.0	0.0	0.0	-726.7	0.00
405	31.2	0.0	0.0	-263.8	0.03
416	31.2	0.0	0.0	-263.8	0.02
428	215.0	0.0	0.0	-1816.5	0.02
470	58.2	0.0	0.0	-491.7	0.01
474	32.4	0.0	0.0	-273.7	0.01
583	19.8	0.0	0.0	-167.5	0.04
510	33.7	0.0	0.0	-284.4	0.02

[a] dN < dS negative selection, dN > dS positive selection, dN = dS neutral; [b] number of synonymous substitutions per synonymous site; [c] number of non-synonymous substitutions per non-synonymous site;

Table 5. Primers used in this study

Primer name	Nucleotide position[a]	Primer sequence
NDV-For1	1-15	5`-GGCCACGCGTCGACTAGTACACCAAACAGAGAATC-3`[b]
NDV-For482	482-501	5'-TYTGAGGAGAGRGCACAGAG-3'
NDV-Rev784	803-784	5`-TGYTGTSWRCARAAYTCRTG-3`
M1[c]	4175-4194	5`-TTCTCTAGCAGTGGGACAGC-3`
M2	4241-4264	5'-TGGAGCCAAACCCGCACCTGCGG-3'
F1	5095-5076	5'-CATCTTCCCAACTGCCACTG-3'
F2	5006-4988	5'-GGAGGATGTTGGCAGCATT-3'
NDV-For4359	4359-4382	5'-CCATTGCTAAATACAATCCTTTCA-3'
NDV-Rev4788	4788-4769	5'-GGGGCTTTYGCACACGCCTC-3'
NDV-For4988	4988-5007	5'-AATGCCGCCAACATCCTCCG-3'
NDV-Rev5261	5261-5241	5'-GTGCCTGGATAGTCAGCTGAG-3'
NDV-For5461	5461-5482	5'-GACYTTATCTGTAAGYACAACC-3'
NDV-Rev5731	5731-5711	5'-CAATTGGCAATAACTGAGCC-3'
NDV-For5918	5918-5940	5'-GTGACAGGCAAYCTTGATATATC-3'
NDV-Rev6204	6204-6185	5'-CTTGTAGTGGCTCTCATCTG-3'
NDV-For6369	6369-6388	5'-AGGCYTCACAACATCYGTTC-3'
NDV-Rev6598	6598-6579	5'-TYGATATGCCTRCGAGRTCG-3'
NDV-For13970	13970-13994	5'-GCAGTGGGATATATCACATCTGTGG-3'
NDV-For14492	14492-14511	5'-CAGCCYGTCCGTCCATTCTG-3'
NDV-Rev14739	14739-14720	5'-CTGAGACCCAGTATTGTGAC-3'

[a] The nucleotide position of primers corresponds with the whole genome nucleotide sequences of NDV strain U.S.(CA)/1083(Fontana)/72 (accession # AY562988);
[b] Twenty nucleotide long of 5'-end of primer NDV-For1 is adapter sequence; [c] M1, M2, F1 and F2 primers are reported by Mase *et al.* [68].

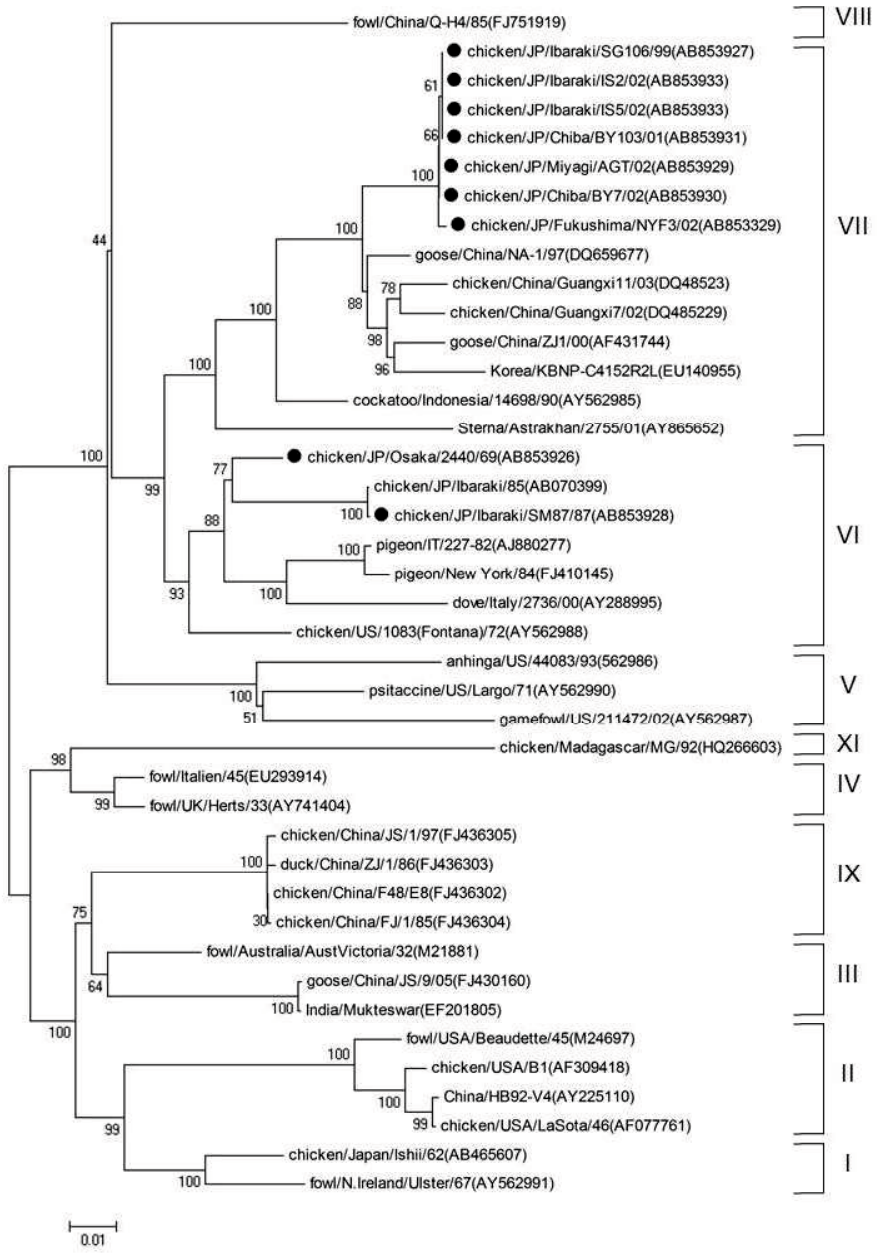


Figure 1. Phylogenetic tree of the complete coding region of F-gene sequences (1-1662 nt). The evolutionary history was inferred using the Neighbor-Joining method. The bootstrap consensus tree inferred from 1000 replicates is taken to represent the evolutionary history of the taxa analyzed [34]. Branches corresponding to partitions reproduced in less than 50% bootstrap replicates are collapsed. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (1000 replicates) are shown next to the branches. The tree is drawn to scale, with branch lengths in the same units as those of the evolutionary distances used to infer the phylogenetic tree. The evolutionary distances were computed using the Maximum Composite Likelihood [102] method and are in the units of the number of base substitutions per site. All positions containing gaps and missing data were eliminated from the dataset (Complete deletion option). Strains used in this study are marked with ●.

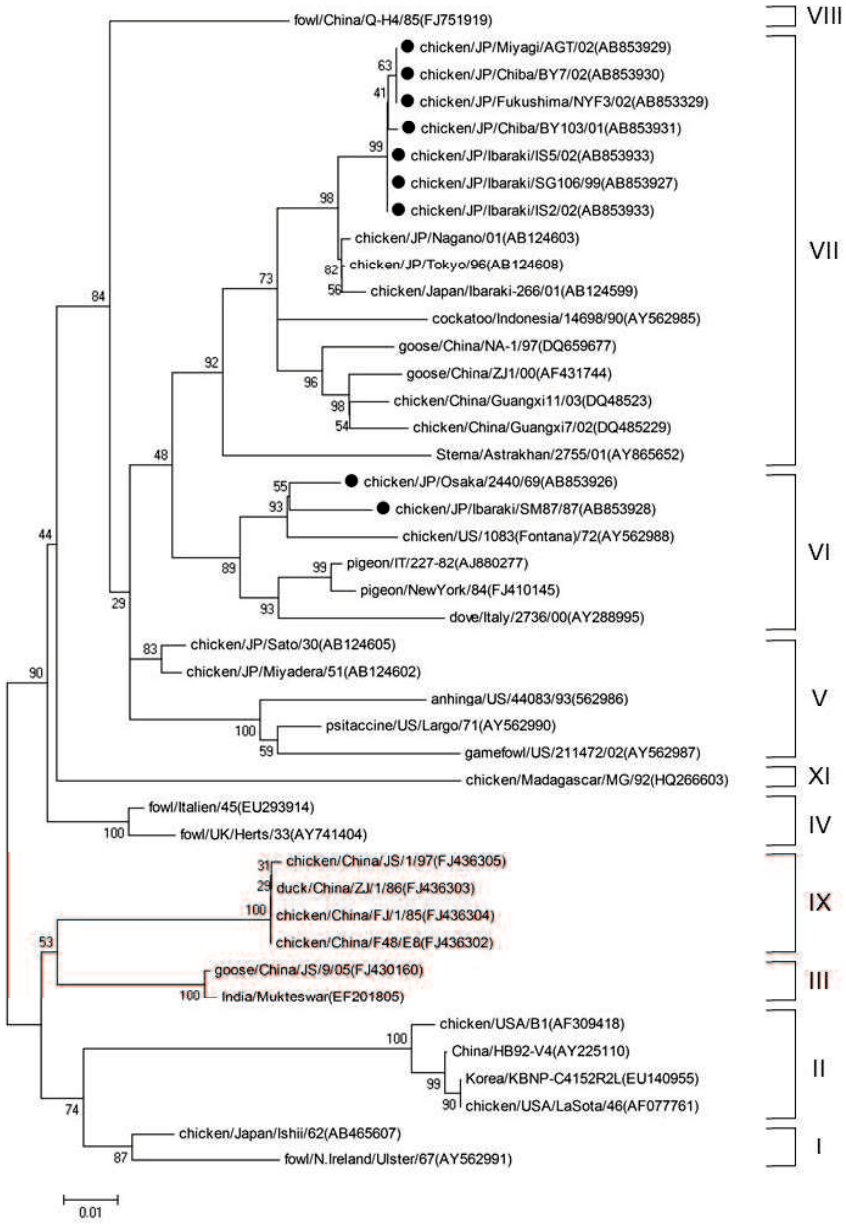


Figure 2. Phylogenetic tree of the 3-prime end of NP-gene sequences (1-622 nt).

The evolutionary history was inferred using the Neighbor-Joining method. The bootstrap consensus tree inferred from 1000 replicates is taken to represent the evolutionary history of the taxa analyzed [34]. Branches corresponding to partitions reproduced in less than 50% bootstrap replicates are collapsed. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (1000 replicates) are shown next to the branches. The tree is drawn to scale, with branch lengths in the same units as those of the evolutionary distances used to infer the phylogenetic tree. The evolutionary distances were computed using the Maximum Composite Likelihood [102] method and are in the units of the number of base substitutions per site. All positions containing gaps and missing data were eliminated from the dataset (Complete deletion option). Strains used in this study are marked with ●.

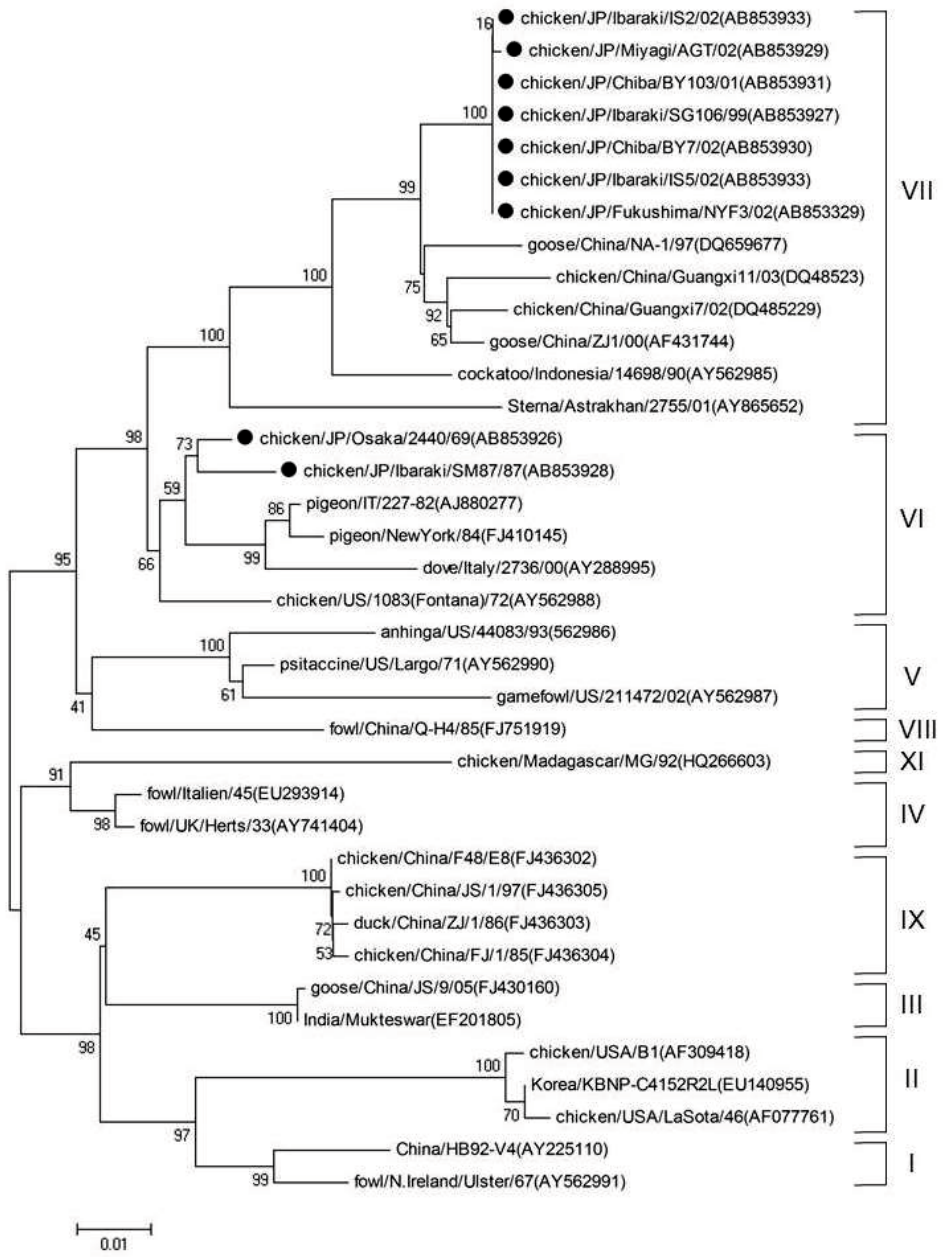


Figure 3. Phylogenetic tree of the 5-prime end of L-gene sequences (5629-6333 nt).

The evolutionary history was inferred using the Neighbor-Joining method. The bootstrap consensus tree inferred from 1000 replicates is taken to represent the evolutionary history of the taxa analyzed [34]. Branches corresponding to partitions reproduced in less than 50% bootstrap replicates are collapsed. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (1000 replicates) are shown next to the branches. The tree is drawn to scale, with branch lengths in the same units as those of the evolutionary distances used to infer the phylogenetic tree. The evolutionary distances were computed using the Maximum Composite Likelihood [102] method and are in the units of the number of base substitutions per site. All positions containing gaps and missing data were eliminated from the dataset (Complete deletion option). Strains used in this study are marked with ●.

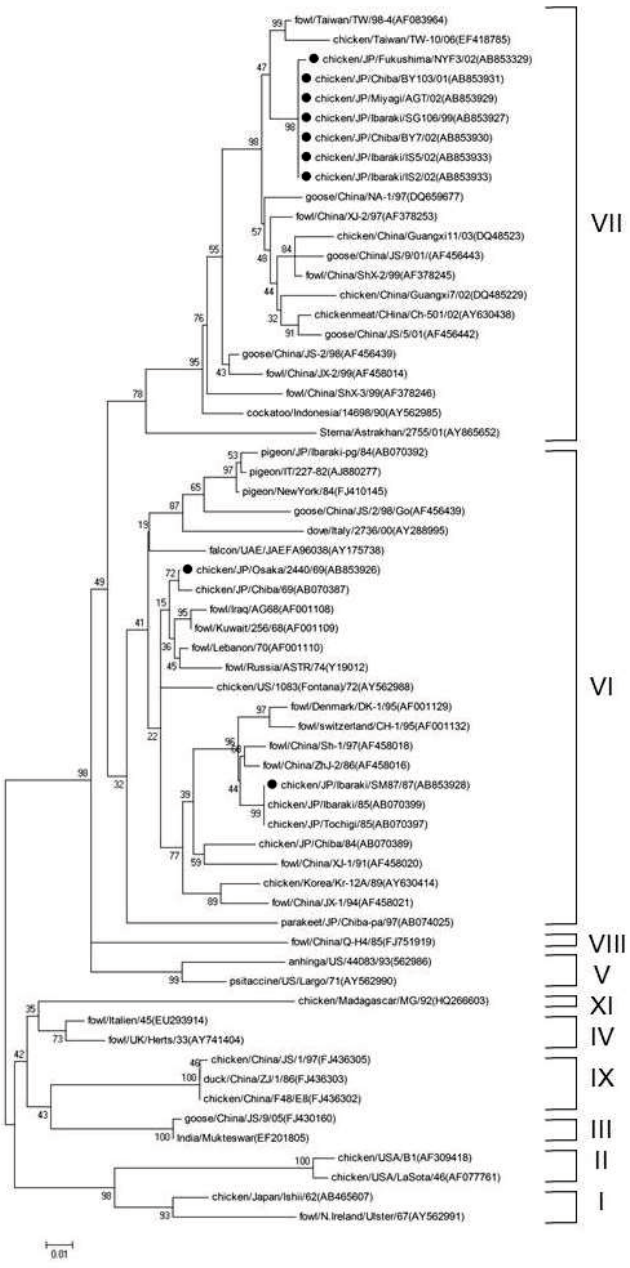


Figure 4. Phylogenetic tree of the variable region of the F-gene sequences (47-421nt).

The evolutionary history was inferred using the Neighbor-Joining method. The bootstrap consensus tree inferred from 1000 replicates is taken to represent the evolutionary history of the taxa analyzed [34]. Branches corresponding to partitions reproduced in less than 50% bootstrap replicates are collapsed. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (1000 replicates) are shown next to the branches. The tree is drawn to scale, with branch lengths in the same units as those of the evolutionary distances used to infer the phylogenetic tree. The evolutionary distances were computed using the Maximum Composite Likelihood [102] method and are in the units of the number of base substitutions per site. All positions containing gaps and missing data were eliminated from the dataset (Complete deletion option). Strains used in this study are marked with ●.

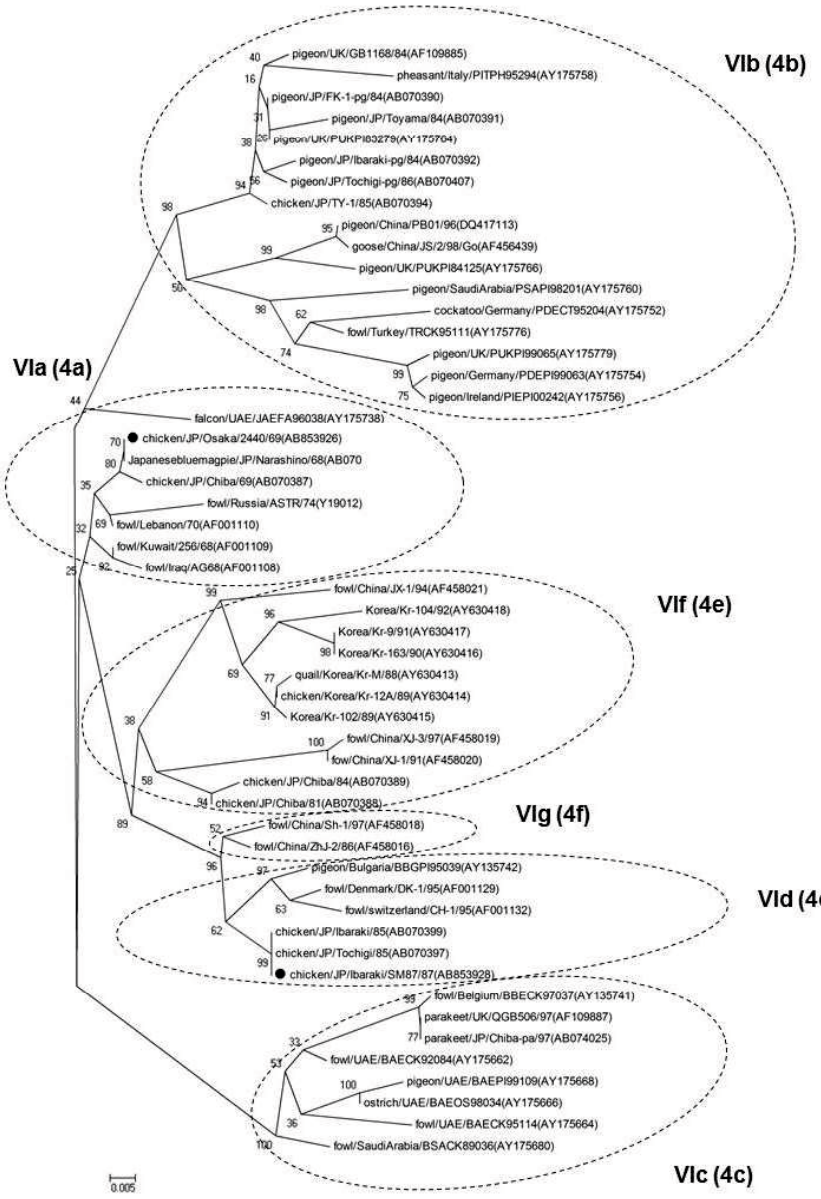


Figure 5. Straight phylogenetic tree of variable region of the F-gene sequences (47-421 nt) of genotype VI NDV.

The evolutionary history was inferred using the Neighbor-Joining method. The bootstrap consensus tree inferred from 1000 replicates is taken to represent the evolutionary history of the taxa analyzed [34]. Branches corresponding to partitions reproduced in less than 50% bootstrap replicates are collapsed. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (1000 replicates) are shown next to the branches. The tree is drawn to scale, with branch lengths in the same units as those of the evolutionary distances used to infer the phylogenetic tree. The evolutionary distances were computed using the Maximum Composite Likelihood [102] method and are in the units of the number of base substitutions per site. All positions containing gaps and missing data were eliminated from the dataset (Complete deletion option). Strains used in this study are marked with ●. Letters inside the parenthesis indicate the sublineage grouping [1].

