Restriction Fragment Length Polymorphism Analysis of the F Gene of Newcastle Disease Viruses Isolated from Chickens and an Owl in Taiwan

Yin-Tai KOU, Ling-Ling CHUEH and Ching-Ho WANG*

Department of Veterinary Medicine, National Taiwan University, 142 Chou-Shan Rd., Taipei 106, Taiwan

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ABSTRACT. To provide information on the epidemiology of Newcastle disease (ND) of poultry in Taiwan, ND virus isolates from chickens and an owl were investigated by restriction site analysis and sequencing of their gene. A 1,349 base fragment of the F (fusion protein) gene was amplified by reverse transcription-polymerase chain reaction (RT-PCR). The PCR products were analyzed using restriction endonucleases, *Hin*fI, *Bst*OI, and *Rsa*I. Three strains isolated from chickens during the 1995 epidemic outbreak had the same restriction sites as that of a 1994 isolate; the number of the restriction sites of *Hin*fI, *Bst*OI, and *Rsa*I were 4, 2, and 4, respectively. In the F gene of the strain isolated from an owl during the same outbreak an additional restriction site of *Hin*fI was found. The 1991 isolate had only 3 restriction sites. The F gene of the owl isolate was amplified by RT-PCR and followed by direct sequencing. The deduced amino acid sequence at the cleavage site of the F protein was of virulent strains, ^{112}R-R-Q-K-R-F^{117}. The F gene of Ow/Tw/2209/95 was phylogenetically most closely related to that of Ck/Tw/2137/95 isolated from the same outbreak. The present results indicate that the causative virus of the 1995 ND outbreak had already been present in Taiwan.—KEY WORDS: chicken, F gene, Newcastle disease, owl, RFLP.

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Newcastle disease (ND) is an economically important and highly contagious disease of poultry [2, 3]. According to the clinical signs, ND are divided into five forms: viscerotropic velogenic, neurotropic velogenic, mesogenic, lentogenic and asymptomatic. This reflects the variation of virulence of different ND virus (NDV) isolates and strains. In order to identify viruses of high and low pathogenicity in the laboratory, three pathogenicity tests, the mean death time (MDT), the intracerebral pathogenicity index (ICPI), and the intravenous pathogenicity index (IVPI), are performed [1]. These pathogenicity tests can distinguish viruses according to the degree of severity of ND caused by different isolates, but do not give much epizootiological information.

Molecular epidemiology has been employed to explore the sequence variation and phylogenic relationship of NDV isolates [6–9]. However, investigation of the nucleotide sequence is time-consuming when the number of samples is large, and a relatively simple way of grouping different isolates with epizootiological relatedness has been demonstrated [4]. After amplifying a 75% region of the F gene by reverse transcription-polymerase chain reaction (RT-PCR) and restriction endonuclease digestion, restriction site analysis established seven major groups of NDV isolates in the world [4, 8].

In Taiwan, there was an epidemic outbreak of ND in 1995. The origin of the causative virus for this outbreak was still unknown. One possibility was that migratory birds carried exotic NDV to Taiwan. In order to explore this possibility, restriction site analysis for the F gene was applied to the NDV strains isolated in Taiwan before and during the 1995 outbreak. The F gene of the NDV isolate (Ow/Tw/2209/95) from a barrel-toe scop owl (*Otus bakkamoena glabripes*) was sequenced. The nucleotide sequence was compared with those of several foreign NDV strains to investigate the evolution of Taiwan isolates. The results indicate the causative virus originated from one that had been present in Taiwan before the 1995 outbreak.

* CORRESPONDENCE TO: WANG, C. H., Department of Veterinary Medicine, National Taiwan University, 142 Chou-Shan Rd., Taipei 106, Taiwan.

MATERIALS AND METHODS

Viruses and pathogenicity tests: The six NDV strains used in this study were isolated from sporadic cases before the 1995 and during the 1995 outbreak in Taiwan. Chicken/Taiwan/970/1991 (Ck/Tw/970/91) and Ck/Tw/2066/94 were isolated before the 1995 outbreak, and Ck/Tw/2137/95 (Taiwan95, Accession number: U62620, GenBank), Ck/Tw/2164/95, and Ck/Tw/2174/95 were isolated from chickens during the 1995 outbreak. Owl (Ow)/Tw/2209/95 was isolated from a dead owl in Taipei City Zoo in April 1995 [13]. A virulent strain (Sato) used at the Taiwan Animal Health Research Institute (TAHRI, Tansui) for ND vaccine potency test as the challenge strain was also used.

