J Microbiol Immunol Infect
2002;35:223-228

Phylogenetic analysis of Newcastle disease virus in Taiwan

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Received: February 8, 2002 Revised: March 11, 2002 Accepted: May 13, 2002

The virulent forms of Newcastle disease virus cause a devastating disease of poultry. Between 1998 and 2000, sporadic outbreaks of Newcastle disease occurred in Taiwan despite vaccination. The causes of the failure of the vaccination remain unclear. The purpose of this study was to investigate the possible factors causing these outbreaks by serologic and virologic methods. Anti-Newcastle disease virus hemagglutination-inhibition titers were measured for serum samples obtained from a breeder farm and a broiler farm. The serologic data showed continued presence of virulent Newcastle disease viruses in the field during inter-outbreak periods. Phylogenetic analysis demonstrated that the field virulent Newcastle disease viruses were genetically similar and were grouped into genotype VIIa. Efficacy testing by virulent Newcastle disease virus challenge revealed that the vaccines used were effective for protecting chickens from infections. This investigation demonstrated that the Newcastle disease virus strain can spread quickly and widely throughout a large geographic area, and that the sporadic cases originate from virulent Newcastle disease viruses present in the field.

Key words: Chicken, epidemiology, fusion gene, hemagglutination-inhibition, Newcastle disease

Newcastle disease (ND) is a significant poultry disease throughout the world. Control of this disease in epizootic areas is dependent upon vaccination [1]. In spite of vaccination in some areas, outbreaks of this disease still occur occasionally. According to the clinical signs, Newcastle disease virus (NDV) is divided into 5 pathogenic forms: viscerotropic velogenic, neurotropic velogenic, mesogenic, lentogenic, and asymptomatic [2]. These categories reflect the variation of virulence of different NDV strains. Despite the different classifications, all NDVs belong to a single serotype, which allows the low virulent strains to be used as a vaccine to protect chickens against high virulent strain challenge. The primary molecular determinant for NDV pathogenicity is the fusion protein cleavage site amino acid sequence. Collins *et al* [3] compared the deduced amino acid sequences of the F0 precursor of NDV and found that viruses that were pathogenic for chickens had the sequence of multiple basic amino acids at the C-terminus of the F2 protein (^{112}RRQRR^{116} or ^{112}RRQKR^{116}) and phenylalanine at residue 117, the N-terminus of the F1 protein; whereas those of low virulence had sequences in the same region of ^{112}GRQGR^{116} and leucine at residue 117. Thus, there appears to be a requirement of a double pair of basic amino acids residues, 112 and 113 together with 115 and 116, plus a phenylalanine at residue 117 if the virus is to show virulence for chickens.

In Taiwan, there have been several reported outbreaks of NDV infection in 1968, 1984, and 1995 [4,5]. Newcastle disease is endemic in Taiwan. The vaccination programs are the same for chickens during outbreak and inter-outbreak periods, although poor vaccination in some farms was suspected [5]. However, ND has still occurred in some farms that followed the vaccination program guidelines. The reason for the outbreaks in vaccinated farms is unknown. Improper vaccination or intrusion of a new virus is another possible reason for these outbreaks. The emergence of variant strains, which may not be protected by the vaccines used in the field, has been suspected as a cause of the recent outbreaks. This study investigated the possible causes of outbreaks by serologic profiling of the infected farms during outbreak and inter-outbreak periods, by challenge test with a representative virulent virus, and by phylogenetic analysis of the F gene of recent NDV isolates.

Materials and Methods

Serologic profile in breeders and broilers

One infected breeder farm in Ilan was selected for the anti-NDV hemagglutination-inhibition (HI) titer determination. Serum samples were collected and sent by mail to the Northern Laboratory of the Poultry Health Center located in the Animal Hospital of National

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Taiwan University. There were 19 lots of various ages at a time in the farm. Ten to 20 blood samples were collected each time at 2-month intervals. The HI titers were measured according to a previously described method [6] within 2 days of arrival. In addition, one infected broiler farm in Ilan, which is 8 km away from the above breeder farm, was selected for the HI measurement. The sampling and measuring methods used in the infected broiler farm were the same as for the breeder farm.