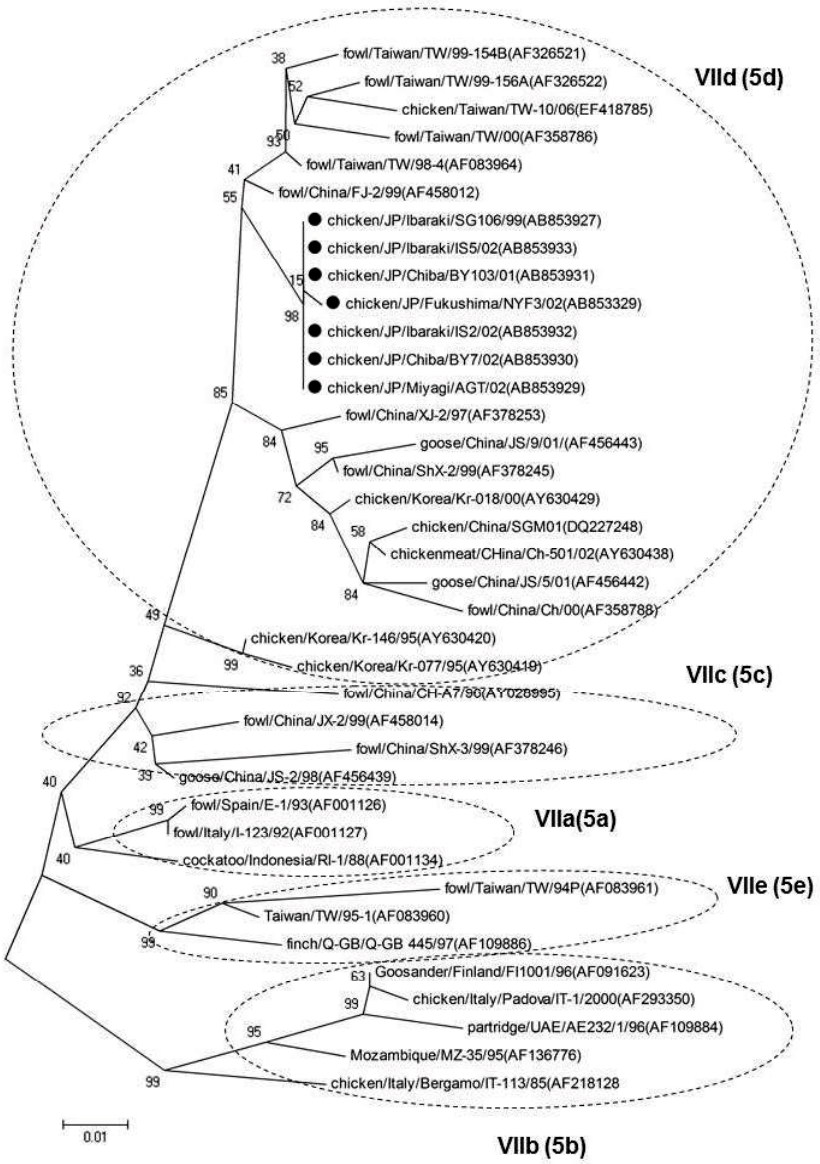


Figure 6. Straight phylogenetic tree of variable region of the F-gene sequences (47-421 nt) of genotype VII NDV.

The evolutionary history was inferred using the Neighbor-Joining method. The bootstrap consensus tree inferred from 1000 replicates is taken to represent the evolutionary history of the taxa analyzed [34]. Branches corresponding to partitions reproduced in less than 50% bootstrap replicates are collapsed. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (1000 replicates) are shown next to the branches. The tree is drawn to scale, with branch lengths in the same units as those of the evolutionary distances used to infer the phylogenetic tree. The evolutionary distances were computed using the Maximum Composite Likelihood [102] method and are in the units of the number of base substitutions per site. All positions containing gaps and missing data were eliminated from the dataset (Complete deletion option). Strains used in this study are marked with ●. Letters inside the parenthesis indicate the sublineage grouping [1].

CHAPTER II

Atypical velogenic Newcastle disease in a commercial layer flock

Introduction

Avian infectious bronchitis (IB) is a highly contagious viral disease of poultry which affects the respiratory and urogenital tract of chickens. It is caused by avian infectious bronchitis virus (IBV), a member of the family Coronaviridae. The disease is characterized by respiratory signs such as rales, coughing and sneezing. In layers and breeders, IB infection may cause decreased egg production or soft shelled, uneven eggs and eggs with poor internal quality [15].

Newcastle disease (ND) is a serious and economically devastating poultry disease. It is caused by Newcastle disease virus (NDV) which belongs to the genus *Avulavirus* in the family Paramyxoviridae. Different NDV strains characteristically show great variation in their pathogenicity [3, 47, 51-55, 74, 107]. NDV isolates can be broadly grouped into five pathotypes known as viscerotropic velogenic, neurotropic velogenic, mesogenic, lentogenic and asymptomatic enteric, based on the clinical signs seen in infected chickens [2, 4-6, 51-55, 82, 107]. Several studies have reported that the fusion (F) -gene of NDV is the main genetic virulence determinant and the best predictor of NDV pathotype [5, 36, 78, 83, 100, 114].

In the field, poultry clinicians normally rely on production performance, clinical signs, pathological lesions and serological profiles to make a diagnosis. Although poultry clinicians are well-trained in recognizing “textbook” cases and disease profiles under controlled experimental conditions, management and environmental factors such as vaccination, nutrition, housing, climate, weather patterns and other related factors may distort the clinical profile of a disease making diagnosis difficult and error prone. Hence, data on actual field cases is important to

guide poultry clinicians in their understanding of disease profiles in actual commercial poultry operations. Case studies, such as the one reported here, are valuable in identifying hidden disease risk factors in the field.

In this study, a commercial layer flock in Japan in 2002 which was initially thought to be infected with IBV based on clinical signs, virus isolation and serological analysis was, using molecular techniques, later diagnosed as an atypical infection with velogenic NDV. This is the first report which documents the possible existence of atypical velogenic ND in poultry operations in Japan.

Materials and Methods

Farm history

The affected commercial layer farm was located in Fukushima prefecture in 2002. It had a population of approximately 120,000 chickens divided into seven flocks housed in conventional open-sided layer facilities. The farm employed standard management and rearing procedures throughout its operations. Routine vaccination were performed against Marek`s disease, infectious bursal disease, fowl pox, IB, ND, infectious coryza, infectious laryngotracheitis and egg drop syndrome. Vaccination against ND was performed using live B1 spray at 10 and 28 days of age, the killed Ishii strain in aluminum adjuvant at 45 days, live B1 spray at 60 days and the killed Ishii strain in aluminum adjuvant again at 90 days of age. Vaccination against IB was performed by spray with the live IBV H120 strain at 10 days of age, spray with live IBV ON strain at 28 days, killed IBV Beaudette/42 strain at 45 and 90 days and the live IB H120 strain again at 105 days.

Egg production performance

Production performance was recorded as follows: Egg production for each poultry house was recorded every day. All eggs were weighed and the number of downgraded eggs and mortality were also recorded daily.

Serological tests

Serial blood collection for serological profiling of antibodies against IBV and NDV was performed routinely. Twelve blood samples were randomly collected per flock once a month from 30 days of age until culling. Haemagglutination inhibition (HI) tests for NDV were performed as described by Salk [90]. Serum samples showing HI titres ≥ 3 ($\log_2$ titres) were defined as positive. Agar gel precipitation (AGP) tests were performed on slides using 1% Noble agar (Difco, USA) gel containing 8.5 % NaCl (Nacalai Tesque, Inc., Kyoto, Japan) as described by Beard [10] to check for IBV titres and to cross-check for NDV HI titres.

Virus isolation

For attempted virus isolation, a 20% suspension of trachea, spleen and kidney (pooled) from five sick birds was made in buffered saline solution with antibiotics (penicillin 100 IU/ml; streptomycin 0.1 mg/ml), and centrifuged at 2,000 g for 20 min. Organs were pooled separately per bird. Then, 0.1 ml of the supernatant was inoculated into the allantoic cavity of five 12-day-old embryonated specific pathogen free (SPF) eggs. Eggs dead within 24 hours were discarded. Allantoic fluid from the inoculated eggs was harvested after 48 hours incubation, diluted two-fold and reinoculated into five 12-day-old embryonated SPF eggs (2^{nd} passage) as described previously [81]. Inoculated eggs were examined for IBV after 5-7days by checking

for dwarfing and curling of the embryos. Isolated viruses were kept in -80C until further use.

Reverse Transcription Polymerase Chain Reaction (RT-PCR)

Isolates were propagated once in 10-day-old embryonated SPF eggs. Viral RNA from infected allantoic fluid was extracted directly by using a QIAamp® Viral RNA Mini Kit (Qiagen, West Sussex, UK). Viral RNA was transcribed to cDNA by using random hexamers and Primescript® Reverse Transcriptase (Takara Bio-Inc, Shiga, Japan).

For molecular diagnosis of IBV, cDNA samples were used to amplify a 440bp partial sequence of the RNA dependent RNA polymerase (RdRp) gene (14127 - 14566 nt of whole genome of IBV H120 strain) by two-step nested PCR using KOD dash® (Toyobo, Osaka, Japan) and 5uM of external and internal primers [24] (Table 2) according to manufacturer`s instructions. Thermocycling conditions were as follows: prewarming at 94^0C for 2 min (1 cycle), denaturation at 94°C for 30 sec, annealing at 50°C for 30 sec and extension at 72°C for 1 min. Amplification steps were performed for 35 cycles with final extension at 72°C for 30 sec (1 cycle). The PCR product was used for direct nucleotide sequencing.

For molecular diagnosis of NDV, a two-step nested PCR was performed to amplify the region comprising the 3' end of the M gene and the 5' end of the F-gene [68-69] using KOD dash® (Toyobo), 5uM of external and internal primers (Table 2) and cDNAs that were transcribed previously. Thermocycling conditions were similar to those previously described. A 921-bp primary product and a 766-bp secondary product were obtained in NDV positive samples. Any confirmed NDV strains were subjected to additional RT-PCR amplification to characterize the open reading frame

of the F-gene sequence (4504-6295 nt of whole NDV genome) using SapphireAmp® Fast PCR Master Mix (Takara Bio), 5uM of forward and reverse primers (Table 2) and cDNAs that were transcribed previously. The thermocycling conditions consisted of initial denaturation at 95^0C for 2 min followed by 35 cycles of 98^0C for 10 sec (denaturation), 55^0C for 10sec (annealing), 72^0C for 10sec (extensions) and final extension at 72^0C for 2min.

Nucleotide sequence analysis and phylogenetic studies

PCR products were analyzed by electrophoresis with 1.2% agarose gel and purified by using QIAquick® Gel Extraction Kit (Qiagen, Valencia, CA). Nucleotide sequences of PCR products were determined using Big Dye terminator cycle-sequencing kit version 3.1 (Applied Biosystems Inc., Foster City, CA) and an ABI Prism 3130 Genetic Analyzer (Applied Biosystems). DNA products were sequenced in both directions.

Sequence assembly and editing were performed using CodonCode Aligner® (version 3.7.1, CodonCode Corporation, MA) and ClustalX® (version 2.1, Conway Institute UCD Dublin, Ireland). Deduced amino acid sequences were determined using the Bioedit® software package version 7.1.3.0 [40]. Confirmation of identity and homology were performed using BLAST http://www.ncbi.nlm.nih.gov. Phylogenetic analyses were conducted using MEGA version 4 [101]. Phylogenetic trees of the complete coding region (1-1662 nt) and variable region (47-421 nt) of the NDV F-gene sequence and partial sequence of the coding region of the RNA dependent RNA polymerase (14127 - 14566 nt of whole genome of H120 strain) of IBV were constructed by the neighbor-joining method with the maximum composite likelihood substitution model at 1000 bootstrap replicates.

Biological characterization of NDV

Pathotypical and biological characterization of confirmed NDV isolate was performed using mean death time (MDT) in 10-day-old embryonated SPF chicken eggs and the intracerebral pathogenicity index (ICPI) in 1-day old chicks as described previously [82]. Confirmation of pathotypes was performed by analysis of the deduced amino acid sequence of the F0 proteolytic cleavage site (residues 112-117).

Results and Discussion

During the investigation period, one flock consisting of approximately 16,000 layers was affected with the disease. The flock was initially performing well (Figure 1) achieving peak production of 95% at 26 weeks of age and with over 90% egg production from 24 to 50 weeks of age. From 56 to 64 weeks, a gradual decline (8%) in egg production (from 84 to 76%) and an increase (2.5%) in soft shelled eggs (from 7.5 to 10%) was observed. Mortality was minimal and within normal industry standards. A three-fold increase in IB−AGP positive results was noted at 61 weeks of age (Figure 2). Based on clinical signs, virus isolation and AGP tests, the flock was diagnosed as infected with IB. The flock was force molted at 65 weeks and production was restarted. Good performance was noted in the second cycle (Figure 1) but similar production problems recurred at 75 weeks. Slight gasping was observed in some birds and a second wave of a gradual decrease of 8% in egg production (from 83 to 75% in four weeks) was noted. Serological tests showed a three-fold increase in IB AGP test positive sera (Figure 2) and a two-fold increase in the reciprocal log2 NDV HI titres at 78 weeks (Figure 3). Two viruses were isolated at 75 weeks and both caused dwarfing in 12-day old chicken embryos after inoculation via the

chorioallantoic cavity. Based on these clinical findings, the case was initially diagnosed as IB.

Ten years after this case was first observed, the virus isolates were repropagated in 10 day old embryonated eggs to confirm their identity by molecular diagnostics and to make new stocks for storage after long periods of time at -80 ^{0}C. One of the isolated strains was confirmed to be IBV by yielding a 440 bp PCR product. Phylogenetic analysis using the RdRp gene showed that this isolate formed a separate clade distinct from vaccine and vaccine-variant viruses, which may indicate that the isolate could be a wild IBV strain. Nucleotide sequence identity showed that this isolate was closely related to CU570 (99%), an IBV isolated from chickens in the US and ck/CH/LJL/111054 (99%), an isolate from a chicken in China. Surprisingly, the second virus isolate yielded a 766-bp product in the nested PCR for the NDV F-gene. Nucleotide sequence analyses of the complete coding region of the F-gene (1-1662 nt) was performed which showed multiple basic amino acids at the F0 proteolytic cleavage site (residues 112-117). The predicted amino acid sequence of the F0 cleavage site was ^{112}RRQKR^{116} at the F2 protein and phenylalanine (^{116}F^{117}) at the N-terminus of the F1 protein. The isolate exhibited MDT of 48 hours in embryonated chicken eggs while the ICPI value was 1.8. These results indicated that the NDV isolate was velogenic. Phylogenetic analysis showed that this NDV isolate belongs to genotype VIId (Figure 4 and 5) and was closely related (99% nucleotide sequence identity) to local strains from Japan and 98% similar to some chicken NDV strains from China and Taiwan and to a goose isolate from China.

This study demonstrated that in some cases, velogenic ND might appear as non-velogenic, especially in Japanese commercial farms where stringent poultry husbandry practices are in place. During this period, similar cases were observed in

two other farms located in Ibaraki and Chiba prefectures that had mild respiratory problems. These farms were diagnosed as infected with IB, but velogenic NDVs were isolated (data available upon request). It may be hypothesized that in the presence of high antibody titres but non-uniform flock immunity (Figure 2), these NDV strains might have been slowly proliferating in susceptible individuals instead of following the typical, deadly pathophysiological course. The presence of other infections or stressful husbandry factors, such as molting could have then activated the disease, but good management procedures might have prevented its progression to a full blown velogenic ND, resulting instead in an atypical infection. Cases such as these may constitute a potential threat to commercial poultry since this disease may go unnoticed and be left uncontrolled. Hence poultry clinicians are well-advised to be watchful for atypical velogenic ND, especially in vaccinated commercial chicken flocks, since it is possible that they may harbour hidden NDV infection. Therefore, strict vaccination and monitoring procedures must always be undertaken.

Table 1. Nucleotide sequence similarity of the present NDV isolate to other isolates of the same genotype

Strain name	Sequence homology (%) with JP/Fukushima/NYF-3/02[a]
JP/Ibaraki/00	99.7
JP/Ibaraki-16/01	99.7
JP/Gunma/01	99.7
JP/Ibaraki-254/01	99.5
JP/Ibaraki-266/01	99.5
FJ-2/99	98.4
GD/1/98/Go	98.4
JP/Tokyo/96	98.1
JP/Ibaraki-ph/97	98.1
TW/98-1	97.9
TW/98-2	97.9
TW/98-4	97.9
GX-3/98	97.9
XJ-2/97	97.7
JP/Kanagawa/99	97.3
GS-2/98	97.1
SHX-2/99	97.1

[a] The complete coding region of the F-gene sequence (1-1662 nt) of the present isolate was compared with that of each other isolate of genotype VIId.

Table 2. Primers used in this study

Primer name	Primer sequence
IBV-RdRp-F1	5`-GGKTGGGAYTAYCCKAARTG-3`
IBV-RdRp-R1	5`-TGYTGTSWRCARAAYTCRTG-3`
IBV-RdRp-F2	5`-GGTTGGGACTATCCTAAGTGTGA-3`
IBV-RdRp-R2	5`-CCATCATCAGATAGAATCATCAT-3`
NDV-Mgene-F1	5`-TTCTCTAGCAGTGGGACAGC-3`
NDV-Fgene-R1	5'-CATCTTCCCAACTGCCACTG-3'
NDV-Fgene-F2	5'-TGGAGCCAAACCCGCACCTGCGG-3'
NDV-Fgene-R2	5'-GGAGGATGTTGGCAGCATT-3'
NDV-For4359	5'-CCATTGCTAAATACAATCCTTTCA-3'
NDV-Rev4788	5'-GGGGCTTTYGCACACGCCTC-3'
NDV-For4988	5'-AATGCCGCCAACATCCTCCG-3'
NDV-Rev5261	5'-GTGCCTGGATAGTCAGCTGAG-3'
NDV-For5461	5'-GACYTTATCTGTAAGYACAACC-3'
NDV-Rev5731	5'-CAATTGGCAATAACTGAGCC-3'
NDV-For5918	5'-GTGACAGGCAAYCTTGATATATC-3'
NDV-Rev6204	5'-CTTGTAGTGGCTCTCATCTG-3'
NDV-For6369	5'-AGGCYTCACAACATCYGTTC-3'
NDV-Rev6598	5'-TYGATATGCCTRCGAGRTCG-3'

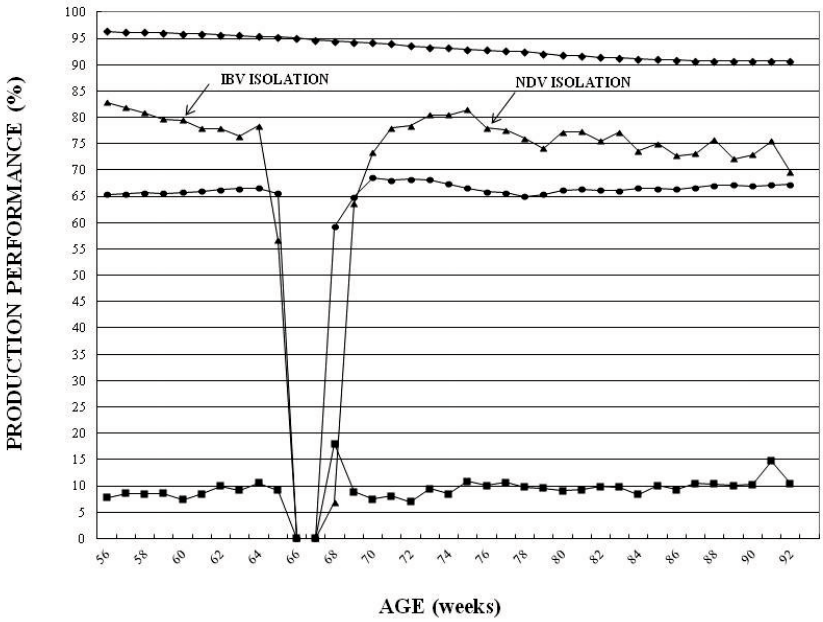
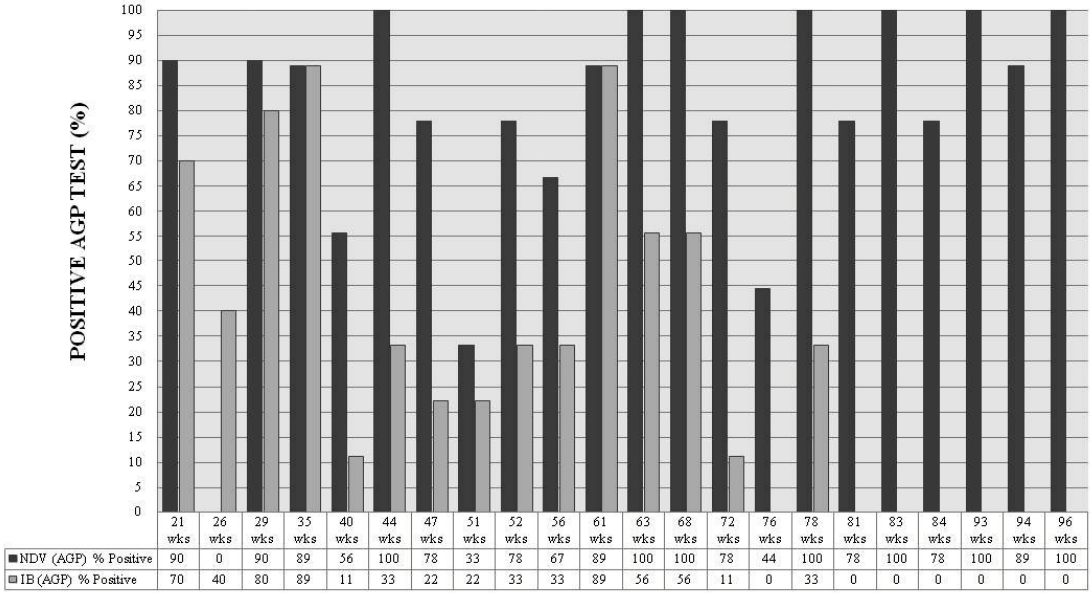


Figure 1. Production performance of the layer chicken flock investigated in this study. Egg production rate (▲), Average egg weight (●), Livability rate (◆), Incidence of down-graded eggs (■). IBV and NDV were isolated at 60 and 75 weeks of age respectively. A gradual decrease in egg production without marked mortality was observed thereafter. An increase in soft shelled eggs was also noted.



	21 wks	26 wks	29 wks	35 wks	40 wks	44 wks	47 wks	51 wks	52 wks	56 wks	61 wks	63 wks	68 wks	72 wks	76 wks	78 wks	81 wks	83 wks	84 wks	93 wks	94 wks	96 wks
NDV (AGP) % Positive	90	0	90	89	56	100	78	33	78	67	89	100	100	78	44	100	78	100	78	100	89	100
IB (AGP) % Positive	70	40	80	89	11	33	22	22	33	33	89	56	56	11	0	33	0	0	0	0	0	0

Figure 2. Serial AGP-positive sera from the layer chicken flock. A three-fold increase in IB and ND AGP test positive sera was noted at 61 weeks and 78 weeks respectively.

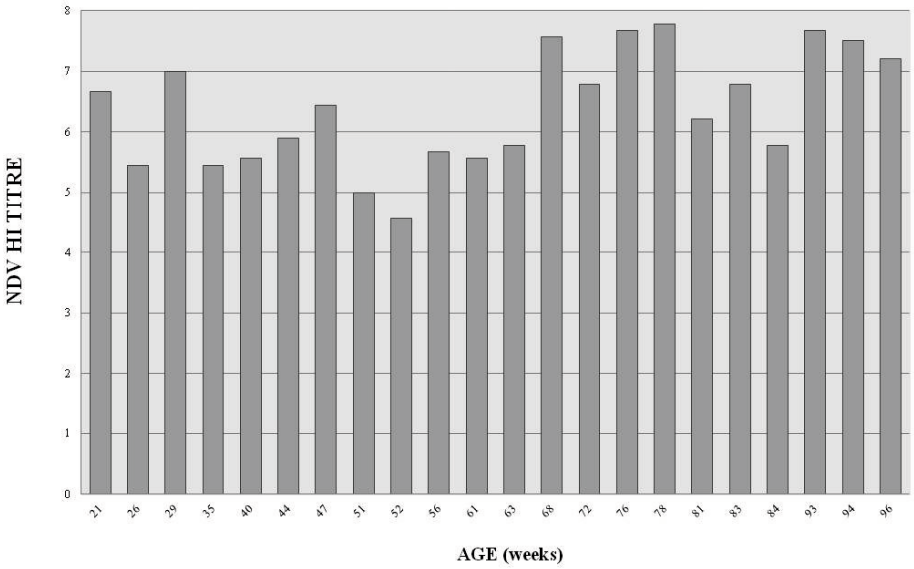


Figure 3. Serial reciprocal $\log_2$ NDV HI titres in this layer chicken flock. An approximately two-fold increase in HI titre was noted at 78 weeks.