After inoculation of the sample filtrates to SPF chicken embryos (TAHRI), viruses were recovered from the allantoic cavity and confirmed by electron microscopy and the hemagglutination-inhibition test with specific anti-NDV antiserum (produced in SPF chickens, TAHRI). The pathogenicity of the isolates was determined by the intracerebral pathogenicity index (ICPI) and intravenous pathogenicity index (IVPI) [1].

RNA extraction: All glassware and double distilled water

(ddH_2O) were treated with 0.1% diethyl-pyrocarbonate (Sigma) for removing RNase contamination. SDS (Sigma), RNasin® (Promega), proteinase K (Sigma) and EDTA were added to the allantoic fluid, and extracted with phenol-chloroform-isoamylalcohol [5]. The RNA was further precipitated in absolute ethanol with sodium acetate and washed with 70% ethanol. The final RNA pellet was dissolved in ddH_2O (at least 20 μl) and frozen at −70°C until used.

Oligonucleotide primers: Five sets of primers were used in RT-PCR or PCR. The first set, Fd3 (5'-CGATTCCATCCGCAAGATCCAAGGGTCTG-3') and Fd5 (5'-GATCTAGGGTATTATTCCCAAGCCA-3'), was from a published primer set [4] and modified according to the F gene of a Taiwan isolate, Ck/Tw/2137/95. A 1,349 base pairs (bp) PCR product was amplified for restriction site analysis. After amplification, the second set of primers, FCa3 (5'-GCCTTAACTCAGTTGACTATCCAGGC-3') and FCa5 (5'-CAAGCAATAAATGCCCGG-3'), was applied to confirm the PCR product.

RT-PCR: Synthesis of cDNA was carried out in 40 μl volumes containing 2.5 μl ddH_2O, 20 μl RNA (1 μg) sample, 8 μl 1.25 mM dNTP (Promega), 1 μl 100 pmol/μl primer (Fd3), 0.2 μl 40 U/μl RNasin, 0.3 μl 10 U/μl AMV reverse transcriptase, and 8 μl 5X reaction buffer (Promega) [5]. The reaction was conducted in a FTS-960 thermal cycler (Corbett Research) and performed at 25°C for 10 min, 42°C for 50 min, and 95°C for 5 min. For PCR, amplification was carried out in 100 μl volumes containing 5 μl cDNA obtained from RT, 74.5 μl ddH_2O, 1 μl 25 mM $MgCl_2$ (Promega), 8 μl 1.25 mM dNTP, 1 μl 100 pmol/μl primer (Fd5), 0.5 μl 5 U/μl Taq DNA polymerase, 10 μl 10X Magnesium-free Thermophilic Buffer (Promega) and 1 drop of mineral oil (ICN). The reaction was performed as 5 cycles of 94°C for 45 sec, 67°C for 20 sec, 72°C for 1 min, and then 30 cycles of 94°C for 45 sec, 48°C for 1 min, 72°C for 3 min. The final polymerization step was conducted at 72°C for 7 min. The PCR products were analyzed on a 1.8% agarose gel (Gibco BRL) containing 0.5 μg/m*l* ethidium bromide (Sigma), and the amplified DNA was detected using an ultraviolet transilluminator. The QIAquick Gel Extraction Kit (Qiagen) was used to recover the target PCR product from the gel.

Nested PCR: The reaction was carried out in 100 μl volumes containing 1.5 μl eluted PCR product, 78 μl ddH_2O, 8 μl 1.25 mM dNTP, 1 μl 100 pmol/μl of each primer (FCa3 and FCa5), 0.5 μl 5 U/μl Taq DNA polymerase, 10 μl 10X Buffer (Promega) and 1 drop of mineral oil [5]. The reaction was performed as 30 cycles of 94°C for 1 min, 67°C for 1 min, 72°C for 1 min 30 sec. The final polymerization step was conducted at 72°C for 3 min.