Virus isolation

Spleen, trachea, or lung from infected chickens was homogenized and inoculated into the allantoic cavity of 9- to 11-day-old specific-pathogen-free (SPF) embryonating chicken eggs (National Institute for Animal Health [NIAH], Taiwan). Inoculated eggs were further incubated for 7 days. The allantoic fluid of the dead embryos as well as the surviving embryos was identified by HI test with specific antiserum for the presence of NDV. A finding of NDV positive by HI test was reconfirmed by reverse transcription-polymerase chain reaction (RT-PCR) and direct sequencing. Samples were considered negative after 2 passages in eggs.

Viruses

Twenty-three NDVs were isolated mostly from northern Taiwan (Table 1) between 1998 and 2000. In addition, one isolate (TW-1/95, from the 1995 outbreak) and one standard challenge strain for vaccine efficacy test (Sato from NIAH) were included in this study. A representative isolate, TW-1/98, was selected for the vaccine efficacy test.

Reverse transcription-polymerase chain reaction, sequencing, and phylogenetic analysis

Viral RNA was extracted with TRIzol LS reagent (Life Technologies, Frederick, MD, US) according to the method described by the manufacturer. Two degenerate primers, 5'-ATGGGC(C/T)CCAGA(C/T)CTTCTAC-3' (sense) and 5'-CTGCCACTGCTAGTTGTGATAATCC-3' (antisense) were used for the amplification of the F gene of NDV by RT-PCR [5]. The PCR products were sent for direct sequence using the Applied Biosystems prism Cycle Sequencing Kit (Perkin Elmer, Branchburg, NJ, US). Forward and reverse sequences were performed to ensure correct results. The nucleotide sequences of the isolates were compared with several Taiwan and China NDV stains in GenBank (Table 2). The sequences were aligned using the Clustal multiple alignment

Table 1. Designation and origin of 23 Taiwan NDV strains

Designation of NDV strain		Origin		
NDV strain	Original	Date of isolation	Location	Host
TW-1/98	2489-2	February 1998	Ilan	Broiler
TW-2/98	2490-5	February 1998	Ilan	Broiler
TW-3/98	2492-2	February 1998	Ilan	Broiler breeder
TW-4/98	2502-4	March 1998	Ilan	Broiler
TW-5/98	2511-1	April 1998	Hsinchu, zoo	Show chicken
TW-6/98	2512-1	April 1998	Hsinchu, zoo	Silver pheasant
TW-7/98	2517-4	April 1998	Taoyuan	Broiler breeder
TW-8/98	2518-3	April 1998	Taoyuan	Broiler breeder
TW-9/98	2523-2	April 1998	Ilan	Broiler breeder
TW-10/98	2526-4	April 1998	Ilan	Broiler breeder
TW-11/98	2529-4	April 1998	Ilan	Broiler
TW-12/98	2530-3	April 1998	Hsinchu	Broiler breeder
TW-13/98	2570-2	July 1998	Taoyuan	Broiler breeder
TW-14/98	2576-3	July 1998	Taichung	Broiler breeder
TW-15/98	2577-2	July 1998	Yunlin	Broiler breeder
TW-16/98	2625-2	November 1998	Taoyuan	Broiler breeder
TW-17/98	2568-1	June 1998	Taipei	Pheasant
TW-18/98	2631-1	November 1998	Ilan	Broiler
TW-1/99	2696-2	April 1999	Ilan	Broiler
TW-2/99	2666-2	January 1999	Ilan	Broiler
TW-1/00	2860-2	May 2000	Ilan	Broiler breeder
TW-2/00	2872-1	June 2000	Ilan	Broiler
TW-3/00	2879-1	August 2000	Ilan	Broiler

Abbreviation: NDV = Newcastle disease virus

Table 2. Reference NDV strains from GenBank used for gene analysis

NDV strain	Origin	Accession number	Genetic grouping
TW/69	Taiwan	AF083959	III
TW/84P	Taiwan	AF083967	VIIa
TW/84C	Taiwan	AF083965	VIIa
TW/94P	Taiwan	AF083961	VIIa
TW/95-1	Taiwan	AF083960	VIIa
TW/95-2	Taiwan	AF083972	VIIa
TW/95-3	Taiwan	AF083970	III
TW/95-4	Taiwan	AF083969	VIIa
TW/95-7	Taiwan	AF083968	VIIa
TW/95-9	Taiwan	AF083966	VIIa
Taiwan 95	Taiwan	NDU62620	VIIa
Ow/Tw/2209/95	Taiwan	AF164966	VIIa
TW/96P	Taiwan	AF083971	VIIa
TW/98-1	Taiwan	AF083963	VIIa
TW/99-154	Taiwan	AF234030	VI
TW/99-157	Taiwan	AF234032	VIIa
TW/99-158	Taiwan	AF234033	VIIa
TW/99-159	Taiwan	AF234034	VIIa
GX-3/98	China	AF378256	VIIa
GPMY/QY97-1	China	AF162714	VIIa
HLJ-4/95	China	AF378259	VI
QH-4/85	China	AF378252	VIII
LaSota	Vaccine	AF077761	II
B1	Vaccine	AF375823	II