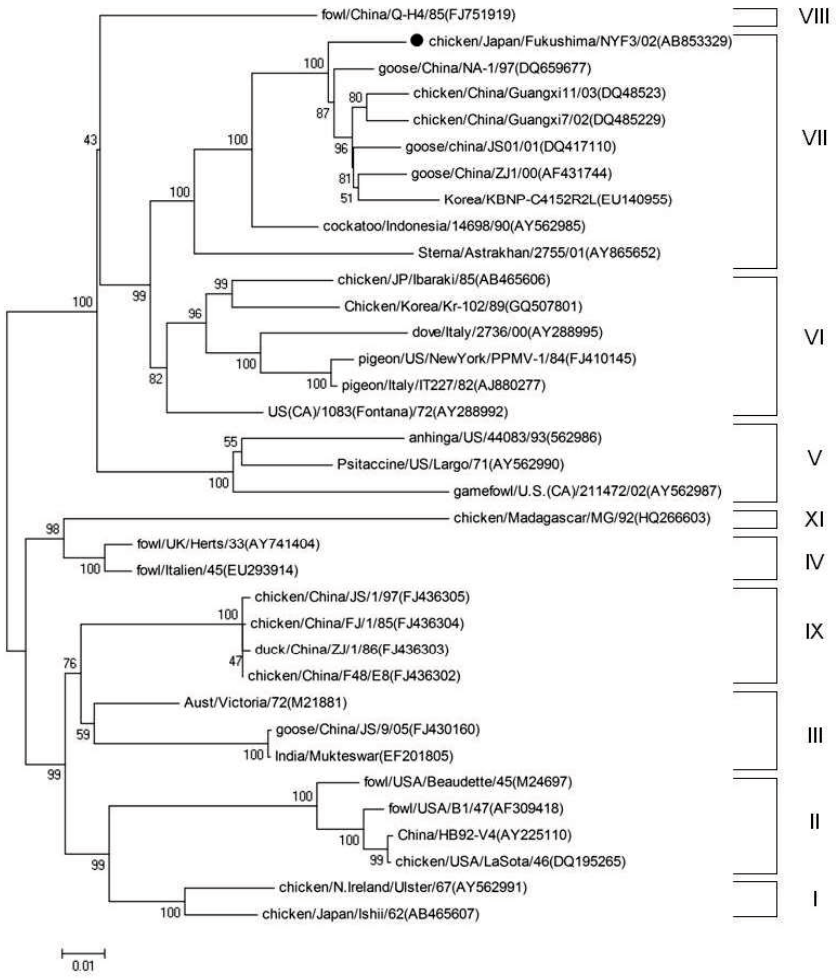


Figure 4. Phylogenetic analysis of the present NDV isolate using the nucleotide sequence of the complete coding region of the F-gene (1-1662 nt) sequence. The strain isolated in this study is marked with ●. The bootstrap consensus tree inferred from 1000 replicates [34] is taken to represent the evolutionary history of the taxa analyzed. Branches corresponding to partitions reproduced in less than 50% bootstrap replicates are collapsed. The percentages of replicate trees in which the associated taxa clustered together in the bootstrap test (1000 replicates) are shown next to the branches. The tree is drawn to scale, with branch lengths in the same units as those of the evolutionary distances used to infer the phylogenetic tree.

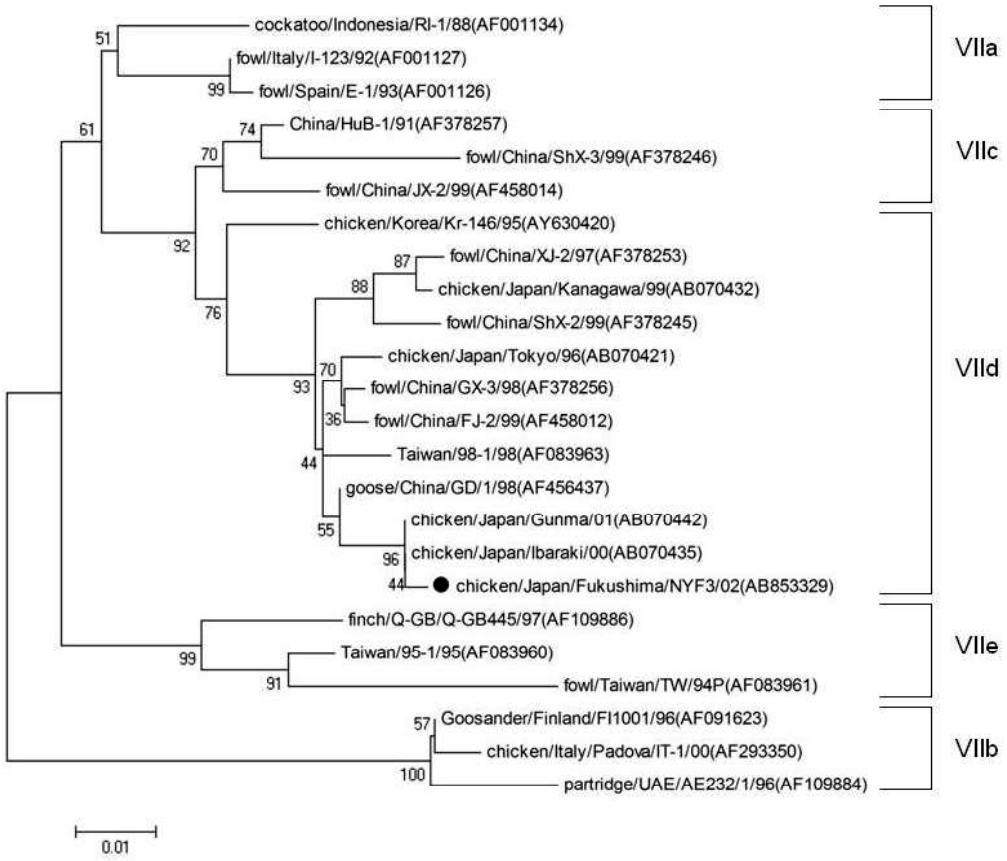


Figure 5. Phylogenetic analysis of the present NDV isolate using the nucleotide sequence of the variable region of the F-gene (47-421 nt). The strain isolated in this study is marked with ●. The bootstrap consensus tree inferred from 1000 replicates [34] is taken to represent the evolutionary history of the taxa analyzed. Branches corresponding to partitions reproduced in less than 50% bootstrap replicates are collapsed. The percentages of replicate trees in which the associated taxa clustered together in the bootstrap test (1000 replicates) are shown next to the branches. The tree is drawn to scale, with branch lengths in the same units as those of the evolutionary distances used to infer the phylogenetic tree.

CHAPTER III

Characterization of complete genome sequence of genotype VI and VII velogenic Newcastle disease virus from Japan

Introduction

Newcastle disease virus (NDV) is an enveloped, non-segmented single stranded, negative-sense RNA virus belonging to the genus Avulavirus of the subfamily Paramyxovirinae, family Paramyxoviridae. It is the causative agent of the highly contagious and economically important Newcastle disease (ND) [4].

Depending on its class and genotype, NDV has 15,186, 15,192 or 15,198 nucleotides comprising of six genes in the order of 3'-NP-P-M-F-HN-L-5' [4, 66]. These genes encode for at least eight proteins, the nucleoprotein (NP), phosphoprotein (P), matrix (M) protein, fusion (F) protein, hemagglutinin-neuraminidase (HN) protein, RNA dependent RNA polymerase (L) and two additional non-structural proteins, V and W, which are generated through RNA editing of the P-gene. Flanking these genes are extracistronic regions called leader and trailer sequence located at the 3-prime and 5-prime end of the NDV genome respectively. These sequences are involved in the replication, transcription and packaging of the genomic and antigenomic RNAs [112]. At the beginning and end of each genes are conserved transcriptional control sequences known as gene start (GS) (3`-UGCCCAUCU/CU-5`) and gene-end (GE) (3`-AAU/CUUUUUU-5`), which functions as transcriptional promoter and terminator [43]. Located between the boundaries of each gene are non-coding segments called intergenic sequences (IGS) that may be 1- 47 nucleotides in length [20, 21, 59].

Through numerous investigations of NDV subsequent to emergence and identification of ND in Java, Indonesia and Newcastle-upon-Tyne in England, NDVs have been grouped into two classes (Class I and II) and into several genotypes and sub-genotypes [4]. Based on phylogenetic analysis of the hypervariable region of the F-gene sequence, Class II NDVs comprising the majority of virulent and some avirulent NDV strains, have been classified into at least eleven genotypes (I-XI) with genotypes I, II, VI and VII being further subdivided into subgenotypes Ia and Ib, II and IIa, VIa to VIh and VIIa to VIIh [14, 42, 63, 65, 106]. A similar classification system by Aldous *et al.* [1] classified NDVs into seven lineages and several sublineages. Recently, a unified classification system using the complete coding sequence of the F-gene was proposed in which, class II NDVs were classified into at least 15 genotypes [ten from previously established genotypes (I – IX and XI) and five new genotypes (X, XII - XV)] with several sub-genotypes] [32].

Previous studies have reported that NDV strains from Japan could be classified into six genotypes (genotypes I-III, VI, VII-VIII) based on phylogenetic analyses of the hypervariable region of the F-gene [68-69]. NDVs affecting poultry flocks from 1960 up to the mid-1980's and pigeons and wild birds from mid-1980's to late 2000 belong to genotype VI while from 1985 up to the present, the predominant genotype affecting the domestic poultry flocks and wild birds in Japan were from genotype VII. These two genotypes can therefore be considered as the most recent NDV strains circulating in Japan [68-69]. Genotype VII is also the most predominant genotype responsible for most outbreaks in East Asian countries including Taiwan, Korea and China [4, 61, 106, 114] since the 1980's.

Previous to the current study, whole genome sequencing of wild strains of NDVs has not been performed in Japan. Molecular characterization of NDVs

especially from recent outbreaks in vaccinated commercial poultry flocks is essential, as it may provide important insights on the genomic properties of NDVs involved in cases of breakthrough infections. In the present study, complete genome sequences of three NDVs isolated from vaccinated commercial layer flocks were determined, analyzed and compared to different field and vaccine NDV strains from various parts of the world. This is the first study to characterize the complete genome sequences of NDV strains isolated in Japan.

Materials and Methods

Virus

Three NDV strains isolated from three vaccinated commercial layer farms in Japan were used in this study. The oldest strain was isolated from three dead layer birds from Osaka Prefecture in 1969 [APMV1/chicken/JP/Osaka/2440/1969 (2440/69) (Accession number AB853926)]. The second strain was isolated from a layer flock with a mild respiratory disease without significant mortalities in Ibaraki Prefecture in 1987 [APMV1/chicken/JP/Ibaraki/SM87/1987 (SM87/87) (Accession number AB853928)]. The third strain was isolated from dead spent layer hens from Ibaraki Prefecture in 1999 [APMV1/chicken/JP/Ibaraki/SG106/1999 (SG106/99) (Accession number AB853927)]. The complete history and clinical profile of these cases were reported in chapter I. Isolates in this study were provided by Poultry Products Quality Control Co. Ltd. (Fukushima, Japan).

Whole genome sequencing

NDV isolates were propagated through a single passage in 10-day-old embryonated SPF eggs. Viral RNA from infected allantoic fluids was extracted using QIAamp® Viral RNA Mini Kit (Qiagen, West Sussex, UK). Viral RNA was transcribed to cDNA by using random hexamers and Primescript® Reverse Transcriptase (Takara Bio-Inc, Shiga, Japan). PCR was performed using SapphireAmp® Fast PCR Master Mix (Takara Bio-Inc, Shiga, Japan), with the following thermocycling conditions; initial denaturation at 95°C for 2 min followed by 35 cycles of 98°C for 10 sec (denaturation), 55°C for 10sec (annealing), 72°C for 10-20sec (extensions) and final extension at 72°C for 2min. PCR products were analyzed by electrophoresis with 1.2% agarose gel and purified by using QIAquick® Gel Extraction Kit (Qiagen, Valencia, CA). Nucleotide sequences of PCR products were determined by Big Dye terminator cycle-sequencing kit version 3.1 (Applied Biosystems Inc., Foster City, CA) and an ABI Prism 3130 Genetic Analyzer (Applied Biosystems). DNA products were bidirectionally sequenced. The cDNAs of 3' and 5'-terminal end of viral RNA were amplified according to the rapid amplification of cDNA ends (RACE) method as described by Li *et al.* [60] and used for the determination of nucleotide sequences. Nucleotide sequence information on the partial NP-gene (nucleotide positions at 15-622), complete coding region of F-gene (4,550-6,211) and partial L-gene (14,015-14,719) were reported previously. Primer sequences used for nucleotide sequencing are available upon request.

Sequence analyses and phylogenetic studies

Sequence assembly and editing were performed using CodonCode Aligner® (version 3.7.1, CodonCode Corporation, MA) and ClustalX® (version 2.1, Conway Institute UCD Dublin, Ireland). Confirmations of identity were performed using BLAST http://www.ncbi.nlm.nih.gov. Nucleotide and deduced amino acid sequences for all reference strains were obtained from GenBank. The consensus deduced amino acid sequence of 100 velogenic, mesogenic and lentogenic NDVs representing the different NDV genotypes and subgenotypes was determined and compared with that of field isolates using Bioedit® software package version 7.1.3.0 [40]. Phylogenetic and molecular evolutionary analyses were conducted according to the unified NDV classification system [32] using MEGA version 4 [101] using the neighbor-joining method with the maximum composite likelihood substitution model at 1000 bootstrap replicates.

Determination of recombination events

Intragenic and intergenic recombination events in the complete genome and full-sequence of all genes of the Japanese field strains were determined using RDP v3.44 program [67]. Seven different algorithms integrated in the program namely RDP, GeneConv, Bootscan, MaxChi, Chimaera, SiScan and 3Seq (window size = 20, highest acceptable p-value = 0.05; Bonferonni correction) were applied to detect any putative recombination breakpoints and to estimate the occurrence of any recombination events in the Japanese field strains.

Virus Neutralization Test

Monolayer of primary chick embryo fibroblast tissue culture (CEF) was prepared using 10-day-old chick embryos as reported previously [56]. Three anti-NDV serum, commercial reference antisera again Ishii strain (Kaketsuken, Kumamoto, Japan), anti-B1 antisera from a 40-days-old layer chicken vaccinated with commercial B1 strain (Kyoritsu Seiyaku, Tokyo, Japan) at 10 days of age by drinking water and 28 days of age by spray, field NDV antisera from a commercial layer flock vaccinated with live B1 at 10 and 28 days of age by spray (Kyoritsu Seiyaku, Tokyo, Japan) and killed Ishii strain in aluminum adjuvant (Kyoto Biken Laboratories Inc, Kyoto, Japan) at 45 and 105 days of age and negative antiserum from a 12-weeks-old specific pathogen-free (SPF) chicken were used. The hemagglutination-inhibition (HI) titers of these antisera were determined as reported previously prior to use [82]. Virus neutralization test was performed using a slight modification of a previously described method [110]. In brief, heat inactivated and two-fold diluted serum was mixed with equal amounts of 100TCID50/50ul of field NDVs and commercial live B1 strain (Nisseiken Co., Ltd., Tokyo, Japan). The virus-serum suspensions were gently mixed and incubated for 1h at 37°C at 5% CO_2. 100uL of the virus-serum suspension were subsequently inoculated to CEF monolayer 96 wells plates. After 1h at 37°C incubation, the inoculum was removed and 200uL of Eagle's Minimum Essential Medium (Nissui Pharmaceutical Co. Ltd., Tokyo, Japan) were added. The plates were incubated for 4 days at 37°C at 5% CO_2 and observed daily for cytopathic effects (CPE). The virus neutralization titer was computed as the reciprocal $\log_2$ of the highest dilution of the serum showing a 50% endpoint of neutralization [89].

Results

General genomic characteristics

All three Japanese strains analyzed had genome lengths of 15,192 nucleotides organized in the order of 3'-NP-P/V/W-M-F-HN-L-5' (Table 1). Insertions of six nucleotides (^{1648}TCCCAA^{1653} for 2440/69; ^{1648}CCCCAA^{1653} for SM87/87; and ^{1648}TCCCAC^{1653} for SG106/99, indicated by antigenome sense) at the 5-prime end of the non-coding region of the NP gene were observed, in contrast to NDV strains belonging to early genotypes (genotypes I-IV). G+C contents were 46.2% for 2440/69 and SM87/87 and 46.5% for SG106/99. The protein-coding capacity of the genome of all Japanese field strains was 90.5%.

Characteristic of the non-coding regions

All of the Japanese strains had leader and trailer sequences composed of 55 and 114 nucleotides, respectively. Except for the GS of the L-gene (ACGGGTAGGA), the GS of the NP, P, M, F and HN were identical [ACGGGTAGAA (indicated by antigenome sense)] in all strains. The GE of the NP genes (TTAGAAAAAAA) was also identical; however, the GE of M and L-genes (TTAGAAAAAA) and P, F and HN genes (TTAAGAAAAAA) were slightly different. Variations in the length of the IGS of the N-P, P-M and M-F IGS (1 nucleotide) F-HN (31 nucleotides) and HN-L IGS (47 nucleotides) were observed. Analyses of the 3-prime and 5-prime untranslated regions (UTR) showed that the 5'-UTRs were always longer than 3'-UTRs and that the lengths of the 5'-UTRs may vary among NDV strains (Table 1).

Gene identities and characteristics of the F and HN proteins

Analyses of the coding sequence of each gene in comparison with widely-used vaccine and closely-related NDV strains showed that among all the genes, the NP and L-genes were the most conserved with sequence identities that ranged from 85.1-99.0% (nucleotide) and 91.2-99.6% (amino acid) in all strains (Table 2). In contrast, the P-gene was the most variable with sequence identities that ranged from 81.7-99.0% (nucleotide) and 79.2-98.7% (amino acid), depending on the strain. Analysis of sequence identities using available complete genome sequences from the GenBank showed that 2440/69, SM87/87 and SG106/99 were most closely related to US(CA)/1083(Fontana)/72 (96.7%), ZhJ-3/97 (97.8%) and to Guangxi/14/02 (97.4%), respectively. In contrast, comparison of the complete genome with vaccine strains showed that the Japanese strains only had 83.5 to 85.5% sequence identities with B1 and La Sota strains.

Analysis of the functional domains of the F-gene sequence of the Japanese strains showed that the deduced amino acid sequences were mostly similar with the consensus amino acid sequences derived from a total of 100 velogenic, mesogenic and lentogenic NDV strains; except that the F protein of 2440/69, SM87/87 and SG106/99 had 1 (at the transmembrane domain), 5 (3 at the signal peptide and 1 each at the fusion peptide and transmembrane domain) and 5 (4 at the signal peptide and 1 at the transmembrane domain) substitutions, respectively. Analysis of the three heptad repeat regions (HR) showed a total of 6 substitutions in HRa (143-185 amino acids), HRb (268-299 amino acids) and HRc (471-500 amino acids) in the Japanese field strains (2 in HRc of 2440/69; 1 in HRc of SM87/87; 1 each in HRa, HRb and HRc of SG106/69) (Table 3).

Strains in this study had HN protein of 571 amino acids, which is a characteristic feature of virulent NDVs. The transmembrane domain of the HN protein from 2440/69 and SG106/99 had 1 and 3 amino acid substitutions, in comparison with the consensus amino acid sequences. Cysteine residue and potential N-glycolysation sites were almost completely conserved except for the loss of N-glycolysation site at amino acid 538 in SM87/87 (Table 5). Ten amino acids constituting the sialic acid binding site [29] were completely conserved (Table 5). Analysis of the ten neutralizing epitopes in the HN protein [18, 45, 46] identified a total of two amino acid substitutions in 2440/69 and three amino acid substitutions in SM87/87 and SG106/99 (Table 4).

Phylogenetic analysis

Phylogenetic analysis using the complete genome sequences revealed that the Japanese NDV strains belong to two distinct genotypes (genotype VI and VII). 2440/69 and SM87/87 were from genotype VI, while SG106/99 belongs to genotype VII (Figure 1). Phylogenetic analysis using the complete coding sequences of NP, P, M, F, HN and L genes yielded the same phylogenetic groupings (Figures 2-7). Subgenotype analyses using the unified NDV classification system recently proposed by Diel *et al.* [32] showed that 2440/69 and SM87/87 belong to subgenotype VIc and SG106/99 to subgenotype VIIe (Figure 8). Analysis of recombination in the field isolates using all the described methods in the RDP v3.44 program showed no recombination events ($p < 0.05$).

Virus neutralization test

Virus neutralization test showed that field NDV strains were completely neutralized at virus neutralization titers (VNT) of at least 4.5 to 6.5 $\log_2$ depending on the antisera that were used. In contrast B1 strain was completely neutralized at VNT of 6.3 to 6.5 $\log_2$ units. Regardless of the antisera, a one unit difference in the $\log_2$ VNTs of B1 and 2440/69 were observed as compared to SM/87 and SG106/99. At this VNT, the corresponding reciprocal $\log_2$ HI titer of the reference and field antisera were 1 to 2 HI units. VNT results and the initial HI titers of the different antisera were presented in Table 6.

Discussion

The complete genome sequences of the three NDV strains isolated from three vaccinated commercial poultry farms in the span of three decades (1969 to 1999) were determined, compared and analyzed. These isolates belong to recent NDV genotypes affecting pets, wild birds and commercial poultry flocks in Japan [68, 69]. To the authors' knowledge, this is the first report characterizing the whole genome sequences of field NDV strains isolated from vaccinated chickens in Japan. Characterization of NDV field isolates from Japan may ultimately prove useful in the prevention, control and management of future ND cases in vaccinated poultry flocks.

The complete genome of the three Japanese strains were comprised of 15,192 nucleotides that constitute six genes, a leader and trailer sequence, twelve UTRs and five IGS. Overall, these general genomic characteristics were consistent with previously characterized class II NDVs, except for those belonging to early genotypes (genotype I to IV), which lack the six nucleotides insertion at nucleotides 1648-1653 of the NP gene (Table 1). Recently, recombination events in the NDV genome have

been reported [19, 23, 41, 76, 87, 113, 116, 117]. In chapter I, no recombination was also observed in 2440/69, SM87/87 and SG106/99 based on the nucleotide sequences of the partial NP- and L-gene and complete coding region of F-gene. By phylogenetic analysis based on complete genome and full-length coding region of each genes (NP, P, M, F, HN and L genes), 2440/69 and SM87/87 fell into the genotype VI and SG106/99 fell into the genotype VII, thus confirming that no recombination had occurred in these viruses. In addition, analysis of complete genome of Japanese isolates by RDP3.44 software did not detect any recombination events.

As reported previously, the F-gene of Japanese isolates had high levels nucleotide sequence identity with contemporary isolates, 2440/69 had 97.3-100% identities with NDV isolates from the Middle East, Japan and Russia; SM87/87 had 98.7-100% identities with Chinese and Japanese isolates; SG106/99 had 97.8-100% identities with isolates from Taiwan, China and Japan. In comparison between the complete genome sequence of Japanese isolates and NDVs from Genbank, 2440/69, SM87/87 and SG106/99 had the highest sequence identities with chicken/US(CA)/1083(Fontana)/72 (96.7%), ZhJ-3/97 (97.8%) and to Guangxi/14/02 (97.4%), respectively (Table 2). These close similarities were also observed by phylogenetic analysis (Figures 1-8). However, relatively low nucleotide identities in the complete genome as compared to the F-gene were observed. It could be due to the small number of complete sequences registered in the database. To resolve these differences, further accumulation of complete nucleotide sequence analysis of NDV, particularly from old isolates, is recommended. It is also possible that the relatively low nucleotide identities in the complete genome sequences may be due to the low nucleotide identities in the other region(s) (e.g. non-coding regions, P and/or HN-genes). Over-all, these data confirmed that commercial poultry farms in Japan

were affected with velogenic ND during the second, third and fourth panzootic, which were characterized by co-circulation of genetically distinct virus lineages predominant in that particular time period (1969-1999) due to involvement of infected wild birds as described previously.

According to the recently proposed unified classification system of NDVs [32], 2440/69 and SM87/87 and SG106/99 belonged to subgenotype VIc and VIIe (Figure 2). By phylogenetic analyses using the hypervariable region (47-421 nucleotides) of F-gene, 2440/69, SM87/87 and SG106/99 were classified into subgenotype VIa, VId and VIId. These disagreements in the classification of the Japanese field strains in this study and in chapter I may be explained by differences in input criteria of the different classification systems. In the conventional system [28, 65, 92], about 400 nucleotides of 5'-end region of F-gene were used; on the other hand, in the unified genotyping system [32], the full-length F-gene sequence (1,662 nucleotides) was needed. The eight and ten subgenotypes of genotypes VI and VII in the conventional system were also reclassified into four and five subgenotypes, respectively. As a consequence, several strains (e.g. Kuwait256, Lebanon70 and Iraq AG86), which were previously classified into subgenotype VIa together with 2440/69 by the conventional genotyping system, could not be classified in the unified genotyping system because of the unavailability of full-length F-gene sequence. Thus, to identify a more accurate genetic grouping, more accumulation of nucleotide sequences data, particularly for early NDV isolates, are needed.