Restriction endonuclease digestion and electrophoresis: The digestion was carried out in 30 μl volumes which comprised 500 ng eluted PCR products [5], 3 μl respective restriction endonuclease buffer and 1.5 μl 10 U/μl *Hin*fI, *Bst*OI or *Rsa*I (Promega), and ddH_2O up to 30 μl. The restriction fragments were observed following electrophoresis on a 2.5% MetaPhor agarose gel (FMC) at 5 V/cm for 6 hr in 0.5X tris-borate/EDTA electrophoresis buffer.

Direct nucleotide sequencing and sequence analysis: The three sets of primers used for sequencing the F gene of the owl isolate (Ow/Tw/2209/95) were Sb3: 5'-ATATGGGCTCCGAACCTTCTACCAGGG-3' and Sb5: 5'-TTTATACAGTCCAATTCTCGCGCCG-3'; Sc3: 5'-TAATACAAGCCAACCAGAATGCCGCC-3' and Sc5: 5'GCTCAAGCAGGAATAAATGCCCGG-3'; and Sd3: 5'-GGGCACCTAAATAATATGCGTGCC-3' and Sd5: 5'TCGCTCTTTGGTTGCTTGTACCC-3'. PCR was carried out in 100 μl volumes containing 5 μl cDNA (RT product), 77.5 μl ddH2O, 8 μl 1.25 mM dNTP, 1 μl 100 pmol/μl of the counterpart of each primer (Sb5, Sc5 and Sd5, respectively), 1 μl 2.5 U/μl cloned *Pfu* DNA polymerase, 10 μl cloned *Pfu* Buffer (Stratagene), and 1 drop of mineral oil. The reaction was performed as 30 cycles of 94°C for 1 min, 67°C for 1 min, 72°C for 1 min 30 sec. The final polymerization step was conducted at 72°C for 3 min. Three different PCR products were sequenced by the dideoxynucleotide method using Taq DyeDeoxy Terminator Cycle Sequencing kit (ABI 3370, Applied Biosystems, U.S.A.) [5]. Four kinds of fluorescent dye-labeled ddNTPs were added with a single-strand primer. The sequence was analyzed by an automatic DNA sequencer (Applied Biosystems). Both strands of three different PCR products were sequenced at least 4 times for the owl isolate to ensure the accuracy of the results. Nucleotide sequence editing, analysis, deduced amino acid sequences were completed using Naling in PC/GENE for aligning two sequences or Megalign program (pairwise sequence alignment) in Lasergene (Wisconsin) for phylogenetic analysis. The sequences of NDV Ck/Tw/69 [5], NDV CK/TW/2137/95, and other foreign NDV strains from GenBank were used.

RESULTS

Pathogenicity of NDV strains: The pathogenicity of the NDV strains isolated from birds in Taiwan is shown in Table 1, except Ck/Tw/69. Ck/Tw/970/91 and Ck/Tw/2066/94 were isolated before the 1995 endemic outbreak; the others were isolated during the outbreak. The pathogenicity tests revealed that all these isolates were velogenic. In addition, the Taiwan isolates are viscerotropic, since they

Table 1. Pathogenicity indexes of NDV isolates

Isolate	Year	Bird	ICPI	IVPI
Ck/Tw/970/91	1991	hen	2.00	2.44
Ck/Tw/2066/94	1994	broiler	1.93	2.63
Ck/Tw/2137/95	1995	broiler	1.90	2.58
Ck/Tw/2164/95	1995	fighting chicken	1.75	ND
Ck/Tw/2174/95	1995	broiler	1.70	2.32
Ow/Tw/2209/95	1995	barrel-toe scop owl	2.00	2.54

ND: Not Done.