Abbreviation: NDV = Newcastle disease virus

algorithm in the MegAlign program (DNASTAR, Madison, WI, US). Phylogenetic relationships were established with the computer program TRECON for Windows (version 1.1) [7]. Briefly, a distance matrix was created by the Kimura 2-parameter model and the tree was constructed by the neighbor-joining algorithm. The robustness of the groupings was assessed by bootstrap resampling of 1000 replicate trees.

Vaccine efficacy test

One representative isolate, TW-1/98, was purified 3 times by plaque formation in a baby hamster kidney cell line (BHK-21, ATCC CCL-10). The 50% lethal dose (LD_{50}) was determined in 1-day-old broilers (Pei-I breeder farm, Lin-Kou) without vaccination after hatching, according to a previous method [8].

Two kinds of chickens, layers and broilers, were chosen for vaccine efficacy test. Seventeen female Hyline layers from an uninfected farm were vaccinated with complete ND vaccination (4 times with live B1, LaSota strains, and 2 times with killed vaccines before laying) and kept in different cages for 2 months in separate animal rooms. These chickens were then challenged intramuscularly with 10^8 LD_{50} of TW-1/98 NDV and observed for 15 days. The HI titer and the egg production rates of each bird were calculated before and after challenge. Five 4-week-old broilers that had received 3 times of NDV vaccination at 1, 10, and 23 days old by spraying with B1, Poulvac NDW (Solvay, the Netherlands), and LoSota (Solvay), respectively, were challenged intramuscularly with $10^{5.5}$ LD_{50} of TW-1/98 and observed for 15 days. All of the challenged chickens were necropsied at the end of the experiments.

Statistical analysis

The HI titers, egg production rates, and hatchability rates were compared using Student's *t* test before and after NDV challenge at 95% significant level [9]. The HI titers in different flocks of the broiler farm were compared using one-way analysis of variance and then by Student's *t* test at 95% significant level [10].

Results

Serology and clinical cases in breeders

The geometric mean HI titers of chickens from the breeder farm in Ilan increased at the time of laying and reached a peak (2^9-2^{11}) at the ages of 28 to 48 weeks and then decreased afterwards. The HI titers of these chickens increased again at the ages of 68 to 78 weeks despite the lack of vaccination during this period. Thus, although no clinical manifestations were observed, the

presence of virulent NDV in the flocks was suspected. The HI titers remained high during the inter-outbreak periods. However, sporadic outbreaks of ND were noted. Several NDVs were isolated during sporadic outbreaks (TW-3/98, TW-9/98, TW-1/00). The weekly mortality of infected flocks varied from 0.8% to 4%. Pathologic lesions of the dead chickens indicated that the NDVs were viscerotropic.

Serology and clinical cases in broilers

The distribution of the HI titers among 5-week-old broilers before an outbreak in a representative farm is shown in Table 3. There were 4 two-story-houses, from A to D, in this farm. Flock A1 was located at the floor, flock A2 1st floor, and so on for houses B, C, and D. There was a wide distribution of HI titers in the flocks indicating the presence of virulent NDV, because the variance of HI titer in a flock with vaccination only was much narrower. Although the same vaccination program was given to all flocks, the geometric mean HI titers varied considerably ($p<0.05$) among different flocks, indicating the presence of virulent NDV in the farm even though no clinical ND was found in this lot. The overall production rates of the farm reached 98% to the market at the age of 38 days. However, NDV infection occurred in the next lot. A virulent NDV was isolated from that farm (TW-2/00). The survival rates at market age decreased to 40% to 85% in this farm due to virulent NDV infection.