Cases of velogenic ND in vaccinated chicken flocks have been reported all over the world [12, 50, 75, 85, 108]. However, the possible involvement of genomic factors in cases of breakthrough infection by NDV in vaccinated chickens has never been thoroughly investigated. The HN and F-proteins are essential transmembrane

glycoproteins that form spike-like protrusions on the outer surface of virion, making them susceptible to mutations due to immune pressures. To understand the genetic properties of NDVs affecting poultry flocks in spite of vaccination, the deduced amino acid sequences of these genes with special emphasis on the functional domains and neutralizing epitopes were investigated. Comparison of the deduced amino acid identities of F and HN proteins of Japanese isolates with vaccine strains commonly used in Japan showed that the Japanese strains had 85.5-93.1% sequence identities with the vaccine strains (Table 2). Samuel *et al.* [91] also observed similar cases with low level amino acid sequence identities for the F and HN proteins between field velogenic strains isolated from vaccinated chickens in Western Africa and vaccine strain La Sota.

Comparison of amino acid sequences of the functional domains of the F and HN proteins between three Japanese field isolates and 100 velogenic, mesogenic and lentogenic NDVs identified several substitutions (Table 3 and 5). In the F protein of the three Japanese isolates, 7, 1, 6 and 3 amino acid substitutions were found in the signal peptide, fusion peptide, heptad repeat region and transmembrane domain, respectively. Amino acid substitutions at the fusion peptide and HR region of F protein were reported to affect the fusion activity of NDV [67, 93]. Gravel *et al.* [39] reported that the replacement of transmembrane domain of NDV to that of Sendai virus and measles virus abolish the fusion activity of the F protein. In the case of HN protein, the Japanese isolates had 4 amino acid substitutions and a loss of a potential N-glycosylation site (at amino acid 538). Mutations in the transmembrane domain and loss of the N-glycosylation of HN protein were reported to affect virus attachment, neuraminidase and fusion promotion activities [70-72]. Several amino acid substitutions in the neutralizing epitopes of the F and HN proteins [18, 45, 46, 80, 105,

115] of the Japanese isolates were also identified (K78R in the F protein, N263K, E347K, E347G and I514V of HN protein) (Table 4). Amino acid substitution(s) in these neutralizing epitopes were previously reported to result to neutralizing escape variants [18, 20-22, 44, 88, 96]

To investigate the effects of the observed mutations on antigenicity and their possible role in the mechanism of breakthrough infection of NDV in vitro, virus neutralization test was performed using commercial reference and NDV antisera from the field. It was observed that the field NDV strains were completely neutralized at VNTs of at least 4.5 to 6.5 $\log_2$ units. These VNTs corresponds to reciprocal $\log_2$ HI titers of approximately less than 3 units. Based on these results, poor flock immunity due to vaccination failure or partial and non-uniform immunization maybe the major factors involved in the mechanism of breakthrough infection of the Japanese field strains. However, a one unit difference in the $\log_2$ VNTs of SM87/87 and SG106/99 were observed as compared to B1 and 2440/69, regardless of the antiserum that was used. Comparison of the neutralizing epitopes of these strains revealed the presence of mutation at the linear epitope of the HN-gene (E347K or E347G) of SM87/87 and SG106/99 (Table 4). Although these differences in VNT may be inconclusive at this point due to small sample size, these results were consistent with observed differences in the antigenicity of field NDVs from vaccinated flocks possessing an E347K mutation in the HN protein as reported previously [20, 22, 44]. It was observed that residue 347 of the HN protein may be a critical determinant for formation of antigenic epitope and may be involved in antigenic selection during virus shedding of NDV in vaccinated flocks [20, 22, 44]. Although *in vitro* results demonstrated that poor immunization may be the most plausible reason in the breakthrough infection of the field strains, *in vivo* and cross-protection experiments using field chickens are

recommended to further elucidate the effects of these mutations not only in the presence or absence of clinical signs but most especially on the production performance of the infected flocks.

In summary, this study described the whole genome characteristics of three strains of NDV belonging to recent genotypes affecting vaccinated commercial poultry flocks in Japan. Several unique mutations were identified in the neutralizing epitopes and functional domains of the field strains. Data obtained from this study may be a useful reference in characterizing future NDV outbreaks in vaccinated chickens. The complete genome sequences of these strains may be use as a genetic map for future studies regarding vaccine designs, reverse genetics systems, recombinant gene technologies and development of molecular diagnostic tools to characterize and prevent future ND outbreaks in vaccinated poultry flocks.

Table 1. Genome length characteristics of NDVs isolated from commercial layer flocks in Japan[a]

Region	Gene start	3' UTR	Coding Sequence[b]	5' UTR	Gene end	Intergenic region	Nucleotide length	Amino acid length[c]
Leader							55	
NP	56-65	66	122-1591	217	1798-1808	1	1753	489
P	1810-1819	83	1893-3080	180	3250-3260	1	1451	395
M	3262-3271	34	3296-4390	112	4493-4502	1	1241	364
F	4504-4513	46	4550-6211	84	6285-6295	31	1792	553
HN	6327-6336	91	6418-8133	195	8318-8328	47	2002	571
L	8376-8385	11	8387-15001	77	15069-15078		6703	2204
Trailer							114	
Whole genome							15192	

[a] All three isolates used in this study (2440/69, SM87/87 and SG106/99) showed same genome length characteristics; [b] including stop codon; [c] without stop codon

Table 2. Nucleotide (nt) and amino acid (aa) sequence identities (%) between Japanese NDV isolates, vaccine and closely-related strains

Gene	Strain (Genotype)	Ishii/62 (I)[a,b]		B1/47 (II)[b]		LaSota (II)		Sato/30 (III)[b]		US/Fontana/72 (VI)		ZhJ-3/97 (VI)		China/Guangxi 14/02 (VII)	
		nt	aa	nt	aa	nt	VI	nt	aa	nt	aa	nt	aa	nt	aa
Complete genome	2440/69 (VI)	n.d.[c]		85.5		85.3		n.d.		96.7		96.3		91.5	
	SM87/87 (VI)	n.d.		84.7		84.5		n.d.		94.6		97.8		90.1	
	SG106/99 (VII)	n.d.		83.7		83.5		n.d.		91.1		90.4		97.4	
NP	2440/69	90.3	94.9	86.0	92.6	85.9	93.1	94.1	97.3	96.4	98.4	96.8	97.8	92.4	96.9
	SM87/87	89.9	94.9	86.0	92.4	85.8	92.8	93.1	98.0	95.2	98.6	98.4	99.4	92.0	97.8
	SG106/99	89.0	93.7	85.9	91.2	85.8	91.6	93.1	96.5	92.4	96.7	92.1	97.1	99.0	99.2
P	2440/69	n.d.	n.d.	84.2	82.3	84.9	84.8	n.d.	n.d.	96.5	96.7	95.9	95.7	90.6	90.1
	SM87/87	n.d.	n.d.	82.6	80.0	83.0	82.5	n.d.	n.d.	94.0	93.2	96.6	95.7	89.1	88.6
	SG106/99	n.d.	n.d.	81.7	79.2	83.0	82.0	n.d.	n.d.	90.2	88.9	89.4	88.1	99.0	98.7
M	2440/69	n.d.	n.d.	86.9	89.6	86.2	89.3	n.d.	n.d.	97.2	98.4	96.4	98.9	93.0	96.4
	SM87/87	n.d.	n.d.	85.8	89.6	85.0	89.3	n.d.	n.d.	95.8	98.6	97.9	99.5	91.8	96.7
	SG106/99	n.d.	n.d.	85.4	89.0	84.7	89.0	n.d.	n.d.	93.2	97.0	92.4	97.5	98.9	98.9
F	2440/69	89.5	93.1	86.5	89.9	86.2	90.1	86.9	87.9	96.3	98.2	96.0	97.7	92.1	95.8
	SM87/87	88.0	92.8	85.6	89.3	85.1	89.5	85.8	85.5	94.5	97.7	98.4	99.1	89.8	94.9
	SG106/99	86.9	91.0	84.5	88.3	84.1	88.1	85.3	85.5	91.8	95.8	89.9	95.1	98.1	97.1
HN	2440/69	88.3	91.9	84.9	89.7	84.6	88.8	n.d.	n.d.	96.7	97.2	96.8	97.4	91.2	94.6
	SM87/87	87.4	91.8	84.6	89.5	84.3	88.6	n.d.	n.d.	94.9	97.0	98.1	98.8	90.7	94.8
	SG106/99	85.8	90.9	82.9	88.6	82.2	87.7	n.d.	n.d.	90.9	94.8	90.8	95.1	98.8	99.7
L	2440/69	n.d.	n.d.	88.0	93.3	86.8	92.7	n.d.	n.d.	97.3	98.7	96.9	98.3	92.9	96.1
	SM87/87	n.d.	n.d.	87.2	92.7	86.1	92.1	n.d.	n.d.	95.4	97.9	98.2	99.0	91.4	95.2
	SG106/99	n.d.	n.d.	86.3	92.5	85.1	91.9	n.d.	n.d.	92.6	96.5	91.9	96.0	98.2	98.7

[a] Nucleotide sequences for comparison with Japanese isolates were from the following sources (GenBank accession number): the NP, F and HN genes of Ishii/62 (AB124600.1, AB465607.1, AB432886.1), NP and F genes of Sato/30 (AB124605.1, AB070382.1), complete genome sequence of B1/47, LaSota, US/Fontana/72, Zhj-3/97 and China/Guangxi14/02 (AF309418.1, JF950510.1, AY562988.1, FJ766529.1 and JX193075.1); [b] Vaccine strains widely-used in Japan; [c] No data

Table 3. Amino acid substitutions in the functional domains of the F protein

Virus (genotype)	Signal peptide	Fusion peptide	Heptad repeats			Transmembrane domain
			HRa	HRb	HRc	
	1-31	117-141	143-185	268-299	471-500	501-521
Consensus[a]	MGSKPSTRIPVPLMLITRIML ILSCICLTSS	FIGAVIGSVALGV ATAAQITAAAAL	QANQNAANILRLKESIA ATNEAVHEVTDGLSQL AVAVGKMQQF	LITGNPILYDSQT QLLGIQVNLPSV GNLNNMR	NNSISNALDKL AESNSKLDKVN VKLTSTSA	LITYIVLTVISLVFGA LSLVL
2440/69 (VI)	-	-	-	-	D479G, K480R	A516T
SM87/87 (VI)	P10L, L13P, M20T	V121I	-	-	K480R	F514S
SG106/99 (VII)	K4I, V11A, L21P, C27R	-	A176S	N272Y	K494R	L521S

[a] The consensus amino acid sequence was derived from 100 velogenic, mesogenic and lentogenic NDV strains from GenBank

Table 4. Amino acids constituting the neutralizing epitopes of the HN protein

Virus (genotype)	HN protein									
	193-201	263	287	321	332-333	346-353	356	494	513-521	569
Consensus[a]	LSGCRD HSH	N	D	K	GK	DEQDY QIR	K	G/D	RITRV SSSS	D
2440/69 (VI)	-	K	-	-	-	-	-	-	I514V	-
SM87/87 (VI)	-	K	-	-	-	E347K	-	-	I514V	-
SG106/99 (VII)	-	K	-	-	-	E347G	-	-	I514V	-

[a]The consensus amino acid sequence was derived from NDV vaccine strains (B1, Clone 30, LaSota, V4, Ishii/62, Sato/30)

Table 5. Amino acid substitutions in functional domains of the HN protein

Virus (genotype)	Transmembrane domain	Receptor recognition	N-linked glycosylation sites	Cysteine residues
	25-45	174, 175, 198, 236, 258, 299, 317, 401, 416, 498, 526 547		
Consensus[a]	FRIAVLLLIVMTLAISAAALV	R, I, D, K, E, Y, Y, E, R, R, Y, E	119, 341, 433, 481, 508, 538	123, 172, 186, 196, 238, 247, 251, 344, 455, 461, 465, 531, 542
2440/69 (VI)	A41V	-	-	-
SM87/87 (VI)	-	-	lost at 538	-
SG106/99 (VII)	I33M, A41S, V45A	-	-	-

[a] The consensus amino acid sequence was derived from a total of 100 velogenic, mesogenic and lentogenic NDV strains from GenBank

Table 6. Virus neutralization titer of field isolates using different NDV antisera

Virus (genotype)	Anti-Ishii antiserum (HI 7.3)[a]	Anti-B1 antiserum (HI 7)	NDV antiserum from a commercial layer flock (HI 7)	Negative antiserum from SPF chickens (HI <1)
B1 (II)	6.3	6.5	6.5	0
2440/69 (VI)	5.5	6.3	6.5	0
SM87/87 (VI)	4.5	5.7	5.7	0
SG106/99 (VII)	4.7	5.3	5.5	0

[a] indicates the reciprocal $\log_2$ HI titer against B1 strain; [b] indicates the reciprocal $\log_2$ virus neutralization titer

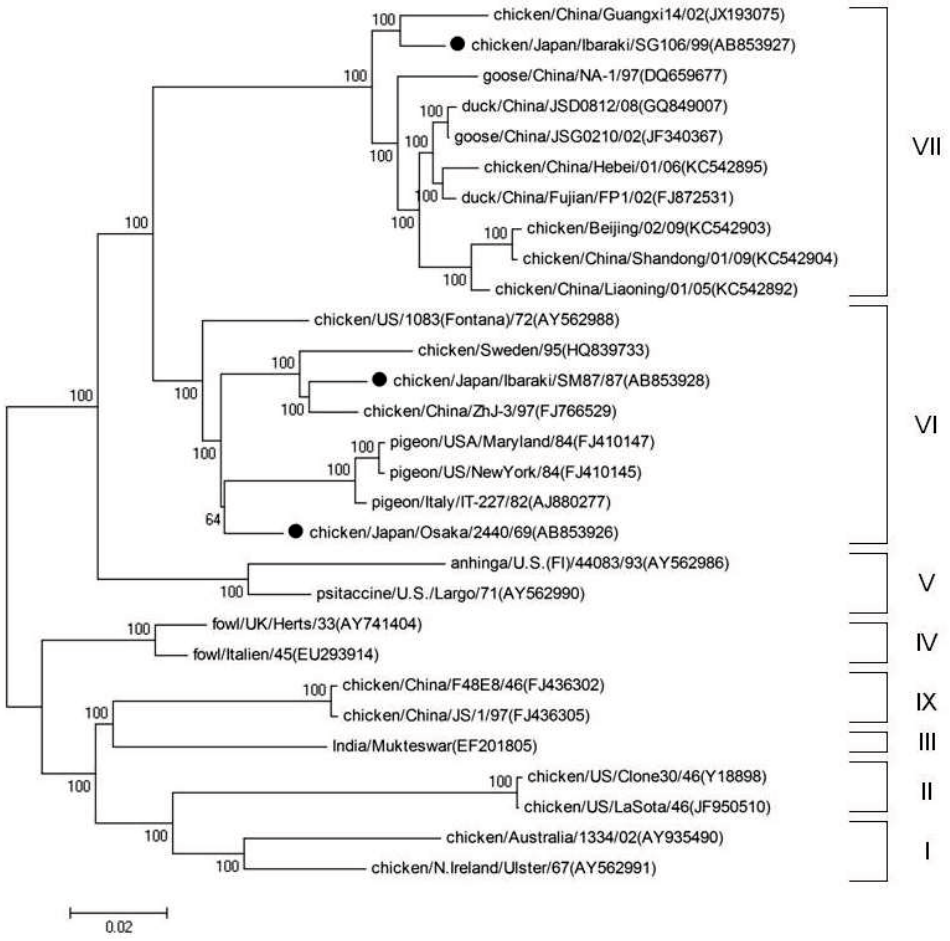


Figure 1. Phylogenetic analysis of NDV field strains from Japan based on the complete genome sequence (1-15,192 nucleotides). The evolutionary history was inferred using the Neighbor-Joining method. The bootstrap consensus tree inferred from 1000 replicates is taken to represent the evolutionary history of the taxa analyzed [34]. Branches corresponding to partitions reproduced in less than 50% bootstrap replicates are collapsed. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (1000 replicates) are shown next to the branches. The tree is drawn to scale, with branch lengths in the same units as those of the evolutionary distances used to infer the phylogenetic tree. The evolutionary distances were computed using the Maximum Composite Likelihood method and are in the units of the number of base substitutions per site. All positions containing gaps and missing data were eliminated from the dataset. Strains used in this study were marked with ●.

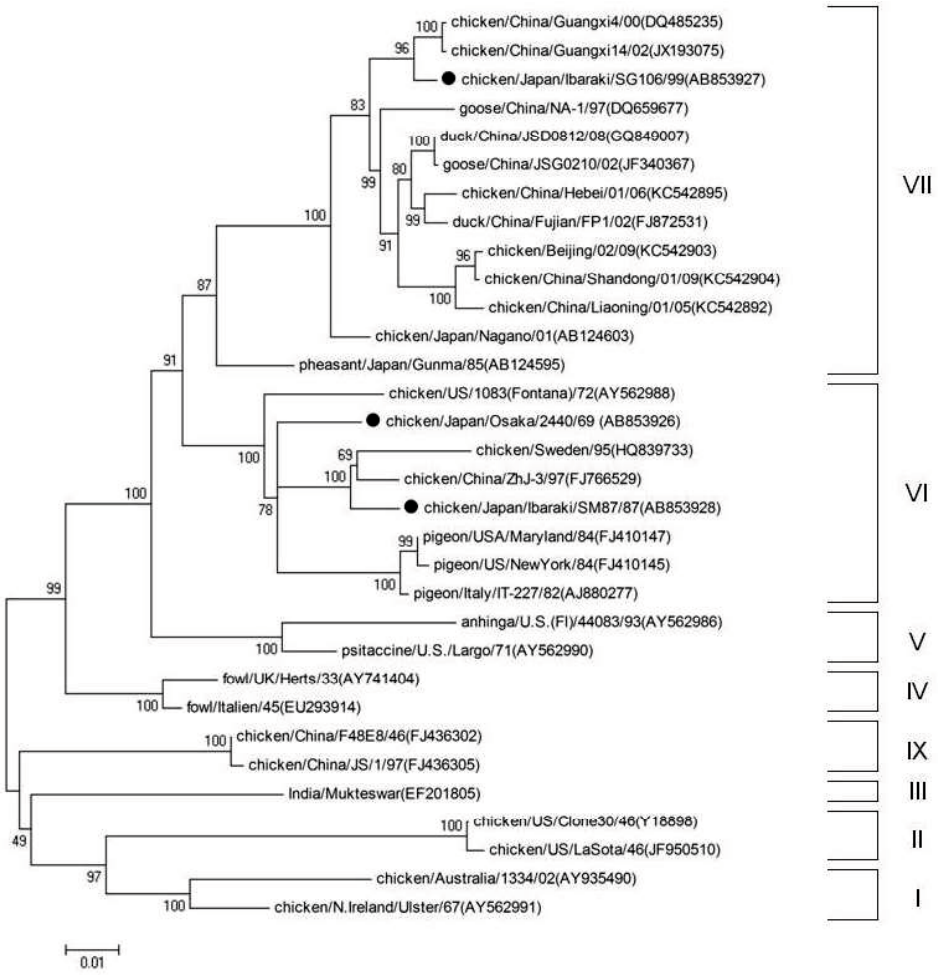


Figure 2. Phylogenetic analysis of NDV field strains from Japan based on the complete coding sequence of the NP-gene (1-1470 nucleotides). The evolutionary history was inferred using the Neighbor-Joining method. The bootstrap consensus tree inferred from 1000 replicates is taken to represent the evolutionary history of the taxa analyzed [34]. Branches corresponding to partitions reproduced in less than 50% bootstrap replicates are collapsed. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (1000 replicates) are shown next to the branches. The tree is drawn to scale, with branch lengths in the same units as those of the evolutionary distances used to infer the phylogenetic tree. The evolutionary distances were computed using the Maximum Composite Likelihood method and are in the units of the number of base substitutions per site. All positions containing gaps and missing data were eliminated from the dataset. Strains used in this study were marked with ●.

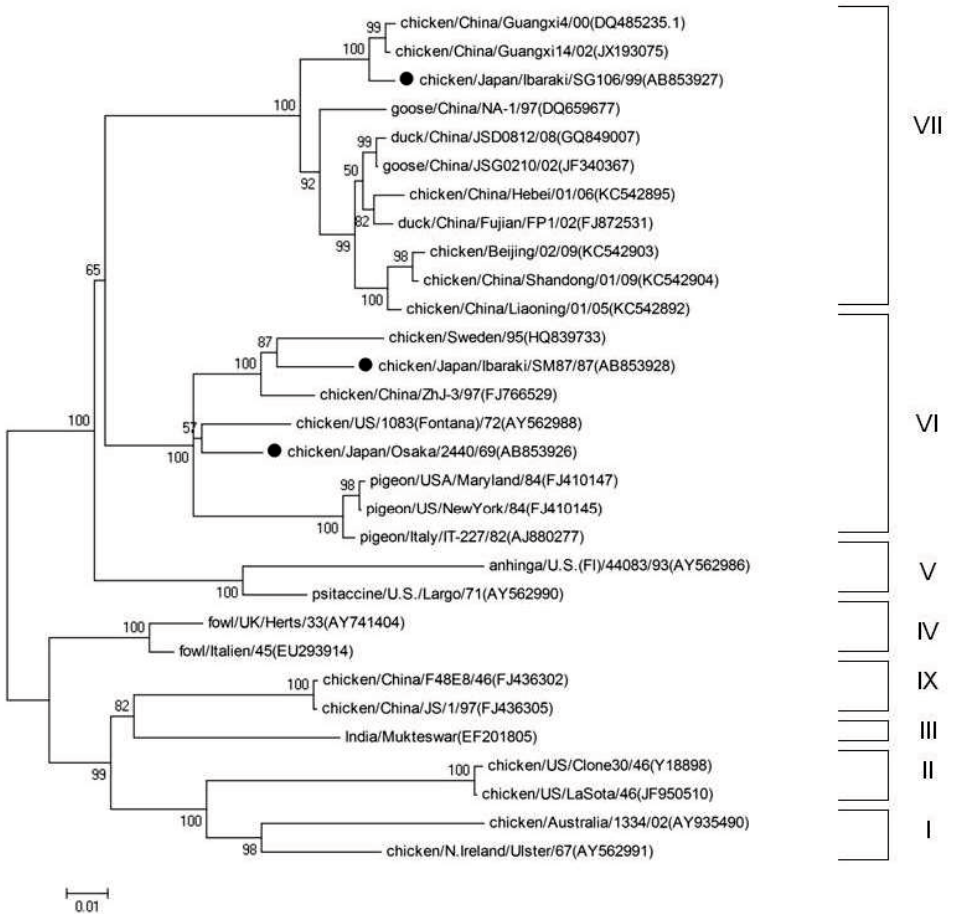


Figure 3. Phylogenetic analysis of NDV field strains from Japan based on the complete coding sequence of the P-gene (1-1188 nucleotides). The evolutionary history was inferred using the Neighbor-Joining method. The bootstrap consensus tree inferred from 1000 replicates is taken to represent the evolutionary history of the taxa analyzed [34]. Branches corresponding to partitions reproduced in less than 50% bootstrap replicates are collapsed. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (1000 replicates) are shown next to the branches. The tree is drawn to scale, with branch lengths in the same units as those of the evolutionary distances used to infer the phylogenetic tree. The evolutionary distances were computed using the Maximum Composite Likelihood method and are in the units of the number of base substitutions per site. All positions containing gaps and missing data were eliminated from the dataset. Strains used in this study were marked with ●.