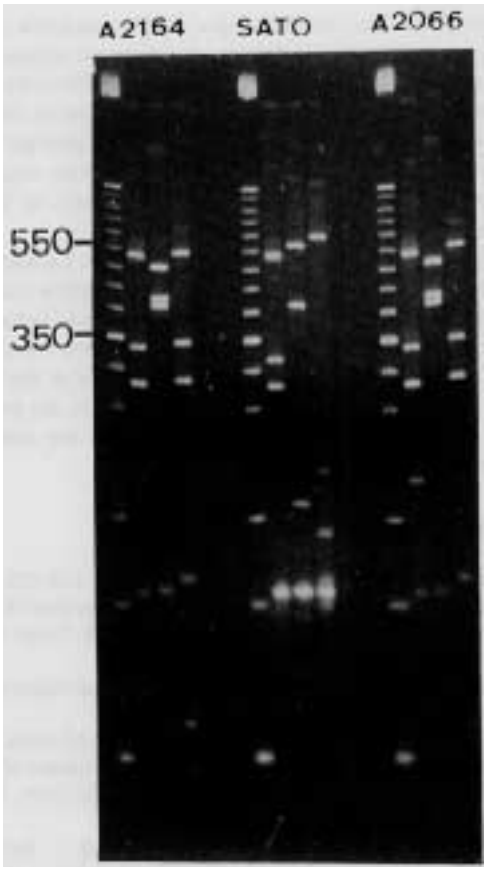

Fig. 1. Restriction fragment length polymorphism patterns of the PCR products of NDV Taiwan isolates and Sato strain. NDV strains are shown on the top of each lane (A2164 means Ck/Tw/2164/95, A2066 means Ck/Tw/2066/94). The first lanes for each isolate are 50 bp DNA ladder (Gibco, BRL), later then, restriction enzymes, *Hin*fI, *Bst*OI and *Rsa*I.

induced hemorrhagic lesions in the digestive tract as observed in the field cases [12].

Grouping of NDV isolates in Taiwan: PCR products were obtained from all isolates listed in Table 1 and the Sato strain with Fd3 and Fd5 primers and confirmed by nested PCR to be the F gene. Following restriction endonuclease digestion, RFLP patterns were similar to each other among the field isolates. The representative profiles are shown in Fig. 1. However, the Sato strain showed a different profile from those of the field isolates. The restriction sites of the F gene of each strain are summarized in Table 2. None of the RFLP patterns was the same as those reported previously [4] and they could be grouped into Group VII [8]. Ck/Tw/2066/94, isolated before the 1995 outbreak, had the same restriction sites as Ck/Tw/2137/95, Ck/Tw/2164/95 and Ck/Tw/2174/95. The isolates from before or during the 1995 outbreak, thus belonged to the same group. Although the 1991 isolate, Ck/Tw/970/91, lacked a restriction site in the position 683 base of *Rsa*I, the other cutting sites were the same as those of the others.

Ck/Tw/69 showed a different RFLP pattern from that of Ck/Tw/2137/95, lacking restriction sites at the positions 1063 base of *Hin*fI and 1625 base of *Rsa*I. It had a unique restriction site at the position 540 base of *Rsa*I but the *Bst*OI at the position of 752 base was common to both isolates.

During the 1995 outbreak, RFLP patterns of isolates from chickens and the owl were the same except one more *Bst*OI cutting site at nucleotide 1116 in the F gene of the latter strain.

Nucleotide sequence of the F gene of NDV: Three different PCR products of 1,624 bases of the F gene were sequenced. The nucleotide sequence in Ow/Tw/2209/95 had 86% and 96% similarities to Ck/Tw/69 and Ck/Tw/2137/95, respectively. The unique restriction site of *Bst*OI in Ow/Tw/2209/95, position 1116 bp, coded for two amino acids. They were Pro-Gly and the same in both Ow/Tw/2209/95 and Ck/Tw/2137/95. The amino acid sequence of the cleavage site for protease to activate the F_0 was ^{112}Arg-Arg-Gln-Lys-Arg-Phe^{117}. It agreed with the character of the velogenic strains: two pairs of basic amino acids and phenylalanine at the 117th amino acid.

Phylogenetic tree of NDV strains: The phylogenetic tree of the F gene from the sequences of the owl isolate (Ow/Tw/2209/95), Ck/Tw/69 [5], and published sequences from GenBank (Ck/Tw/2137/95) are shown in Fig. 2. Taiwan isolates were close to Australia-Victoria, Miyadera and Herts 33. The difference between owl isolate, Ow/Tw/2209/95 (GenBank accession number: AF164966) and a chicken

Table 2. The cleavage sites of F gene fragments of NDV isolates generated by different restriction endonucleases

Isolate	*Hin*fI	*Bst*OI	*Rsa*I
Ck/Tw/69[a]	875, 883, 1198, 1400	629, 752, 953, 1116, 1601	540, 683, 973, 1087
Ck/Tw/970/91	875, 883, 1064, 1400	752, 1260	973, 1087, 1625
Ck/Tw/2066/94	875, 883, 1064, 1400	752, 1260	683, 973, 1087, 1625
Ck/Tw/2137/95	875, 883, 1064, 1400	752, 1260	683, 973, 1087, 1625
Ck/Tw/2164/95	875, 883, 1064, 1400	752, 1260	683, 973, 1087, 1625
Ck/Tw/2174/95	875, 883, 1064, 1400	752, 1260	683, 973, 1087, 1625
Ow/Tw/2209/95	875, 883, 1064, 1400	752, 1116, 1260	683, 973, 1087, 1625

a) The restriction sites of Ck/Tw/69 [5] were obtained from the sequences by using RESTRI in PC/GENE.