Phylogenetic analysis

A 534 bp PCR product was obtained with the primer set Fap-Faq. This fragment was sequenced, and the first 350 bp were compared with the corresponding sequences of reference NDVs from GenBank (Table 2). Among the 23 isolates, 4 were genotype II (TW-17/98, TW-18/98, TW-2/99, TW-3/00; Fig. 1), which is the vaccine strain with ^{112}GRQGR-L^{117} at the fusion cleavage site. The other 19 isolates were virulent NDVs with ^{112}RRQKR-F^{117} at the fusion cleavage site. All of the 19 isolates were genotype VIIa (Fig. 1). Almost all NDV isolates were of this genotype. The 19 isolates were closely related with sequence homology of 97.7% to 100%. The NDV isolates of other laboratories from GenBank were also grouped into genotype VIIa. This finding indicates that a single genotype of NDV is spread widely throughout Taiwan. Interestingly, 2 separate China strains, GPMY/QY97-1 and GX-3/98 isolated in 1997 and 1998, were included in the branch of genotype VIIa and they showed a high genetic similarity (98.6%-99.7%) to isolates in this study.

There were no genotype III isolates, as in the 1969 outbreak, during a period between 1998 and 2000. In addition, one strain, TW/99-154, a reference strain isolated by NIAH Taiwan, was of genotype VI. This was the first NDV isolate from Taiwan that belongs to this genotype since 1969.

Vaccine efficacy test

The HI titers of the 17 layers ranged from 2^8 to 2^{11}, with a geometric mean titer of $2^{9.3} \pm 2^{0.98}$ before challenge, and $2^{11.6} \pm 2^{0.88}$ at 15 days after challenge, the latter of which was significantly higher ($p<0.05$). The egg production rate before challenge was 93% ± 3.2%, and that after challenge was 91% ± 2.8%. No significant difference in egg production rate ($p>0.05$) or hatchability rate ($p>0.05$) was found before and after challenge. Clinical and pathologic investigations were normal in all layers and broilers after challenge, indicating that the vaccines used in this study were still effective for controlling NDV in the field.

Discussion

About 50 000 blood samples have been tested per year

Table 3. Distribution of anti-NDV HI titers in 5-week-old broilers in different flocks before an outbreak of ND

Flock	Anti-NDV HI titer (2^n)												Geometric mean titer
	0	1	2	3	4	5	6	7	8	9	10	11	
A1			2[a]		1	4	1		1	1			34
A2				2	1		3	1	2		1		69
B1				2	1	2	2	2	1				42
B2			1			2		1	2	1	2	1	181[b]
C1					1		1	3	2	2	1		181[b]
C2				3		5			2				32
D1			2		3	3		1			1		28
D2		1	1		1	2	1	1	2		1		49

Abbreviations: NDV = Newcastle disease virus; HI = hemagglutination inhibition

[a]Number of chickens with indicated anti-ND HI titer.

[b]HI titer is significantly higher than those in the other flocks ($p<0.05$).