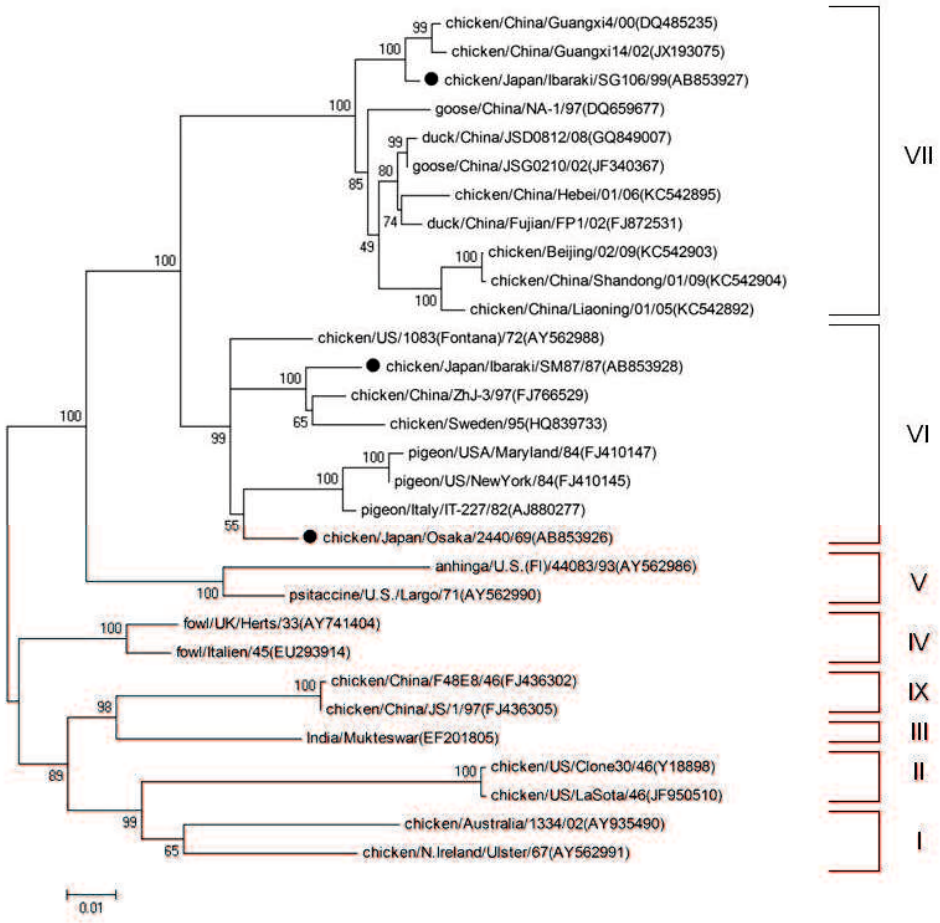


Figure 4. Phylogenetic analysis of NDV field strains from Japan based on the complete coding sequence of the M-gene (1-1095 nucleotides). The evolutionary history was inferred using the Neighbor-Joining method. The bootstrap consensus tree inferred from 1000 replicates is taken to represent the evolutionary history of the taxa analyzed [34]. Branches corresponding to partitions reproduced in less than 50% bootstrap replicates are collapsed. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (1000 replicates) are shown next to the branches. The tree is drawn to scale, with branch lengths in the same units as those of the evolutionary distances used to infer the phylogenetic tree. The evolutionary distances were computed using the Maximum Composite Likelihood method and are in the units of the number of base substitutions per site. All positions containing gaps and missing data were eliminated from the dataset. Strains used in this study were marked with ●.

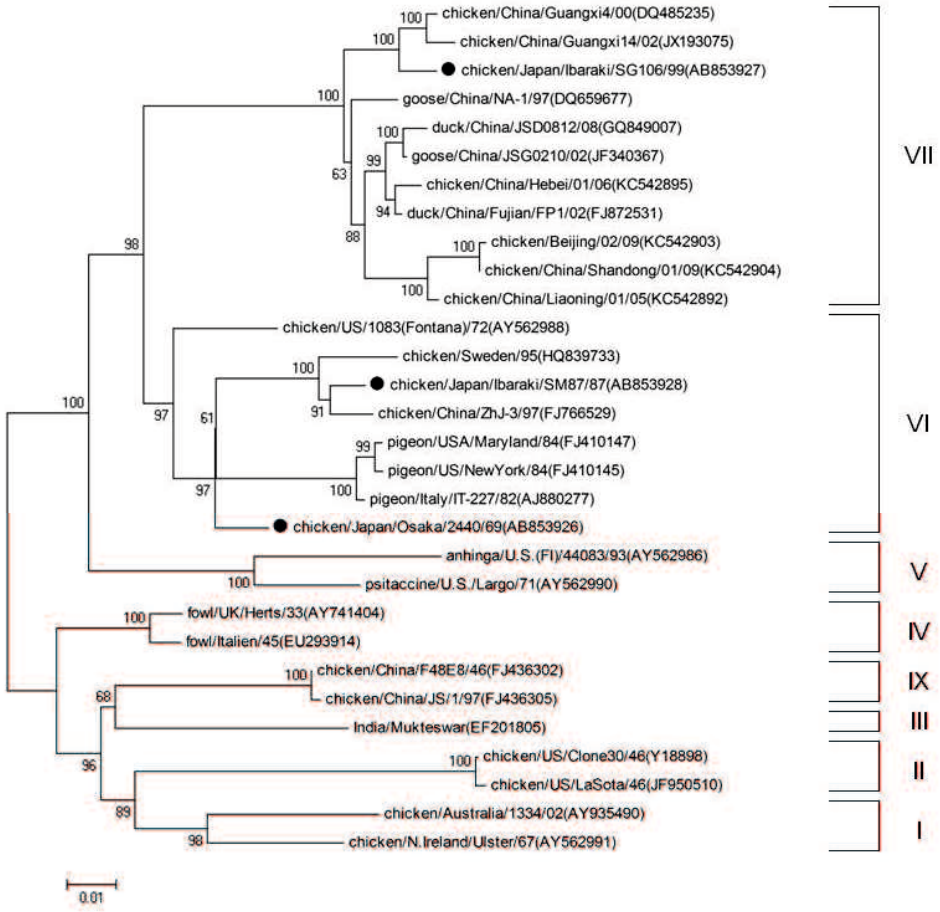


Figure 5. Phylogenetic analysis of NDV field strains from Japan based on the complete coding sequence of the F-gene (1-1662 nucleotides). The evolutionary history was inferred using the Neighbor-Joining method. The bootstrap consensus tree inferred from 1000 replicates is taken to represent the evolutionary history of the taxa analyzed [34]. Branches corresponding to partitions reproduced in less than 50% bootstrap replicates are collapsed. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (1000 replicates) are shown next to the branches. The tree is drawn to scale, with branch lengths in the same units as those of the evolutionary distances used to infer the phylogenetic tree. The evolutionary distances were computed using the Maximum Composite Likelihood method and are in the units of the number of base substitutions per site. All positions containing gaps and missing data were eliminated from the dataset. Strains used in this study were marked with ●.

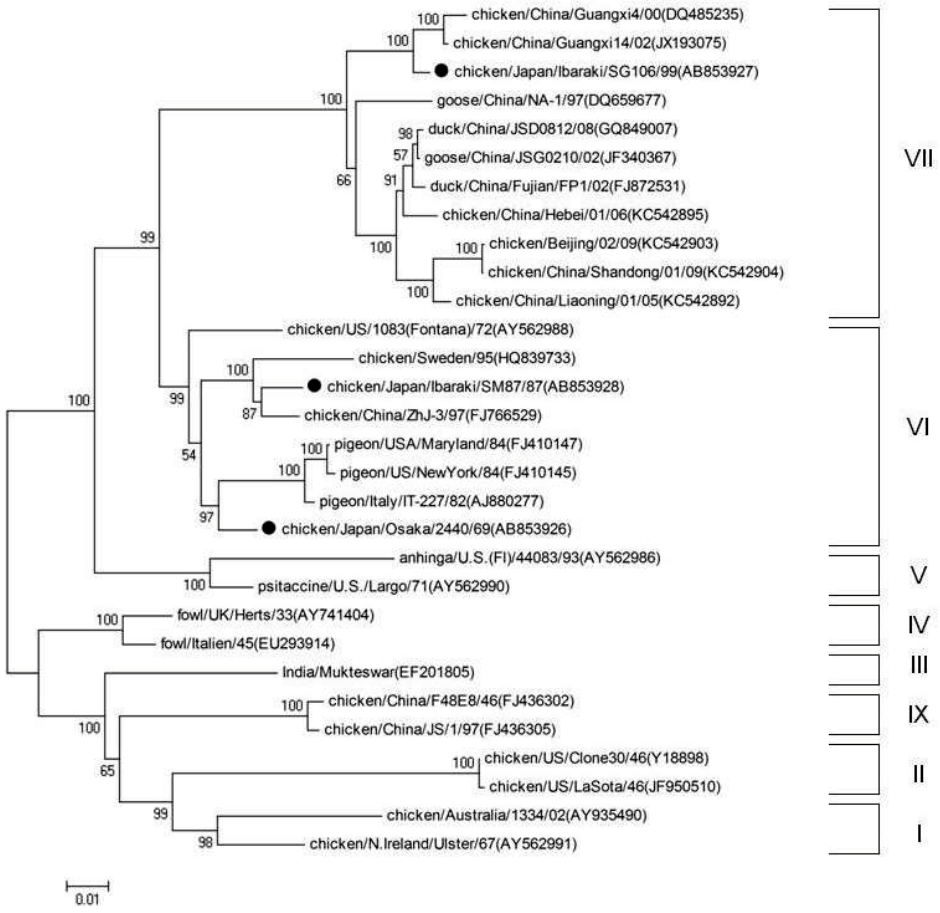


Figure 6. Phylogenetic analysis of NDV field strains from Japan based on the complete coding sequence of the HN-gene (1-1716 nucleotides). The evolutionary history was inferred using the Neighbor-Joining method. The bootstrap consensus tree inferred from 1000 replicates is taken to represent the evolutionary history of the taxa analyzed [34]. Branches corresponding to partitions reproduced in less than 50% bootstrap replicates are collapsed. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (1000 replicates) are shown next to the branches. The tree is drawn to scale, with branch lengths in the same units as those of the evolutionary distances used to infer the phylogenetic tree. The evolutionary distances were computed using the Maximum Composite Likelihood method and are in the units of the number of base substitutions per site. All positions containing gaps and missing data were eliminated from the dataset. Strains used in this study were marked with ●.

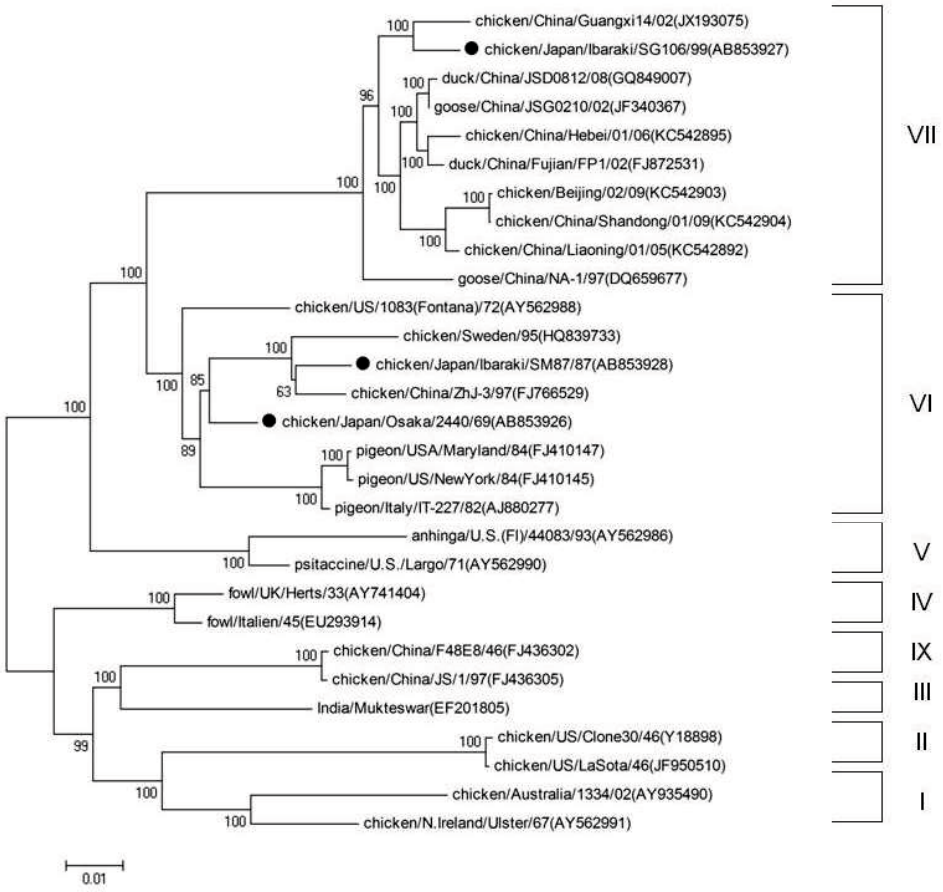


Figure 7. Phylogenetic analysis of NDV field strains from Japan based on the complete coding sequence of the L-gene (1-6615 nucleotides). The evolutionary history was inferred using the Neighbor-Joining method. The bootstrap consensus tree inferred from 1000 replicates is taken to represent the evolutionary history of the taxa analyzed [34]. Branches corresponding to partitions reproduced in less than 50% bootstrap replicates are collapsed. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (1000 replicates) are shown next to the branches. The tree is drawn to scale, with branch lengths in the same units as those of the evolutionary distances used to infer the phylogenetic tree. The evolutionary distances were computed using the Maximum Composite Likelihood method and are in the units of the number of base substitutions per site. All positions containing gaps and missing data were eliminated from the dataset. Strains used in this study were marked with ●.

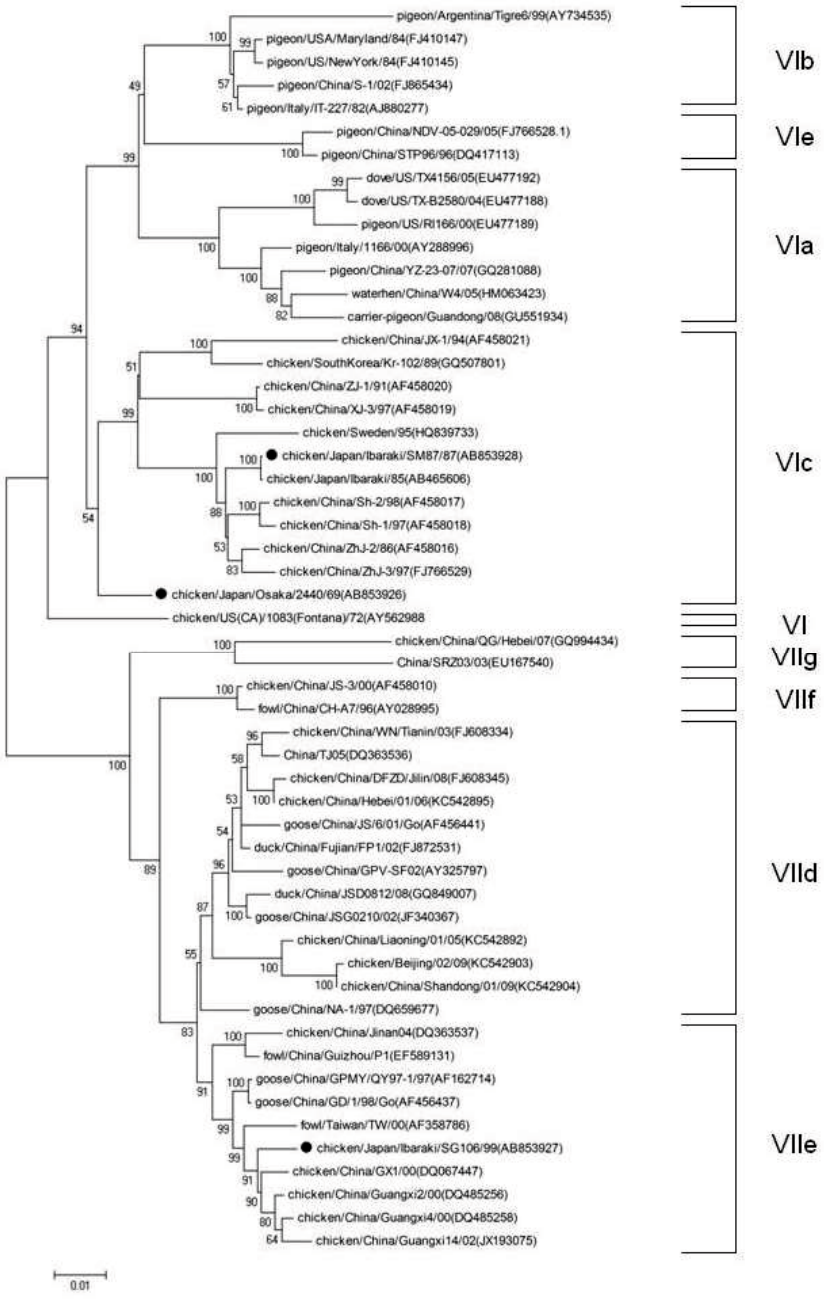


Figure 8. Phylogenetic analysis based on the complete coding sequence of the F-gene (1 – 1662 nt) of genotype VI and VII NDV strains following the unified NDV classification system [32]. The evolutionary history was inferred using the Neighbor-Joining method. The bootstrap consensus tree inferred from 1000 replicates is taken to represent the evolutionary history of the taxa analyzed [34]. Branches corresponding to partitions reproduced in less than 50% bootstrap replicates are collapsed. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (1000 replicates) are shown next to the branches. The tree is drawn to scale, with branch lengths in the same units as those of the evolutionary distances used to infer the phylogenetic tree. The evolutionary distances were computed using the Maximum Composite Likelihood method and are in the units of the number of base substitutions per site. All positions containing gaps and missing data were eliminated from the dataset. Strains used in this study were marked with ●.

CHAPTER IV

Surveillance of avian paramyxovirus from migratory waterfowl in the San-in Region of western Japan from 2006 to 2012

Introduction

Avian paramyxoviruses (APMVs), which belong to the genus Avulavirus in the family Paramyxoviridae, comprise nine antigenically distinct serotypes (APMV-1 to 9) [3]. Recently, new serotypes of APMVs, APMV-10, 11 and 12 were proposed respectively [11, 73, 103]. Although APMV-1, which is synonymous with Newcastle disease virus (NDV), is highly pathogenic in poultry, the other APMV serotypes are also known to cause respiratory and reproductive diseases in chickens [109]. All APMV serotypes, except APMV-5, circulate widely in wild bird populations [33, 35, 97]. However, the information on the distribution of APMVs in wild birds is limited, especially in Japan.

Wild birds, particularly waterfowl, are known reservoirs of APMV-1, 4, 6, 8 and 9 [3], and are considered to be important carriers of APMVs. The potential for disease transmission is considered to be particularly high because many waterfowl, such as geese, swans, and ducks, overwinter in Japan after migrating from Alaska, the Russian Far East, eastern Siberia, eastern Mongolia and northeastern China [84].

While very little is known about the molecular and biological characteristics and pathogenicity of APMV serotypes 2-9, extensive research has been conducted on APMV-1, or NDV. NDVs have been divided into three major pathotypes on the basis of pathogenicity; lentogenic (low virulence), mesogenic (moderate virulence) and velogenic (high virulence) [3]. Furthermore, recent phylogenetic analyses have separated NDVs into two distinct sister clades, classes I and II, each of which contain

several genotypes [3, 13]. The majority of viruses that have been reported to be velogenic in domestic poultry have been grouped in class II, while lentogenic strains are dominant in class I [30, 58].

We previously experimentally demonstrated that a non-pathogenic NDV isolate from wild waterfowl became highly pathogenic after several passages in chickens [95]. The findings of that study demonstrated that wild birds were potentially capable of transmitting and spreading precursors of velogenic viral strains to domestic poultry. Consequently, continuous surveillance of APMV, including NDV, in wild birds is important for providing information on the viruses in the field, as well as emerging velogenic viruses.

In this study, we conducted a survey for APMV in populations of overwintering migratory waterfowl from 2006 to 2012 in the San-in region of western Japan where 16 APMV strains were previously isolated. Consequently, we examined the pathogenic and phylogenetic relationships among the collected APMV isolates and compared them with other isolates in the field.

Materials and Methods

Samples

A total of 1,967 fresh fecal samples were collected from tundra swan (*Cygnus columbianus*), mallard (*Anas platyrhynchos*), white-fronted goose (*Anser albifrons frontalis*), common teal (*Anas crecca*), Eurasian wigeon (*Anas penelope*), spot-billed duck (*Anas poecilorhyncha*), gadwall (*Anas strepera*), and unidentified duck spp. (*Anas spp.*) during winter (from November to March) of 2006 to 2012. Samples were collected at eight different sites, Lake Koyama, Pond Nikko, Lake Togo, Tenjin River, Hino River, Ito Coast, Yonago Waterbirds Sanctuary, and rice fields in

the suburbs of Yasugi city, in the San-in region (Tottori and Shimane prefectures) of western Japan. The fecal samples were collected individually, placed in screw-cap tubes, and stored at -80˚C until analysis.

Virus isolation

Virus isolation was performed using a previously described method with a slight modification [94]. Each collected fecal sample was suspended at a concentration of approximately 20% in phosphate-buffered saline (pH 7.2) containing penicillin at 10,000 units/ml and streptomycin at 10 mg/ml. The suspension was centrifuged at 1,000×g for 10 min. Aliquots of 200 µl of supernatant were then used to inoculate into the allantoic cavities of two 9- to 11-day-old embryonated chicken eggs, which were then incubated at 37˚C for 3 days unless the embryo died. The inoculated eggs were then chilled to 4˚C and the allantoic fluid of each egg was tested for hemagglutination activity.

Serotyping

All hemagglutinating agents were identified in a hemagglutination inhibition (HI) test using reference antisera against APMV strains: APMV-1/goose/Alaska/415/91, APMV-2/Chicken/California/Yucaipa/56, APMV-3/turkey/Wisconsin/68, APMV-4/duck/ Mississippi/320/75, APMV-6/duck/Hong Kong/18/199/77, and APMV7/dove/Tennessee/4/75 [57]. Samples that tested positive for at least one of these antisera were identified as APMV. The methods used in the HI test followed established procedures [94].

Sequencing and BLAST search

Viral RNA was isolated from infected allantoic fluid by using QIAamp Viral RNA Mini Kit (Qiagen, CA). The F genes coding full-length ORFs were amplified using PrimeScriptTM Reverse Transcriptase (TaKaRa, Shiga, Japan) for RT and KOD Dash polymerase (Toyobo, Osaka, Japan) for PCR. After extraction from an agarose gel using a QIAquick Gel Extraction Kit (Qiagen), viral cDNA fragments were sequenced using a BigDye Terminator v3.1 Sequencing Kit (Applied Biosystems, CA) on a 3130xl Genetic Analyzer (Applied Biosystems). The F gene-specific primer sequences and conditions employed for RT, PCR and sequencing are available upon request. The DNA sequence data were edited and aligned using BioEdit software (ver. 7.0.8.0) [104] before being subjected to BLAST search analysis using the NCBI database.

Pathogenicity test

To assess the virulence of each APMV isolate, the mean death time (MDT in hours) of chick embryos at the minimum lethal dose and the intracerebral pathogenicity index (ICPI) in 1-day-old chicks were measured [9].

Phylogenetic analysis

Phylogenetic analyses of F gene segments were performed using ClustalX implemented in the MEGA 4 software package [101]. The phylogenetic tree was estimated using the Kimura two-parameter nucleotide model, and the robustness of the clusters obtained by the neighbor joining algorithm were assessed using 1,000 bootstrap replicates.

Results

In the winters of 2006 to 2012, a total of 1,967 fecal samples were collected from tundra swan (n=263), mallard (n=683), white-fronted goose (n=61), common teal (n=60), Eurasian wigeon (n=188), spot-billed duck (n=92), gadwall (n=2) and unidentified duck spp. (n=618) in the San-in region of western Japan (Table 1).

A total of 64 hemagglutinating agents were isolated and assayed in the HI tests using anti-APMV reference strain antisera. Of these, 15 samples tested positive for at least one of the antisera were identified as APMV (Table 2). Some minor cross-reactions between two different serotypes of APMVs were found as reported previously [4]. Consequently 3 isolates were identified as NDV, and 12 isolates were AMPV-4, based on the highest titers in HI tests (Table 2 and 3). Another isolate (Tundra swan/Shimane/91-94/2007), which did not react to any APMV antisera (serotypes 1-4, 6, and 7), was identified as APMV-8 by fusion (F) -gene sequencing and BLAST analysis (the highest homology was with APMV-8/goose/Delaware/1053/76; 98%).

Furthermore, a BLAST search for other APMV isolates corroborated the serological findings. Briefly, 12 APMV-4 isolates showed the highest homology with APMV-4/KR/YJ/2006 (more than 96%). Duck/Tottori/N12/2006 showed the highest homology with NDV/Pennsylvania/3167/2009 (99%), and Duck/Tottori/453/2009 and Duck/Tottori/481/2009 showed the highest homology with NDV/duck/China/08-046/2008 (98%). Consequently, a total of 16 APMV strains (tundra swan (n=1), mallard (n=2), common teal (n=1), Eurasian wigeon (n=3), spot-billed duck (n=5), unidentified duck spp. (n=4)) were isolated. The overall rate of APMV isolation was 0.8% (Table 1).