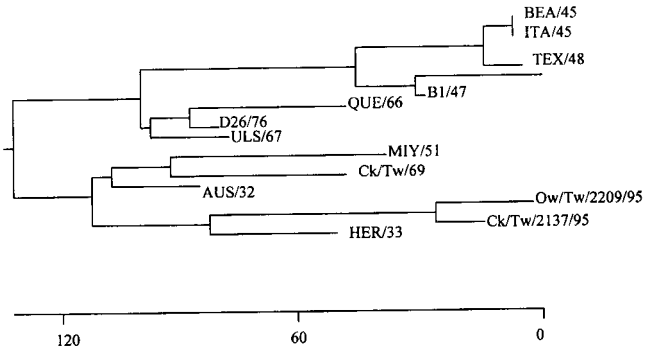

Fig. 2. Phylogenetic tree of Taiwan isolates and foreign strains based on nucleic acid sequence of the F gene. Scale below indicates the number of the difference in the nucleotides.

isolate, Ck/Tw/2137/95 was 4%. Both isolates were obtained in the same period. However, the owl isolate was quite different from the early isolate, Ck/Tw/69.

DISCUSSION

The present results indicate that Taiwan isolates belonged to none of the proposed six groups [4], or rather genotype VII based on the restriction site analysis [8]. However, the *Hin*fI and *BstO*I cutting sites were similar to those of group III NDV strains (AUS/32 and MIY/51). This group includes NDV strains isolated during the 1960s panzootic in the Far East [4, 8]. The present isolates could be the descendants of the strains in that outbreak and those from outbreaks in Western Europe in the 1990s [8].

The nucleotide sequence variation rate in the non-structural gene of Newcastle disease virus is less than 1% [11]. The early isolate, Ck/Tw/69 is close to Ballagi-Pordany's group III (AUS/32 and MIY51) [4] on the basis both of RFLP patterns and phylogenetic analysis and quite different from recent isolates. Between the 1969 and 1995 outbreaks, the introduction of NDV strains from other countries must have occurred since such a great variation is unlikely in 26 years.

The F protein contributes to the pathogenicity of NDV. This protein mediates fusion of viral and cellular membranes for the virus to penetrate into the host cell. The amino acid sequence at the cleavage site of the F protein of the present Taiwan isolates along with their ICPI and IVPI data indicates that these NDV strains are velogenic [2, 10].

Although NDV could evolve through a pet bird [7], 4% variation is unlikely in such a short time (less than 5 months) in the same outbreak. Ck/Tw/2137/95 and Ow/Tw/2209/95 might co-circulate in Taiwan [14]. Ow/Tw/2209/95 was isolated at the end of the 1995 outbreak. Although the difference in the nucleotide sequence between the owl isolate and the chicken isolate, Ck/Tw/2137/95 reached 4%, the owl isolate is closer to Ck/Tw/2137/95 than any other foreign isolates. It is very likely that the owl isolate originated from a different NDV in the same chicken outbreak [14]. Despite the unique *BstO*I restriction site in the F gene of Ow/Tw/2209/95, the deduced amino acid sequence is the same as those of the isolates from chickens. In addition, all other cutting sites were the same as those of chicken isolates. The owl may have been infected from chickens at the end of the 1995 outbreak. The causative virus might have been brought by flying birds to Taipei City Zoo [13].

There was a ND outbreak in early 1995 in Taiwan. The 1995 outbreak began in January on the western coast of Taiwan and spread quickly throughout the island before the disease was brought under control in May of the same year. Since the same RFLP pattern was obtained from the NDV isolates before and after the 1995 outbreak in the present study, it is concluded the causative virus of the outbreak had existed in Taiwan.

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