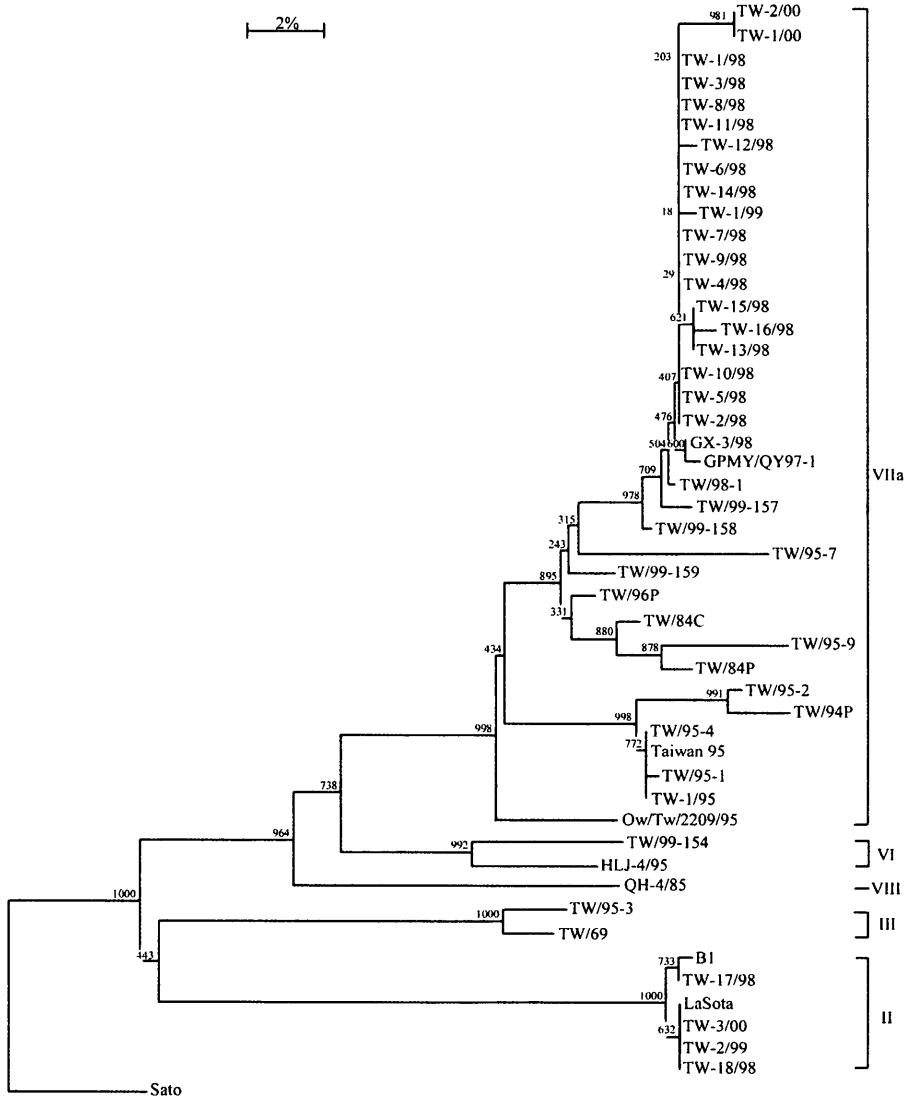

Fig. 1. Phylogenetic tree of the nucleotide sequences of the partial F gene (positions 71-420) of NDV isolated and reference strains form GenBank. Numbers at branching points are bootstrap values. The robustness was assessed by bootstrap re-sampling 1000 replicate trees using Sato strain as the evolutionary root of tree.

for ND HI in Taiwan recently. From March 1993 through December 2001, a total of 452 422 blood samples were assayed for ND HI. The serologic profile of the infected farms indicated that virulent NDVs were present in the farms during inter-outbreak periods. This may explain the sporadic reoccurrence of ND in the field. The emergence of a new strain of NDV is unlikely based on the phylogenetic results and the vaccine efficacy test results, which indicates that the vaccines used in the field still provide protection for chickens infected by virulent viruses. Thus, eradication of ND should focus on eliminating the presence of NDVs in the chicken flocks even when there are no clinical cases. Besides serologic investigation, NDV detection should be regularly performed.

In this study, all virulent isolates fell into genotype VIIa. These isolates were closely related with high sequence homology. These viruses were isolated from various areas of Taiwan, indicating the fact that they spread quickly and widely throughout the island. It is well known that the genotype VII viruses have been present in east Asia since the 1980s and in western Europe since 1990s [11], and have caused outbreaks in Taiwan in 1995 [4,5]. These viruses are still the predominant strains in Taiwan. The reason for this quick spreading and the predominance of this genotype in

1998 is difficult to determine. However, the breeders may have an ongoing role in these outbreaks because the earlier isolates between 1998 and 2000 were recovered from some broiler breeder farms. These breeders may have spread the infection to progeny boiler ranches. Although true transovarian transmission was not demonstrated in this study, the transmission of these viruses may have been from hatching chicks of breeder farms to their progeny [12,13]. Thus, strict control in the breeder farms is important for the eradication of this disease.

Phylogenetic analysis revealed a close association of the isolates in genotype VIIa between Taiwan and China (GX-3/98, GPMY/QY97-1), indicating that NDVs from these 2 areas were from the same progenitor virus. Since the import of agricultural products from China into Taiwan is prohibited, travelers or smuggling of birds might have contributed to viral transmission. The government of Taiwan should pay more attention to prevent the illegal intrusion of agricultural products. However, migratory birds may also play a role in viral transmission.

Another China strain from genotype VI, HLJ-4/95, was included for comparison with a Taiwan strain, TW/99-154. The bootstrap value of the Taiwan strain was high (992/1000), indicating that it was also of genotype VI. Before 1998, there were no reports of genotype VI in Taiwan [5]. The strains in this genotype were mostly found in some European and Middle Eastern countries. Several years after NDV isolation was noted in several laboratories in Taiwan, this genotype first appeared in 1999, and thus has likely originated from areas outside Taiwan. Further study to determine the source of this genotype is required.

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