Virulence of virus isolates was assessed by pathogenicity tests with chicken embryos and chicks (Table 4). The MDT of the 6 representative isolates was more than 168 hr, which is typical for avirulent viruses. The ICPI of these samples ranged from 0.00 to 0.16, which is also within the expected values for avirulent viruses. The amino acid sequence at the cleavage site of the F protein was deduced from the nucleotide sequence of the corresponding gene. Two of the 3 NDV isolates, Duck/Tottori/453/2009 and Duck/Tottori/481/2009, possess ^{112}ERQER-LV^{118}, with the remaining isolate Duck/Tottori/N12/2006 possessing ^{112}GKQGR-LI^{118} at the fusion cleavage site; these characteristics were all typical of avirulent viruses [26].

A phylogenetic tree was constructed based on the partial sequences of the F genes together with those from Genbank (Figs. 1 and 2). The NDV isolates in the present study were divided into the two sister clades, Duck/Tottori/453/2009 and Duck/Tottori/481/2009, which belonged to the class I genotype c (Fig. 1), and the remaining isolate, Duck/Tottori/N12/2006, was included in the class II genotype I (Fig. 2).

To investigate the relationship between the NDV isolates from wild birds and field isolates from poultry farms in Japan, a phylogenetic tree was constructed (Fig. 3). The result showed that three NDV isolates in the present study were clearly distinguishable from any of the NDV isolates that had caused NDV outbreaks in Japan in recent years.

Discussion

In the present study, a total of 16 APMVs were isolated from wild birds (Table 3). Of these isolates, APMV-4 strains were isolated at different sites in the San-in region in Japan relatively frequently during the study period. Conversely, APMV-8, which was isolated in 2007, was considerably rare, even at a global scale [25, 111]. Stanislawek *et al.* [98] isolated NDV and APMV-4 from wild ducks in New Zealand in 1997. In the United States, Goekjian *et al.* [35] reported that NDV, APMV-4, and APMV-6 were isolated from migratory waterfowl from 2004 to 2006. In this study, we found different serotypes of APMVs in wild bird species that had migrated into the San-in region, western Japan.

In the present surveillance study, the overall isolation rate of APMV was 0.8%, which is slightly lower than our previous survey in the same region in 1997-2000 (1.4%, 5 isolates/359 fecal samples) [94]. Another study in Japan reported that 11 NDV strains (prevalence rate: 0.46%) were isolated from 2,381 fecal samples of northern pintail from 2006 to 2008 in the Tohoku region in northeastern Japan [48]. The findings of these studies also support the notion that migratory waterfowl play an important role in the maintenance of APMVs in nature [3].

In cases where APMV-4 was isolated from waterfowl, the birds rarely exhibited clinical signs of viral infection [3, 25, 37, 97]. However, in chickens experimentally infected with APMV-4, all of the birds manifested symptoms of microscopic lesions in the trachea, lung, gut, and pancreas [109]. Viral replication in chickens was also confirmed by isolation of the virus in embryonated eggs. It is therefore possible that the non-pathogenic APMV-4 that is maintained in populations of wild waterfowl has the potential to become pathogenic after transmission to, and circulation within, domestic chicken populations.

Our previous report showed that a lentogenic NDV isolate from wild waterfowl become velogenic after repeated passage in chickens, causing 100% mortality in the infected birds [95]. The results suggested that circulation of lentogenic NDV isolates in poultry farm can result in viruses becoming velogenic. In the present study, an NDV class II strain, Duck/Tottori/N12/2006, was isolated in the field. Previous studies have shown that the majority of velogenic viruses in domestic poultry belong to class II [30]. Therefore, although the pathogenicity tests conducted in this study showed that the isolate was lentogenic, it could be a possible precursor virus in a future Newcastle disease outbreak in Japan.

Two outbreaks of Newcastle disease on poultry farms in Ireland in 1990 [8] were caused by velogenic isolates that were very similar, both antigenically and genetically, to avirulent viruses isolated from feral waterfowl [27]. Moreover, genetic analysis of viruses isolated during outbreaks in 1998 to 2000 in Australia were also very similar to viruses isolated from birds in the wild [38]. Therefore, to investigate the genetic affiliation among the field isolates in Japan, phylogenetic analyses were conducted. The results revealed that there was no genetic relationship between the isolates obtained from wild birds and isolates from domestic poultry in Japan. Especially, class II NDV isolate, Duck/Tottori/N12/2006, was most closely related to the old isolate, NDV/chicken/Japan/Ishii/1962 (Fig. 3) indicating that the isolate is not a direct ancestor for the recent outbreaks in Japan. However, the pathogenic potential of the isolate to domestic poultry cannot be ignored. It is therefore necessary to continue surveillance of avian paramyxoviruses in wild waterfowl. Continued surveillance over multiple years will allow us to increase our understanding of the role of wild birds in the dissemination of APMVs in the field.

Table 1. Isolation of avian paramyxovirus from fecal samples of migratory waterfowls in the San-in region of western Japan during the winters of 2006 to 2012

Species	Sample year							Total	Isolation rate (%)
	2006	2007	2008	2009	2010	2011	2012		
Spot-billed duck					5/87	0/5		5/92	5.4
Common teal			0/58			1/2		1/60	1.7
Eurasian wigeon	2/6	1/106				0/60	0/16	3/188	1.6
Unidentified duck				2/227	2/350	0/34	0/7	4/618	0.6
Tundra swan	0/17	1/135	0/101	0/2		0/8		1/263	0.4
Mallard	2/127	0/164	0/251	0/51	0/68		0/22	2/683	0.3
White-fronted goose		0/49				0/12		0/61	0
Gadwall	0/2							0/2	0
Total	4/152	2/454	0/410	2/280	7/505	1/121	0/45	16/1967	0.8
Isolation rate (%)	2.6	0.4	0	0.7	1.4	0.8	0		

Table 2. Hemagglutination inhibition titers of avian paramyxovirus (APMV) isolates against reference APMV antisera

Virus	Reference antiserum					
	APMV-1	APMV-2	APMV-3	APMV-4	APMV-6	APMV-7
Homologous	2560[a]	640	1280	5120	640	640
Duck/Tottori/N12/2006	1280[b]	<	80	320	320	320
Duck/Tottori/2/2006	<[c]	<	<	2560	160	160
Duck/Tottori/126/2006	<	<	<	640	40	40
Duck/Tottori/T99/2006	<	<	<	1280	<	80
Duck/Tottori/140/2007	<	<	<	1280	<	40
Tundra swan/Shimane/91-94/2007	<	<	<	<	<	<
Duck/Tottori/453/2009	640	<	160	320	320	320
Duck/Tottori/481/2009	640	<	160	320	320	320
Duck/Tottori/114-115/2010	<	<	<	640	40	40
Duck/Tottori/99/2010	<	<	<	640	<	160
Duck/Tottori/237-238/2010	<	<	<	1280	40	160
Duck/Tottori/250/2010	<	<	<	640	40	<
Duck/Tottori/251-252/2010	<	<	<	640	40	40
Duck/Tottori/264/2010	<	<	<	640	<	40
Duck/Tottori/267-268/2010	<	<	<	1280	80	80
Duck/Tottori/22/2011	<	<	<	640	<	40

[a]Expressed as a reciprocal of the highest dilution of the antiserum inhibiting hemagglutination units of the virus.
[b]Underlined numbers represent the highest titers of each virus in HI test using a panel of reference antisera prepared against 6 subtypes of reference strains of APMVs (APMV-1-4, APMV-6, and -7).
[c]< : less than 1:40.

Table 3. Avian paramyxovirus isolates from migratory waterfowl in western Japan

Virus	Collection date	Sample site	Host	Subtype
Duck/Tottori/N12/2006	2006.12.13	Pond Nikko, Tottori	Eurasian Wigeon	NDV(class II)
Duck/Tottori/2/2006	2006.12.13	Pond Nikko, Tottori	Eurasian Wigeon	APMV-4
Duck/Tottori/126/2006	2006.12.18	Lake Togo, Tottori	Mallard	APMV-4
Duck/Tottori/T99/2006	2006.12.18	Lake Togo, Tottori	Mallard	APMV-4
Duck/Tottori/140/2007	2007.11. 8	Lake Koyama, Tottori	Eurasian Wigeon	APMV-4
Tundra swan/Shimane/91-94/2007	2007. 3.20	Yasugi-city, Shimane	Tundra swan	APMV-8
Duck/Tottori/453/2009	2009. 1.20	Pond Nikko, Tottori	Unidentified Duck	NDV(class I)
Duck/Tottori/481/2009	2009. 1.20	Pond Nikko, Tottori	Unidentified Duck	NDV(class I)
Duck/Tottori/114-115/2010	2010.11.18	Tenjin River, Tottori	Unidentified Duck	APMV-4
Duck/Tottori/99/2010	2010.11.18	Tenjin River, Tottori	Unidentified Duck	APMV-4
Duck/Tottori/237-238/2010	2010.11. 3	Lake Koyama, Tottori	Spot-billed duck	APMV-4
Duck/Tottori/250/2010	2010.11. 3	Lake Koyama, Tottori	Spot-billed duck	APMV-4
Duck/Tottori/251-252/2010	2010.11. 3	Lake Koyama, Tottori	Spot-billed duck	APMV-4
Duck/Tottori/264/2010	2010.11. 3	Lake Koyama, Tottori	Spot-billed duck	APMV-4
Duck/Tottori/267-268/2010	2010.11. 3	Lake Koyama, Tottori	Spot-billed duck	APMV-4
Duck/Tottori/22/2011	2011.11. 5	Pond Nikko, Tottori	Common teal	APMV-4

Table 4. Pathogenicity of avian paramyxovirus (APMV) isolates.

Virus	Serotype (clade)	ICPI[a]	MDT (hr)[b]
Duck/Tottori/237-238/2010	APMV-4	0.16	>168
Duck/Tottori/2/2006	APMV-4	0.04	>168
Tundra swan/Shimane/91-94/2007	APMV-8	0.16	>168
Duck/Tottori/453/2009	NDV (class I)	0.00	>168
Duck/Tottori/481/2009	NDV (class I)	0.00	>168
Duck/Tottori/N12/2006	NDV (class II)	0.00	>168

[a]ICPI: intracerebral pathogenicity index in 1-day-old chicks
[b]MDT: mean death time (hr) for chicken embryos infected with one minimum lethal dose of virus

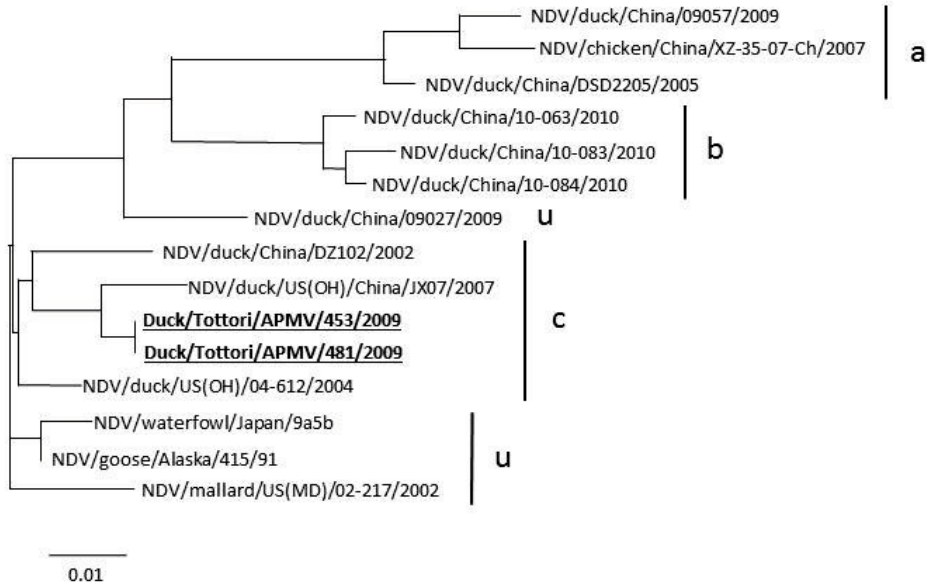


Figure 1. Phylogenetic tree of F gene sequences from NDV isolates (class I). The phylogenetic tree was generated using the neighbor-joining algorithm with 1000 bootstrap replicates in MEGA (4.0.2). Analysis was based on nucleotides 47–420 (372 bp) of the F gene. NDV isolates collected in this study were underlined. Letters a, b, and c represent each sub-group and "u" indicates "unidentified".

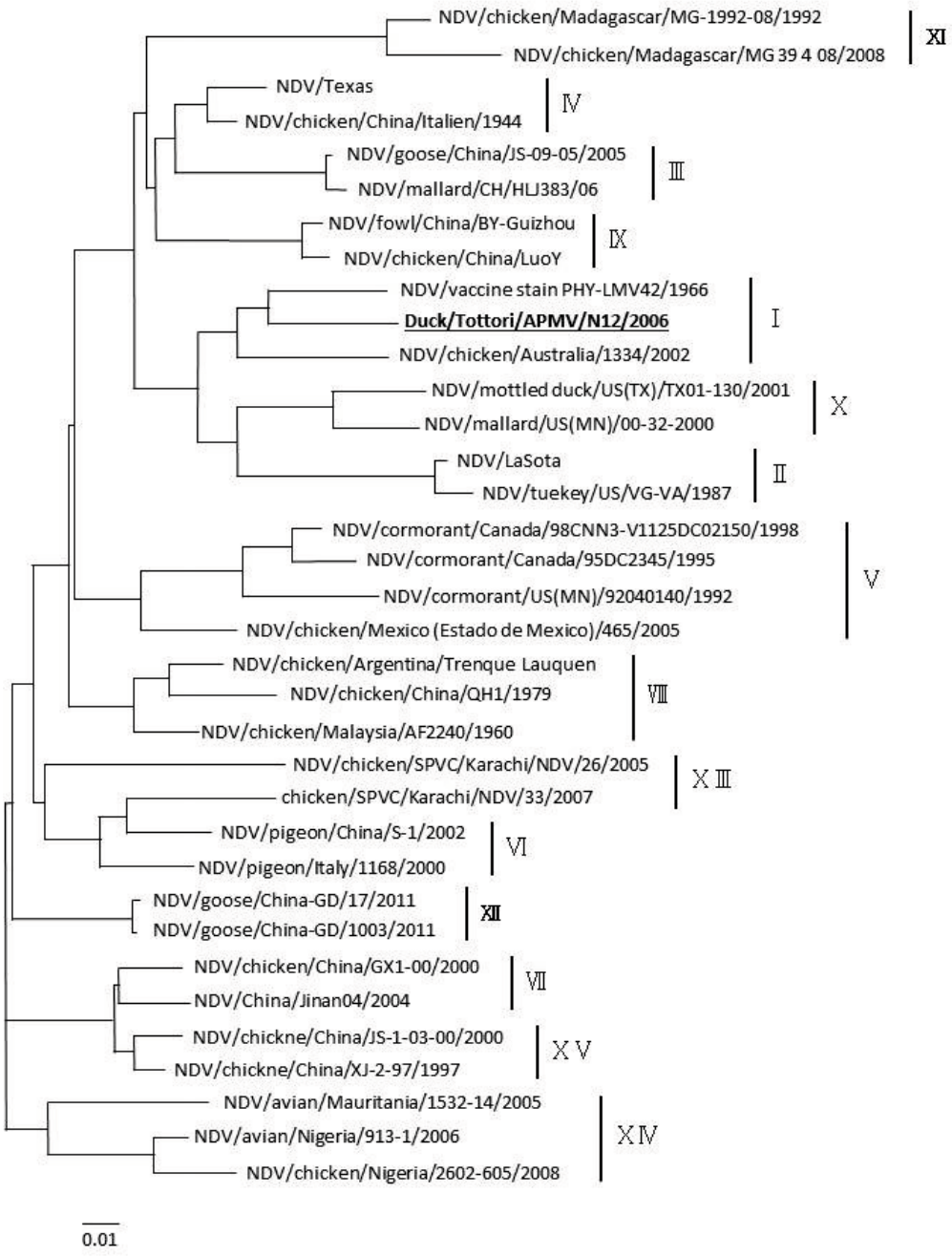


Figure 2. Phylogenetic tree of F gene sequences from NDV isolates (class II). The phylogenetic tree was generated using the neighbor-joining algorithm with 1000 bootstrap replicates in MEGA (4.0.2). Analysis was based on nucleotides 47–420 (372 bp) of the F gene. NDV isolates collected in this study were underlined. Roman numerals I - XV indicate each genotype.

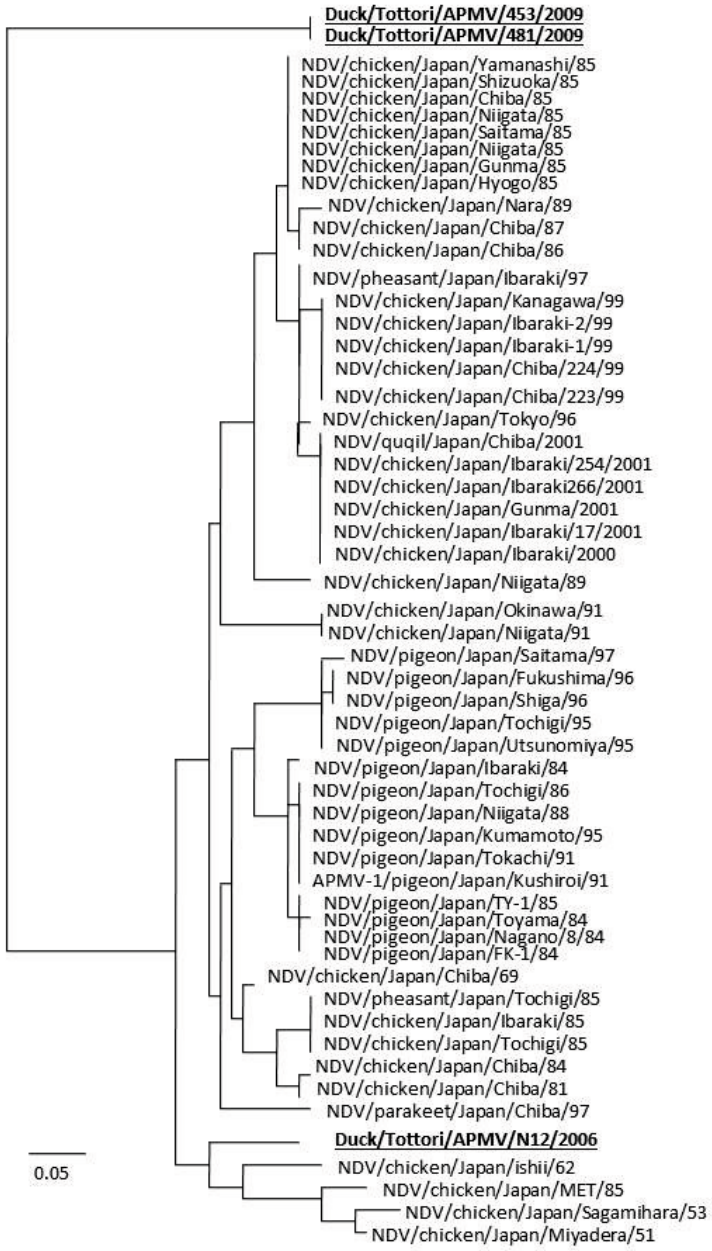


Figure 3. Phylogenetic tree of F gene sequences from recent NDV isolates (class II) in Japan. The phylogenetic tree was generated using the neighbor-joining algorithm with 1000 bootstrap replicates in MEGA (4.0.2). Analysis was based on nucleotides 47–420 (372 bp) of the F gene. NDV isolates collected in this study were underlined.

GENERAL DISCUSSION

ND is a major veterinary concern because of its economically devastating effects on the poultry industry. The global economic impact of ND is enormous. It has been recognized as one of the biggest contributor of economic losses to the global economy than any other animal viruses [3-4]. Epidemiological surveillance and investigations are therefore important to prevent outbreaks and economic losses due to this pathogen.

ND has become endemic in most countries. NDV has been reported to infect 241 species of birds representing 27 of the 50 orders of avian species [3]. In Japan, it was reported that NDV isolates are of multiple origins and that Japanese NDV strains could be classified into six genotypes (genotypes I-III, VI, VII-VIII) [68-69]. However, molecular epidemiological data on the subgenotype classification and relationships of Japanese NDV strains to other strains from different parts of the world are lacking. Studies on the causes of ND outbreaks in vaccinated poultry farms in Japan are also limited. A molecular epidemiological study was therefore conducted using nine strains of NDVs isolated from vaccinated poultry flocks from different prefectures in Japan. The investigation showed that over-all, four ND panzootics occurred in Japan and that those outbreaks were characterized by co-circulation of genetically distinct virus lineages due to virus transmission from infected wild birds either by international bird trade or migration patterns. Comparison with other isolates from different parts of the world demonstrated that all the field isolates from vaccinated poultry were part of much bigger outbreaks extending into provinces, regions and in some cases, continents.

Vaccination has been the standard method of prevention against ND. The use of live lentogenic strains alone or in combination with killed velogenic strains has been the core of ND vaccination programs in Japan and in other parts of the world [68]. However, recently, cases of velogenic ND in vaccinated poultry farms in Japan and in other parts of the world have been reported. Isolation of velogenic ND in apparently healthy flocks has also been observed [12, 50, 75, 77, 85, 108]. To guide poultry clinicians in their understanding of disease profiles in actual commercial poultry operations and to identify hidden NDV disease risk factors in the field, an example of these cases has been described. Despite extensive vaccination, an atypical case of velogenic ND in vaccinated layer flock was reported. However, compared to "textbook" cases, only mild respiratory disease with mild to moderate decrease in egg production similar to IB infection was observed. Chapter II demonstrated that atypical velogenic ND may exist and may become potential threats to commercial poultry in Japan.

Occasional fatal NDV outbreaks in vaccinated commercial poultry flocks have been reported. These cases were mostly attributed to improper vaccination, immunosuppression due to infectious and non-infectious causes and flocks that were challenged with more velogenic viruses [79]. Several studies have demonstrated that although vaccination may minimize the pathologic effects of NDVs in vaccinated chickens, viral infection, replication and shedding may still occur [12, 50, 75, 77, 85, 107]. However, at present very limited studies are available regarding the possible roles of genomic factors in these cases. To determine the possible influence of the genetic make-up of NDV in cases of breakthrough infection, the complete genome sequences of three strains of NDV isolated from vaccinated poultry flocks in the span of three decades in Japan were analyzed. It was demonstrated that NDV strains

infecting vaccinated chicken flocks possess several important amino acid substitutions at the neutralizing epitopes and functional domains of the F and HN proteins. These amino acid substitutions could have altered the function of NDV proteins and diminish the humoral immunity of the host, which then increased the viral growth properties leading to breakthrough infections.

It has been known that wild waterfowl and other aquatic birds are carriers and reservoirs of NDVs. It was previously shown that a non-pathogenic NDV strain from wild waterfowl can become highly pathogenic after several passages in chickens [95]. That result indicated that wild birds may transmit and spread precursors of velogenic viral strains to domestic poultry. Chapter 1 and chapter 3 also demonstrated that wild birds played an important role in the transmission and dynamics of spread of NDVs not only in Japan but in the Far East region. Because of the important role that wild birds play in the circulation of NDV, a seven-year surveillance of NDVs from wild waterfowl in the San-in region was conducted. A total of 16 avian paramyxoviruses consisting of three lentogenic Newcastle disease viruses (NDVs), 12 APMV-4, and one APMV-8 were isolated. The results showed that NDV and APMV-4 are relatively widely distributed among wild waterfowl that migrate to Japan from northern regions. It is therefore necessary that continuous surveillance of APMV in wild waterfowl must be conducted regularly due to the pathogenic potential of these isolates in domestic poultry.

This study was able to elucidate the molecular epidemiology of NDV isolates from vaccinated commercial poultry farms in Japan, as well as their transmission mechanisms, clinical and production profile, molecular characteristics and infection dynamics. Consequently, a seven-year surveillance of NDVs from wild waterfowl was conducted, in which several NDV and APMV strains were isolated. This study

may be use as useful reference in characterizing future NDV outbreaks in vaccinated poultry flocks and as a genetic map for future investigations regarding vaccine designs, reverse genetics systems and development of molecular diagnostic tools to prevent future ND outbreaks in vaccinated chickens.

ACKNOWLEDGMENT

This dissertation has been a fantastic journey that has taken me to visit a lot of places and live in a foreign country for a significant period of time. It has been a great and amazing experience, a spectacular time of astonishing intellectual and cultural growth that I will forever remember. It is my honor to thank my mentors, colleagues, friends and family members for their invaluable contributions for the completion of this work. This research would not have been possible without their support.

I would like to express my heartfelt appreciation and sincerest gratitude to Mrs. Masako and Dr. Hiromitsu Katoh for their unconditional and never-ending moral and financial support during my stay and study in Japan. Living away from my family, you have become my foster parents extending me all the love, care and support that I need. I will forever be thankful for all the kindness, generosity, patience and understanding that you have always shared to me. I am very much grateful for all the encouragements and inspiration and for being great role models and brilliant mentors. I am very much grateful and indebted to you for giving me the enormous freedom and opportunity to wander culturally and intellectually. I will forever cherish these memories for the rest of my life. Words are not enough to express my feelings and I would like to dedicate this dissertation to you. From the bottom of my heart, thank you very much.

This work was also made possible by Dr. Toshihiro Ito and Dr. Hiroshi Ito. I am very much fortunate to have you as my great advisors. I am very grateful and I owe you my deepest gratitude for accepting me as your student and giving me the opportunity to study in Japan. I am very thankful for the generous and excellent

guidance, assistance and understanding during the conduct of this study. I will never forget the help you have extended to me for patiently improving this work. Despite of your very busy schedule, I appreciate very much the generosity for sharing me your invaluable time, wisdom, knowledge and tremendous effort and for inspiring me to become a better researcher and a veterinarian.

I also owe my deepest gratitude to Dr. Koichi Otsuki for the mentorship and for carefully editing and improving this research work. There have been numerous occasions where I remember feeling disheartened but your messages and words of encouragements always reinvigorate my enthusiasm and raise my spirit to continue and work harder. Thank you very much for the kindness, motivation and inspiration.

This study was also made possible thanks to Dr. Kozo Takase. I would like to express my heartfelt gratitude for dedicating your precious time in carefully reviewing, analyzing and studying this work. Thank you very much for the insightful suggestions and recommendations for the improvement of this study.

It is an honor for me to also thank Dr. Kazutoshi Shirota. Thank you for the support, guidance and supervision. I appreciate very much your great managerial skills and without your help, this study could have been very challenging. Thank you very much for the helpful comments, advices, support and encouragement.

I would like to also express my gratitude to Mrs. Ito (Dr. Hiroshi's lovely wife) and to their adorable children for making my stay in Tottori memorable, fun and comfortable. For the warm accommodations, generous hospitality and delicious meals, thank you very much.

It is my honor to acknowledge and thank all the members of PPQC family namely Dr. Terumasa Suzuki, Mr. Hideki Matsui, Mr. Hirohiko Saitoh, Mr. Ryota Taira, Ms. Makiko Ishii, Ms. Yukari Mutoh, Ms. Misa Saitoh, Ms. Eri Kashima, Ms.

Shiori Watanabe, Mrs. Sanae Mori, Ms. Mika Satoh, Ms. Fumie Ouchi, Mrs. Tomoko Satoh, Mrs. Miyuki Satoh, Mr. Takeo Konno, Mr. Hisao Kikuta, Mrs. Tomomi Sugeno, Ms. Ayumi Satoh, Ms. Chika Nunota, Dr. Fletcher del Valle and especially to Ms. Madoka Katoh and Ms. Mai Katoh. Thank you very much for the immeasurable love and support you gave me during my stay in Japan. I am grateful for the overwhelming support, encouragements, assistance and for making my stay in Japan enjoyable and memorable. This accomplishment will never be possible without your help. Together with Mrs. Masako, Dr. Katoh and Dr. Shirota, I share this work and all my future accomplishments with you. Makoto ni arigatou gozaimashita.

And most especially, my sincerest thanks to my parents, siblings and relatives. I have been blessed with a very loving and supportive family and they have been a constant source of encouragement. I appreciate all of the sacrifices that you have made for me.

To all whom I forgot to mention but had helped me in every way, thank you very much!

Appendix 1. Details of NDV strains used in this study.

NDV isolate	Country of origin	Host	Year	Genotype	Sublineage	Accession number
JP/Osaka/2440/69	Japan	Layer Chicken	1969	VIa	4a	AB853926; AB854728; AB854729
JP/Ibaraki/SM1987/87	Japan	Layer Chicken	1987	VId	4d	AB853928; AB854730; AB854731
JP/Ibaraki/SG106/99	Japan	Layer Chicken	1999	VIId	5d	AB853927; AB854732; AB854733
JP/Chiba/BY103/01	Japan	Layer Chicken	2001	VIId	5d	AB853931; AB854734; AB854735
JP/Chiba/BY7/02	Japan	Layer Chicken	2002	VIId	5d	AB853930; AB854736; AB854737;
JP/Fukushima/NYF3/02	Japan	Layer Chicken	2002	VIId	5d	AB853329; AB854740; AB854741
JP/Ibaraki/IS5/02	Japan	Layer Chicken	2002	VIId	5d	AB853933; AB854744; AB854745
JP/Ibaraki/IS2/02	Japan	Layer Chicken	2002	VIId	5d	AB853932; AB854742; AB854743
JP/Miyagi/AGT/02	Japan	Layer Chicken	2002	VIId	5d	AB853929; AB854738; AB854739
JP/Ishii/62	Japan	Chicken	1962	I	1	AB070385
Ulster-2C/67	Northern Ireland	Domestic fowl	1967	I	1	M24694
Beaudette/45	USA	Domestic fowl	1945	II	2	M24697
LaSota/46	USA	Domestic fowl	1946	II	2	M24696
JP/Miyadera/51	Japan	Chicken	1951	II	2	AB070383
JP/Sagamihara/53	Japan	Chicken	1953	II	2	AB070384
JP/MET/95	Japan	Chicken	1995	II	2	AB074018
JP/Sato/30	Japan	Chicken	1930	III	3a	AB070382
AusVictoria/32	Australia	Domestic Fowl	1932	III	3a	M21881
Herts/33	UK	Domestic fowl	1933	IV	3b	M24702
IT 50/73	Italy	Turkey	1973	V	3c	AF218135
SG-4H/65	Singapore	Domestic fowl	1965	VIII	3d	AF136786
ZA 5/68	South Africa	Domestic fowl	1968	VIII	3d	AF136762
ZA 34/94	South Africa	No Data	1994	VIII	3d	AF136773
QH 1/79	Western China	Domestic fowl	1979	VIII	3d	AF378250
QH-4/85	Western China	Domestic fowl	1985	VIII	3d	AF378252
JP/Okinawa/91	Japan	Chicken	1991	VIII	3d	AB074015

Appendix 1. continued.

NDV isolate	Country of origin	Host	Year	Genotype	Sublineage	Accession number
JP/Kagoshima/91	Japan	Chicken	1991	VIII	3d	AB074014
IT-147/94	Italy	Turkey	1994	VIII	3d	EU604262
JP/Narashino/68	Japan	Japanese Blue Magie	1968	VIa	4a	AB070386
Iraq/AG-68	Iraq	Domestic Fowl	1968	VIa	4a	AF001108
Kuwait/256/68	Kuwait	Domestic Fowl	1968	VIa	4a	AF001109
JP/Chiba/69	Japan	Chicken	1969	VIa	4a	AB070387
Lebanon/70	Lebanon	Domestic Fowl	1970	VIa	4a	AF001110
Israel/70	Israel	Domestic Fowl	1970	VIa	4a	AF001111
ASTR/74	Russia	Domestic Fowl	1974	VIa	4a	Y19012
PTRPI95107	Turkey	Pigeon	1995	VIa	4a	AY 175763
JAEFA96038	United Arab Emirates	Falcon	1996	VIa	4a	AY 175738
PUKPI83279	United Kingdom	Pigeon	1983	VIb	4b	AY 175764
GB 1168/84	Great Britain	Pigeon	1984	VIb	4b	AF109885
PUKPI84125	Great Britain	Pigeon	1984	VIb	4b	AY 175766
JP/Ibaraki-pg/84	Japan	Pigeon	1984	VIb	4b	AB070392
JP/Toyama/84	Japan	Pigeon	1984	VIb	4b	AB070391
JP/FK-1-pg/84	Japan	Pigeon	1984	VIb	4b	AB070390
JP/TY-1/85	Japan	Chicken	1985	VIb	4b	AB070394
JP/Tochigi-pg/86	Japan	Pigeon	1986	VIb	4b	AB070407
PDECT95204	Germany	Cockatoo	1995	VIb	4b	AY 175752
-TRCK95111	Turkey	Domestic Fowl	1995	VIb	4b	AY 175776
PITPH95294	Italy	Pheasant	1995	VIb	4b	AY 175758
PB01/96	China	Pigeon	1996	VIb	4b	DQ417113
JS-2/98/Go	China	Goose	1998	VIb	4b	AF456439
PSAPI98201	Saudi Arabia	Pigeon	1998	VIb	4b	AY 175760
PDEPI99063	Germany	Pigeon	1999	VIb	4b	AY 175754
PUKPI99065	Great Britain	Pigeon	1999	VIb	4b	AY 175779
PIEPI00242	Ireland	Pigeon	2000	VIb	4b	AY 175756
BSACK89036	Saudi Arabia	Domestic Fowl	1989	VIc	4c	AY 175680
BAECK92084	United Arab Emirates	Domestic Fowl	1992	VIc	4c	AY 175662
BAECK95114	United Arab Emirates	Domestic Fowl	1996	VIc	4c	AY 175664
QGB506/97	Great Britain	Parakeet	1997	VIc	4c	AF109887

Appendix 1. continued.

NDV Isolate	Country of Origin	Host	Year	Genotype	Sublineage	Accession Number
JP/Chiba-pa/97	Japan	Parakeet	1997	VIc	4c	AB074025
BBECK97037	Belgium	Domestic Fowl	1997	VIc	4c	AY 135741
BAEOS98034	United Arab Emirates	Ostrich	1998	VIc	4c	AY 175666
BAEPI99109	United Arab Emirates	Pigeon	1999	VIc	4c	AY 175668
JP/Ibaraki/85	Japan	Chicken	1985	VId	4d	AB070399
JP/Tochigi/85	Japan	Chicken	1985	VId	4d	AB070397
DK-1/95	Denmark	Domestic Fowl	1995	VId	4d	AF001129
CH-1/95	Switzerland	Domestic Fowl	1995	VId	4d	AF001132
BBGPI95039	Bulgaria	Pigeon	1998	VId	4d	AY 135742
TW154/99	Taiwan	Domestic Fowl	1999	VId	4d	AF234030
JP/Chiba/81	Japan	Chicken	1981	VIf	Not Applicable	AB070388
JP/Chiba/84	Japan	Chicken	1984	VIf	Not Applicable	AB070389
Kr-M/88	Korea	Quail	1988	VIf	Not Applicable	AY630413
Kr-12A/89	Korea	Chicken	1989	VIf	Not Applicable	AY630414
Kr-102/89	Korea	No Data	1989	VIf	Not Applicable	AY630415
Kr-163/90	Korea	No Data	1990	VIf	Not Applicable	AY630416
Kr-9/91	Korea	No Data	1991	VIf	Not Applicable	AY630417
XJ-1/91	China	Fowl	1991	VIf	Not Applicable	AF458020
Kr-104/92	Korea	No Data	1992	VIf	Not Applicable	AY630418
JX-1/94	China	Fowl	1994	VIf	Not Applicable	AF458021
XJ-3/97	China	Fowl	1997	VIf	Not Applicable	AF458019
Zhj-2/86	China	Fowl	1986	VIg	Not Applicable	AF458016
Sh-1/97	China	Fowl	1997	VIg	Not Applicable	AF458018
RI3/88	Indonesia	Domestic fowl	1988	VIIa	5a	AF001135
JP/Niigata/89	Japan	Chicken	1989	VIIa	5a	AB070410
I123/92	Italy	Domestic fowl	1992	VIIa	5a	AF001128
E1/93	Spain	Domestic fowl	1993	VIIa	5a	AF001126
TW/94P	Taiwan	Fowl	1994	VIIa	5a	AF083961
DE143/95	Germany	Domestic Fowl	1995	VIIa	5a	AF109881
IT-112/84	Italy	Turkey	1984	VIIb	5b	AF218127
IT 113/85	Italy	Chicken	1985	VIIb	5b	AF218128
RI1/88	Indonesia	Cockatoo	1988	VIIb	5b	AF001134
MZ35/95	Mozambique	No Data	1995	VIIb	5b	AF136776

Appendix 1. continued.

NDV Isolate	Country of Origin	Host	Year	Genotype	Sublineage	Accession Number
FI1001/96	Finland	Goosander	1996	VIIb	5b	AF091623
AE2321/96	United Arab Emirates	Partridge	1996	VIIb	5b	AF109884
IT1/2000	Italy	Chicken	2000	VIIb	5b	AF293350
Kr-D/84	Korea	Peafowl	1984	VIIc	5c	AY630412
TW /84P	Taiwan	No Data	1984	VIIc	5c	AF083967
TW /84C	Taiwan	Domestic fowl	1984	VIIc	5c	AF083965
JP/Gunma-ph/85	Japan	Pheasant	1985	VIIc	5c	AB070395
JP/Yamanashi/85	Japan	Chicken	1985	VIIc	5c	AB070396
HUB 1/91	No Data	No Data	1991	VIIc	5c	AF378257
TW/95-7	Taiwan	Domestic Fowl	1995	VIIc	5c	AF083968
Ch-A7/96	China	Fowl	1996	VIIc	5c	AY028995
CZ 3898/96	Czech Republic	Domestic fowl	1996	VIIc	5c	AF109883
Ch62/96	Switzerland	Fowl	1996	VIIc	5c	AF109880
TW/96P	Taiwan	Pigeon	1996	VIIc	5c	AF083971
JS-2/98	China	Fowl	1998	VIIc	5c	AF458013
TW159/99	Taiwan	Domestic Fowl	1999	VIIc	5c	AF234034
SHX3/99	Western China	Domestic fowl	1999	VIIa	5c	AF378246
JX-2/99	China	Fowl	1999	VIIc	5c	AF458014
Kr-279/95	Korea	Broiler Chicken	1995	VIId	5d	AY630421
Kr-146/95	Korea	Broiler Chicken	1995	VIId	5d	AY630420
Kr-077/95	Korea	Broiler Chicken	1995	VIId	5d	AY630419
JP/Tokyo/96	Japan	Chicken	1996	VIId	5d	AB070421
JS-1/97/Go	China	Goose	1997	VIId	5d	AF456435
JP/Ibaraki-ph/97	Japan	Pheasant	1997	VIId	5d	AB070424
GX1/97	Western China	Domestic Fowl	1997	VIId	5d	AF378254
XJ-2/97	China	Fowl	1997	VIId	5d	AF458011
JS-3/98/Go	China	goose	1998	VIId	5d	AF456436
GD-1/98/Go	China	goose	1998	VIId	5d	AF456437
GS-2/98	Western China	Domestic fowl	1998	VIId	5d	AF378249
GX 3/98	Western China	Domestic Fowl	1998	VIId	5d	AF378256
TW /98-1	Taiwan	No Data	1998	VIId	5d	AF083963
TW /98-2	Taiwan	Domestic Fowl	1998	VIId	5d	AF083973

Appendix 1. continued.

NDV Isolate	Country of Origin	Host	Year	Genotype	Sublineage	Accession Number
SHX-2/99	Western China	Domestic fowl	1999	VIIa	5d	AF378245
TW/98-4	Taiwan	Fowl	1998	VIId	5d	AF083964
TW/99-154b	Taiwan	Domestic fowl	1999	VIId	5d	AF326521
TW /99-156a	Taiwan	Domestic fowl	1999	VIId	5d	AF326522
SHD 1/99	No Data	No Data	1999	VIId	5d	AF378260
SHX-6/99	Western China	Domestic fowl	1999	VIIa	5d	AF378247
FJ-2/99	China	Fowl	1999	VIId	5d	AF458012
JP/Kanagawa/99	Japan	Chicken	1999	VIId	5d	AB070432
JP/Ibaraki-1/99	Japan	Chicken	1999	VIId	5d	AB070430
JP/Chiba-222/99	Japan	Chicken	1999	VIId	5d	AB070427
JP/Chiba/2000	Japan	Chicken	2000	VIId	5d	AB070433
JP/Ibaraki/2000	Japan	Chicken	2000	VIId	5d	AB070435
Ch/2000	China	Fowl	2000	VIId	5d	AF358788
TW/2000	Taiwan	Fowl	2000	VIId	5d	AF358786
Kr-018/00	Korea	Layer Chicken	2000	VIId	5d	AY630429
JS-9/01/Go	China	Goose	2001	VIId	5d	AF456443
JS-5/01/Go	China	Goose	2001	VIId	5d	AF456442
SGM/01	China	Broiler Chicken	2001	VIId	5d	DQ227248
JP/Ibaraki-254/2001	Japan	Chicken	2001	VIId	5d	AB070436
JP/Ibaraki-266/2001	Japan	Chicken	2001	VIId	5d	AB070438
JP/Ibaraki-16/2001	Japan	Chicken	2001	VIId	5d	AB070440
JP/Gunma/2001	Japan	Chicken	2001	VIId	5d	AB070442
Ch-501/02	China	Chicken Meat	2002	VIId	5d	AY630438
TW10/06	Taiwan	Broiler Chicken	2006	VIId	5d	EF418785
TW /94P	Taiwan	Domestic fowl	1994	VIIa	5e	AF083961
TW 1/95	Taiwan	No Data	1995	VIIe	5e	AF083960
TW /95-4	Taiwan	Domestic Fowl	1995	VII	5e	AF083969
TW /95-2	Taiwan	Domestic Fowl	1995	VII	5e	AF083972
QGB445/97	Great Britain	Finches	1997	VIIe	5e	AF109886
Zhj-1-85	China	Fowl	1985	IX	Not Applicable	AF458023
F48E9	China	ND	2005	IX	Not Applicable	AY997298

REFERENCES

1. Aldous, E.W., Mynn, J.K., Banks, J. and Alexander, D.J. 2003. A molecular epidemiological study of avian paramyxovirus type 1 (Newcastle disease virus) isolates by phylogenetic analysis of a partial nucleotide sequence of the fusion protein gene. *Avian Path.* **32**: 239-257.

2. Alexander, D.J. 1988. In: Newcastle Disease. pp. 1-10, Kluwer Academic Publishers, Boston.

3. Alexander, D. J. 2003. Newcastle disease, other avian paramyxoviruses**,** and pneumovirus infections. pp. 63–99. In: Diseases of poultry, 11th ed. (Saif, Y. M., Barnes, H. J., Glisson, J. R., Fadly, A. M., McDougald, L. R. and Swayne, D. E. eds.), Iowa State Press, Ames, Iowa.

4. Alexander, D.J. 2008. Newcastle disease, other avian paramyxoviruses and pneumovirus infection. pp. 75-93. In: Diseases of poultry, 12th ed. (Saif YM. ed.) Blackwell Publishing, Iowa.

5. Alexander, D.J. 2009. Ecology and epidemiology of Newcastle disease. pp. 19-26. In: Avian influenza and Newcastle disease (Capua, I. and Alexander, D.J. eds.), Springer-Verlag, Milan.

6. Alexander, D.J. and Allan, W.H. 1974. Newcastle disease virus pathotypes. *Avian Pathol.* **3(4)**: 269-278.

7. Alexander, D.J., Bell J.G. and Alders R.G. 2004. In: Technology review: Newcastle disease with special emphasis on its effect on village chickens. pp. 13-16, Food and Agriculture Organisation of the United Nations, Rome.

8. Alexander, D.J., Campbell G., Manvell R.J., Collins M.S., Parsons G. and McNulty M.S. 1992. Characterization of an antigenically un-usual virus responsible for two outbreaks of Newcastle disease in the Republic of Ireland in 1990. *Vet. Rec.* **130:** 65–68.

9. Allan, W.H., Lancaster, J. E. and Toth, B. 1978. In: Newcastle disease vaccines. pp. 74-79, Food and Agriculture Organisation of the United Nations, Rome.

10. Beard, C. W. 1970. Demonstration of type-specific influenza antibody in mammalian and avian sera by immunodiffusion. *Bull. World Health Organ.* **42**: 779–785.

11. Briand, F.X., Henry, A., Massin, P. and Jestin, V. 2012. Complete genome sequence of a novel avian paramyxovirus. *J. Virol.* 86: 7710.

12. Bwala, D., Fasina, F., Van Wyk, A. and Duncan, N. 2010. Effects of vaccination with lentogenic vaccine and challenge with virulent Newcastle disease virus (NDV) on egg production in commercial and SPF Chickens. *Int. J. Poult. Sci.* **10**: 98-105.

13. Cai, S., Li, J., Wong, M.T., Jiao, P., Fan, H., Liu, D., Liao, M., Jiang, J., Shi, M., Lam, T.T., Ren, T. and Leung, F.C. 2011. Genetic characterization and evolutionary analysis of 4 Newcastle disease virus isolate full genomes from waterbirds in South China during 2003-2007. *Vet. Microbiol.* **152:** 46-54.

14. Cattoli, G., Fusaro, A., Monne, I., Molia, S., Le Menach, A., Maregeya, B., Nchare, A., Bangana, I., Maina, A.G., Koffi, J.N., Thiam, H., Bezeid, O.E., Salviato, A., Nisi, R., Terregino, C. and Capua, I. 2010. Emergence of a new genetic lineage of Newcastle disease virus in west and central Africa – implications for diagnosis and control. *Vet. Microbiol.* **142 (3-4):** 168-76.

15. Cavanagh, D. and Naqi S. 2003. **Infectious bronchitis.** pp. 101-119**.** In: Diseases of Poultry, 11th ed. (Saif Y.M., Barnes H.J, Glisson J.R., Fadly A.M., McDougald L.R. and Swayne D. eds.), Iowa State University Press, Ames, Iowa.

16. Chambers, P., Millar, N.S. and Emerson, P.T. 1986. Molecular cloning of complementary DNA to Newcastle disease virus, and nucleotide sequence analysis of the junction between the genes encoding the haemagglutinin-neuraminidase and the large protein. *J. Gen. Virol.* **67**: 475-486.

17. Chambers, P., Millar, N.S. and Emerson P.T. 1986. Nucleotide sequence of the gene encoding the fusion glycoprotein of Newcastle disease virus. *J. Gen. Virol.* **67**: 2685-2694.

18. Chambers, P., Nesbit, M., Yusoff, K., Millar, N.S., Samson, A.C. and Emmerson, P.T. 1988. Location of a neutralizing epitope for the haemagglutinin-neuraminidase glycoprotein of Newcastle disease virus. *J. Gen. Virol.* **69**: 2115-2122.

19. Chare, E. R., Gould, E. A. and Holmes, E.C. 2003. Phylogenetic analysis reveals a low rate of homologous recombination in negative-sense RNA viruses. *J. Gen. Virol.* **84**: 2691–2703.

20. Cho, S.H., Kim, S.J. and Kwon, H.J. 2007. Genomic sequence of an antigenic variant Newcastle disease virus isolated in Korea. Virus Genes **35(2)**: 293-302.

21. Cho, S.H., Kwon, H.J., Kim, T.E., Kim, J.H.,Yoo, H.S. and Kim, S.J. 2008. Variation of a Newcastle disease virus hemagglutinin-neuraminidase linear epitope. *J. Clin. Microbiol.* **46**: 1541-1544.

22. Choi, K.S., Kye, S.J., Kim, J.Y. and Lee, H.S. 2013. Genetic and antigenic variation of shedding viruses from vaccinated chickens after challenge with virulent Newcastle disease virus. Avian Diseases **57(2)**: 303-306.

23. Chong, Y., Padhi, A., Hudson, P. and Poss, M. 2010. The effect of vaccination on the evolution and population dynamics of avian paramyxovirus-1. *PLos pathog* **6(4)** doi:10.1371/journal.ppat.1000872

24. Chu, D.K., Leung, C.Y., Gilbert, M., Joyner, PH., Ng, EM., Tse, T.M., Guan, Y., Peiris, J.S. and Poon, L.L. 2011. Avian coronavirus in wild aquatic birds. *J. Virol.* **85**: 12815-20.

25. Cloud, S.S. and Rosenberger, J.K. 1980. Characterization of nine avian paramyxoviruses. *Avian Dis.* **24**: 139–152.

26. Collins, M. S., Bashiruddin, J. B. and Alexander, D. J. 1993. Deduced amino acid sequences at the fusion protein cleavage site of Newcastle disease viruses showing variation in antigenicity and pathogenicity. *Arch. Virol.* **128**: 363–370.

27. Collins, M.S., Franklin, S., Strong, I., Meulemans, G. and Alexander, D. J. 1998. Antigenic and phylogenetic studies on a variant Newcastle disease virus using anti-fusion protein monoclonal antibodies and partial sequencing of the fusion protein gene. *Avian Pathol.* **27**: 90–96.

28. Collins, M. S., Strong, I. and Alexander, D.J. 1996. Pathogenicity and phylogenetic evaluation of the variant Newcastle disease viruses termed “pigeon PMV-1 viruses” based on the nucleotide sequence of the fusion protein gene. *Arch. Virol.* **141**: 635-647.

29. Connaris, H., Takimoto, T., Russell, R., Crennell, S., Moustafa, I., Portner, A. and Taylor, G. 2002. Probing the sialic acid binding site of the hemagglutinin-neuraminidase of Newcastle disease virus: identification of key

amino acids involved in cell binding, catalysis, and fusion. *J. Virol.* **76**: 1816-1824.

30. Czegledi, A., Ujvari, D., Somogyi, E., Wehmann, E., Werner, O., Lomniczi, B. 2006. Third genome size category of avian paramyxovirus serotype 1 (Newcastle disease virus) and evolutionary implications. *Virus Res.* **120**: 36-48.
31. Delport, W., Poon, A., Frost, S. and Kosakovsky Pond, S. Datamonkey 2010: a suite of phylogenetic analysis tools for evolutionary biology. *Bioinformatics* **26**: 2455-2457.
32. Diel, D.G., da Silva, L.H., Liu, H., Wang, Z., Miller, P.J. and Afonso C.L. 2012. Genetic diversity of avian paramyxovirus type 1: proposal for a unified nomenclature and classification system of Newcastle disease virus genotypes. *Infect. Genet. Evol.* **12**: 1770–1779.
33. Dormitorio, T.V., Giambrone, J.J., Guo, K. and Hepp, G.R. 2009. Detection and characterization of avian influenza and other avian paramyxoviruses from wild waterfowl in parts of the southeastern United States. *Poult. Sci.* **88**: 851-855.
34. Felsenstein, J. 1985. Confidence limits on phylogenies: An approach using the bootstrap. *Evolution* **39**: 783-791.
35. Goekjian, V.H., Smith, J.T., Howell, D.L., Senne, D.A., Swayne, D.E. and Stallknecht, D.E. 2011. Avian influenza viruses and Avian paramyxoviruses in wintering and breeding waterfowl populations in North Carolina, USA. *J. Wildl. Dis.* **47**: 240-245.
36. Gotoh, B., Ohnishi, Y., Inocencio, N., Esaki, E., Nakayama, K., Barr, P., Thomas, G. and Nagai, Y. 1992. Mammalian subtilisin-related proteinases in cleavage activation of the paramyxovirus fusion glycoprotein: superiority of furin/PACE to PC2 or PC1/PC3. *J. Virol.* **66**: 6391-6397.

37. Gough, R. E. and Alexander, D. J. 1984. Avian paramyxovirus type 4 isolated from a ringed teal (*Calonetta leucophrys*). *Vet. Rec.* **115**: 653.

38. Gould, A.R., Kattenbelt, J.A., Selleck, P., Hansson, E., Della-Porta, A. and Westbury, H.A. 2001. Virulent Newcastle disease in Australia: Molecular epidemiological analysis of viruses isolated prior to and during the outbreaks of 1998–2000. *Virus Res.* **77**: 51–60.

39. Gravel K.A., McGinnes L.W., Reitter J. and Morrison T.G. 2011. The transmembrane domain sequence affects the structure and function of the Newcastle disease virus fusion protein. *J. Virol.* **85**: 3486–3497.

40. Hall, TA. 1999. BioEdit: a user-friendly biological sequence alignment editor and analysis program for Windows 95/98/NT. *Nucleic Acids Symp. Ser.* **41:** 95-98.

41. Han, G.Z., He, C.Q., Ding, N.Z. and Ma. L.Y. 2008. Identification of a natural multi-recombinant of Newcastle disease virus. *Virology* **371**: 54-60.

42. Herczeg, J., Wehmann, E., Bragg, R.R., Travassos Dias, P.M., Hadjiev, G., Werner, O. and Lomniczi, B. 1999. Two novel genetic groups (VIIb and VIII) responsible for recent Newcastle disease outbreaks in Southern Africa, one (VIIb) of which reached Southern Europe. *Arch. Virol.* **144**: 2087-2099.

43. Huang, Z., Krishnamurthy, S., Panda, A. and Samal, S.K. 2003. Newcastle disease virus V protein is associated with viral pathogenesis and functions as an alpha interferon antagonist. *J. Virol.* **77 (16)**: 8676-8685.

44. Hu, S., Wang, T., Liu, Y., Meng, C., Wang, X., Wu, Y. and Liu, X. 2010. Identification of a variable epitope on the Newcastle disease virus hemagglutinin-neuraminidase protein. *Vet. Microbiol.* **140(1-2)**: 92-97.

45. Iorio, R.M., Glickman, R.L. and Sheehan, J.P. 1992. Inhibition of fusion by neutralizing monoclonal antibodies to the hemagglutinin-neuraminidase glycoprotein of Newcastle disease virus. *J. Gen. Virol.* **73**: 1167-1176.

46. Iorio, R.M., Syddall, R.J., Sheehan, J.P., Bratt, M.A., Glickman, M.A. and Riel, A.M. 1991. Neutralization map of the hemagglutinin-neuraminidase glycoprotein of Newcastle disease virus: domains recognized by monoclonal antibodies that prevent receptor recognition. *J. Virol.* **65**:4999-5006.

47. Islam, M., Ito, T., Takakuwa, H., Takada, A., Itakura, C. & Kida, H. (1995). Acquisition of pathogenecity of a Newcastle disease virus isolated from a Japanese quail by intracerebral passage in chickens. *Jpn. J. Vet. Res.* **42**: 147-156.

48. Jahangir, A., Ruenphet, S., Ueda, S., Ueno, Y., Shoham, D., Shindo, J., Okamura, M., Nakamura, M. and Takehara, K. 2009. Avian influenza and Newcastle disease viruses from northern pintail in Japan: isolation, characterization and inter-annual comparisons during 2006-2008. *Virus Res.* **143**: 44-52.

49. Jindal, N., Chander, Y., Chockalingam, A.K., de Abin, M., Rdig, P.T. and Goyal, S.M. 2009. Phyogenetic analysis of Newcastle disease viruses isolated from waterfowl in the Upper Midwest Region of the United States. *Virol. J.* **6**: 191.

50. Kapczynski, D. and King, D. 2005. Protection of chickens against overt clinical disease and determination of viral shedding following vaccination with commercially available Newcastle disease virus vaccines upon challenge with highly virulent virus from the California 2002 exotic Newcastle disease outbreak. *Vaccine* **23**: 3424-3433.

51. Katoh, H. and Yoshimura, S. 1970. Pathological studies on Newcastle disease I: Central nervous system lesions accompanied by necrotic foci caused by Asian type Newcastle disease. *J. Jpn. Vet. Med. Assoc.* **23(5)**: 298-304.

52. Katoh, H. 1971. Pathological studies on Newcastle disease II: Lesions induced by TCND Strain. *J. Jpn. Vet. Med. Assoc.* **24(6)**: 309-314.

53. Katoh, H. 1974. Pathological studies on Newcastle disease lymphatic lesions in the fowl with Asian type Newcastle disease. *J. Jpn. Vet. Med. Assoc.* **27(4)**: 175-182.

54. Katoh, H. 1976. Pathological studies on Newcastle disease IV. Lesions in experimental infection with American type or lentogenic virus. *J. Jpn. Vet. Med. Assoc.* **29(6)**: 331-334.

55. Katoh, H. and Yoshimura, S. 1977. Pathological studies on Newcastle disease: laryngotracheal and conjunctival lesions caused by so-called Asian type Newcastle disease virus. *Jpn. J. Vet Sci.* **39(1)**:15-26.

56. Katz, D., Moshe, H.B. and Alon S. 1976. Titration of Newcastle disease virus and its neutralizing antibodies in microplates by modified hemadsorption and modified hemadsorption-inhibition method. *J. Clin. Microbiol.* **3(3)**: 227-232.

57. Kida, H. and Yanagawa, R. 1979. Isolation of a new avian paramyxovirus from a rock pigeon *(Columba livia)*. *Zentralbl. Bakteriol. Orig. A.* **245**: 421-428.

58. Kim, L. M., King, D. J., Curry, P. E., Suarez, D. L., Swayne, D. E., Stallknecht, D. E., Slemons, R. D., Pedersen, J. C., Senne, D. A., Winker, K. and Afonso, C. L. 2007. Phylogenetic diversity among low-virulence Newcastle disease viruses from waterfowl and shorebirds and comparison of genotype distributions to those of poultry-origin isolates. *J. Virol.* **81**:12641–12653.

59. Krishnamurthy, S. and Samal, S.K. 1998. Nucleotide sequence of the trailer, nucleocapsid protein gene and intergenic regions of Newcastle disease virus strain Beaudette C and completion of the entire genome sequence. *J. Gen. Virol.* **79**: 2419–2424.

60. Li, Z., Yu, M., Zhang, H., Wang, H.Y. and Wang, L.F. 2005. Improved rapid amplification of cDNA ends (RACE) for mapping both the 5′ and 3′ terminal sequences of paramyxovirus genomes. *J. Virol. Methods* **130**: 154–156.

61. Liang, R., Cao, D.J., Li, J.Q., Chen, J., Guo, X., Zhuang, F.F. and Duan, M.X. 2002. Newcastle disease outbreaks in western China were caused by the genotypes VIIa and VIII. *Vet. Microbiol.* **87**:193-203.

62. Lien, Y., Lee, J., Su, H., Tsai, H., Tsai, M., Hsieh, C. and Tsai, S. 2007. Phylogenetic characterization of Newcastle disease viruses isolated in Taiwan during 2003–2006. *Vet. Microbiol.* **123**:194-202.

63. Liu, X.F., Wan, H.Q., Ni, X.X., Wu, Y.T. and Liu W.B. 2003. Pathotypical and genotypical characterization of strains of Newcastle disease virus isolated from outbreaks in chicken and goose flocks in some regions of China during 1985–2001. *Arch. Virol.,* **148**: 1387-1403.

64. Liu, X.F., Wang, X., Wu, S., Hu, S., Peng, Y., Xue, F. and Liu, X. 2009. Surveillance for avirulent Newcastle disease viruses in domestic ducks (*Anas platyrhynchos* and *Cairina moschata*) at live bird markets in Eastern China and characterization of the viruses isolated. *Avian Pathol.* **38**: 377-391.

65. Lomniczi, B., Wehmann, E., Herczeg, J., Ballagi-Pordany, A., Kaleta, E.F., Werner, O., Meulemans, G., Jorgensen, P.H., Mante, A.P., Gielkens, A.L., Capua, I. and Damoser, J. 1998. Newcastle disease outbreaks in recent years in western

Europe were caused by an old (VI) and a novel genotype (VII). *Arch. Virol.* **143**:49-64.

66. Maminiaina, O.F., Gil, P., Briand, F.X., Albina, E., Keita, D., Andriamanivo, H., Chevalier, V., Lancelot, R., Martinez, D., Rakotondravao, R., Rajaonarison, J., Koko, M., Andriantsimahavandy, A., Jestin, V. and de Almeida, R. 2010. Newcastle disease virus in Madagascar: Identification of an original genotype possibly deriving from a died out ancestor of genotype IV. *PLoS ONE* **5**: e13987. doi:10.1371/journal.pone.0013987

67. Martin, D.P., Lemey, P., Lott, M., Moulton, V., Posada, D. and Lefeuvre, P. 2010. RDP3: a flexible and fast computer program for analyzing recombination. *Bioinformatics* **26**: 2462-2463.

68. Mase, M., Imai, K., Sanada, Y., Sanada, N., Yuasa, N., Imada, T., Tsukamoto, K. and Yamaguchi, S. 2002. Phylogenetic analysis of Newcastle disease virus genotypes isolated in Japan. *J. Clin. Microbiol.* **40**: 3826-3830.

69. Mase, M., Murayama, K., Karino, A. and Inoue, T. 2011. Analysis of the fusion protein gene of Newcastle disease viruses isolated in Japan. *J. Vet. Med. Sci*. **73**: 47-54.

70. McGinnes, L.W. and Morrison, T.G. 1995. The role of individual oligosaccharide chains in the activities of the HN glycoprotein of Newcastle disease virus. Virology **212(2)**: 398–410.

71. McGinnes, L. W., Sergel, T., Chen, H., Hamo, L., Schwertz, S. and Morrison, T.G. 2001. Mutational analysis of the membrane proximal heptad repeat of Newcastle disease virus fusion protein. *Virology* **289**: 343-352.

72. McGinnes, L., Sergel, T. and Morrison, T. 1993. Mutations in the transmembrane domain of the HN protein of Newcastle disease virus affect the structure and activity of the protein. *Virology* **196(1)**: 101–110.

73. Miller, P., Afonso, C.A., Spackman, E., Scott, M.A., Pedersen, J.C. Senne, D.A., Brown, J.D., Fuller, C.M., Uhart, M.M., Karesh, W.B., Brown, I.H., Alexander, D.J., and Swayne, D.E. 2010. Evidence for a new Avian paramyxovirus serotype 10 detected in Rockhopper penguins from the Falkland islands. *J. Virol.* **84**: 11496-11504.

74. Miller, P., Decanini, L. and Afonso, C. 2010. Newcastle disease: Evolution of genotypes and the related diagnostic challenges. *Infection, Genetics and Evolution* **10**: 26–35.

75. Miller, P., King, D., Afonso, C. and Suarez, D. 2007. Antigenic differences among Newcastle disease virus strains of different genotypes used in vaccine formulation affect viral shedding after a virulent challenge. *Vaccine* **25**: 7238-7246.

76. Miller, P., Mia Kim L., Ip, H. and Afonso C. 2009. Evolutionary dynamics of Newcastle disease virus. *Virology* **391**: 64-72.

77. Munir, M., Abbas, M., Khan, M., Zohari S. and Berg, M. 2012. Genomic and biological characterization of a velogenic Newcastle disease virus isolated from a healthy backyard poultry flock in 2010. *Virol. J.* **9:** 46.

78. Nagai, Y., Klenk, H. & Rott, R. 1976. Proteolytic cleavage of the viral glycoproteins and its significance for the virulence of Newcastle disease virus. *Virology* **72**: 494-508.

79. Nakamura, K., Ohtsu, N., Nakamura, T., Yamamoto, Y., Yamada, M., Mase, M., Imai, K. 2008. Pathologic and immunohistochemical studies of Newcastle

disease in broiler chickens vaccinated with ND: severe nonpurulent encephalitis and necrotizing pancreatitis. *Vet Pathol.* **45**: 928-933.

80. Neyt, C., Geliebter, J., Slaoui, M., Morales, D., Meulemans, G. and Burny, A. 1989. Mutations located on both F1 and F2 subunits of the Newcastle disease virus fusion protein confer resistance to neutralization with monoclonal antibodies. *J. Virol.*, **63**: 952-954.

81. Office International des Epizooties (OIE). 1996. Avian infectious bronchitis. pp. 539-548. In: Manual of standards for diagnostic tests and vaccines, 3rd ed. (Truszczynski, M., Pearson, J.E. and Edwards, S. eds.), Office International des Epizooties, Paris.

82. Office International des Epizooties (OIE). 2010. Newcastle disease. In: Manual of Diagnostic Tests and Vaccines for Terrestrial Animals. Chapter 2.3.14. http://www.oie.int/fileadmin/Home/eng/Health_standards/tahm/2.03.14_NEWCASTLE_DIS.pdf

83. Ogasawara, T., Gotoh, B., Suzuki, H., Asaka, J., Shimokata, K., Rott, R. and Nagai, Y. 1992. Expression of factor X and its significance for the determination of paramyxovirus tropism in the chick embryo. *EMBO Journal* **11**: 467-472.

84. Otsuki, K., Takemoto, O., Fujimoto, R., Yamazaki, K., Kubota, N., Hosaki, H., Mitani, T. and Tsubokura, M. 1987. Isolation of influenza A viruses from migratory waterfowls in San-in district, western Japan in the winter of 1983-1984. *Res. Vet. Sci.* **43**: 177-179.

85. Parede, L. and Young, PL. 1990. The pathogenesis of velogenic Newcastle disease virus infection of chickens of different ages and different levels of immunity. *Avian Dis.* **34**: 803-808.

86. Pond, S. and Frost, S. 2005. Datamonkey: rapid detection of selective pressure on individual sites of codon alignments. *Bioinformatics* **21**: 2531-2533.

87. Qin, Z., Sun, L., Ma, B., Cui, Z., Zhu, Y., Kitamura, Y. and Liu, W. 2008. F gene recombination between genotype II and VII Newcastle disease virus. *Virus Res.* **131:** 299-303.

88. Qin, Z., Tan, L., Xu, H.Y., Ma, B.C., Wang, Y.L., Yuan, X.Y. and Liu W.J. 2008. Pathotypical characterization and molecular epidemiology of Newcastle disease virus Isolates from different hosts in China from 1996 to 2005. *J. Clin. Microbiol.* **46(2)**: 601–611.

89. Reed, L.J. and Muench, H. 1938. A simple method of estimating fifty percent endpoints. *Am. J. Hyg.* **27**: 493-497.

90. Salk, J. E. 1944. Simplified procedure for titrating hemagglutinating capacity of influenza virus and the corresponding antibody. *J. Immunol.* **49**: 87-98.

91. Samuel, A., Nayak, B., Paldurai, A., Xiao, S., Aplogan, G.L., Awoume, K.A., Webby, R.J., Ducatez, M.F., Collins, P.L. and Samala, S.K. 2013. Phylogenetic and pathotypic characterization of Newcastle disease viruses circulating in West Africa and efficacy of a current vaccine. *J. Clin. Microbiol.* **51**: 771-781.

92. Seal, B.S., King, D.J. and Bennett, J.D. 1995. Characterization of Newcastle disease virus isolates by reverse transcription PCR coupled to direct nucleotide sequencing and development of sequence database for pathotype prediction and molecular epidemiological analysis. *J. Clin. Microbiol.* **33**: 2624–2630.

93. Sergel-Germano, T., McQuain, C. and Morrison, T. 1994. Mutations in the fusion peptide and heptad repeat regions of the Newcastle disease virus fusion protein block fusion. *J. Virol.* **68**: 7654–7658.

94. Shengqing, Y., Shinya, K., Otsuki, K., Ito, H. and Ito, T. 2002. Isolation of myxoviruses from migratory waterfowls in San-in district, western Japan in winters of 1997-2000. *J. Vet. Med. Sci.* **64**: 1049-1052.

95. Shengqing, Y., Kishida, N., Ito, H., Kida, H., Otsuki, K., Kawaoka, Y. and Ito, T. 2002. Generation of velogenic Newcastle disease viruses from a nonpathogenic waterfowl isolate by passaging in chickens. *Virology* **301**: 206-211.

96. Snoeck, C.J., Owoade, A.A., Hymann, E.C., Alkali, B.R., Okwen, M.P., Adeyanju, A.T., Komoyo, G.F., Nakaoune, E., Faou, A.L. and Muller, C.P. 2013. High genetic diversity of Newcastle disease virus in poultry in west and central Africa: Cocirculation of genotype XIV and newly defined genotypes XVII and XVIII. *J. Clin. Microbiol.* **51(7)**: 2250-2260

97. Stallknecht, D. E., Senne, D. A., Zwank, P. J., Shane, S. M. and Kearney, M. T. 1991. Avian Paramyxoviruses from Migrating and Resident Ducks in Coastal Louisiana. *J. Wildl. Dis.* **27**: 123-128.

98. Stanislawek, W.L., Wilks, C.,R., Meers, J., Horner, G.W., Alexander, D.J., Manvell, R.J., Kattenbelt, J.A., Gould, A.R. 2002. Avian paramyxoviruses and influenza viruses isolated from mallard ducks (*Anas platyrhynchos*) in New Zealand. *Arch. Virol.* **147**: 1287-1302.

99. Steward, M., Vipond, I., Millar, N. and Emmerson, P. 1993. RNA editing in Newcastle disease virus. *J. Gen. Virol.* **74(12)**: 2539-47.

100. Takakuwa, H., Ito, T., Takada, A., Okazaki, K. & Kida, H. 1998. Potentially virulent Newcastle disease viruses are maintained in migratory waterfowl populations. *Jpn. J. Vet. Res.* **45**: 207-215.

101. Tamura, K., Dudley, J., Nei, M. and Kumar, S. 2007. MEGA4: Molecular Evolutionary Genetics Analysis (MEGA) software version 4.0. *Mol. Biol. Evol.* **24**: 1596-1599.

102. Tamura, K., Nei, M. and Kumar, S. 2004. Prospects for inferring very large phylogenies by using the neighbor-joining method. *Proc. Natl. Acad. Sci. U.S.A* **101**: 11030-11035.

103. Terregino, C., Aldous, E.W., Heidari, A., Fuller, C.M., De Nardi, R., Manvell, R.J., Beato, M.S., Shell, W.M. Monne, I., Brown, I.H., Alexander, D.J. and Capua, I. 2013. Antigenic and genetic analyses of isolate APMV/wigeon/Italy/3920-1/2005 indicate that it represents a new avian paramyxovirus (APMV-12). *Arch. Virol.* **158**: 2233-2243.

104. Tippmann, H. F. 2004. Analysis for free: comparing programs for sequence analysis. *Brief Bioinform.* **5**: 82-87.

105. Toyoda, T., Gotoh, B., Sakaguchi, T., Kida, H. and Nagai, Y. 1988. Identification of amino acids relevant to three antigenic determinants on the fusion protein of Newcastle disease virus that are involved in fusion inhibition and neutralization. *J. Virol.* **62**: 4227-4430.

106. Tsai, H., Chang, J.K.H., Tseng, C.H., Frost, K.M., Manvell, R.J. and Alexander, D.J. 2004. Antigenic and genotypical characterization of Newcastle disease viruses isolated in Taiwan between 1969 and 1996**.** *Vet. Microbiol.* **104**:19-30.

107. Tsunekuni, R., Ito, H., Kida, H., Otsuki, K. and Ito, T. 2010. Increase in the neuraminidase neuraminidase activity of a nonpathogenic Newcastle disease virus isolate during passaging in chickens. *J. Vet. Med. Sci.* **72**: 453-457.

108. Van Boven, M., Bouma, A., Fabri, T., Katsma, E., Hartog, L. and Koch, G. 2008. Herd immunity to Newcastle disease virus in poultry by vaccination. *Avian Pathol.* **37**: 1-5.

109. Warke, A., Stallknecht, D., Williams, S.M., Pritchard, N. and Mundt, E. 2008. Comparative study on the pathogenicity and immunogenicity of wild bird isolates of avian paramyxovirus 2, 4, and 6 in chickens. *Avian Pathol.* **37**: 429-434.

110. World Health Organization (WHO), WHO Manual on Animal Influenza Diagnosis and Surveillance (2002)
http://www.who.int/csr/resources/publications/influenza/en/whocdscsrncs20025rev.pdf

111. Yamane, N., Arikawa, J., Odagira, T. and Ishida, N. 1982. Characterization of avian paramyxoviruses isolated from feral ducks in northern Japan: the presence of three distinct viruses in nature. *Microbiol. Immunol.* **26**: 557–568.

112. Yan, Y. and Samal, S.K. 2008. The role of intergenic sequences in Newcastle disease virus RNA transcription and pathogenesis. *J. Virol.* **82**: 1323-1331.

113. Yin, Y., Cortey, M., Zhang, Y., Cui, S., Dolz, R., Wang, J., Gong, Z. 2011. Molecular characterization of Newcastle disease viruses in Ostriches (Struthiocamelus L.): further evidences of recombination within avian paramyxovirus type 1. *Vet. Microbiol.* **149**:324-329.

114. Yu, L., Wang, Z, Jiang, Y., Chang, L. and Kwang, J. 2001. Characterization of newly emerging Newcastle disease virus isolates from the People's Republic of China and Taiwan. *J. Clin. Microbiol.* **39**: 3512–3519.

115. Yusoff, K., Nesbit, M., Mc Cartney, H., Meulemans, G., Alexander, D.J., Collins M.S., Emmerson, P.T. and Samson AC. 1989. Location of neutralizing epitopes on the fusion protein of Newcastle disease virus strain Beaudette C. *J. Gen. Virol.* **70**: 3105-3109.

116. Zhang R., Wang X., Su J., Zhao J. and Zhang G. 2010. Isolation and analysis of two naturally occurring multi-recombination Newcastle disease viruses in China. *Virus Res.* **151**: 45–53.

117. Zhang R., Pu J., Su J., Zhao J., Wang X. and Zhang S. 2010. Phylogenetic characterization of Newcastle disease virus isolated in the mainland of China during 2001-2009. *Vet. Microbiol.* **141(3-4)**: 246-257.