

Retrospective characterization of Newcastle Disease Virus Antrim '73 in relation to other epidemics, past and present

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SUMMARY

In November 1973 Newcastle disease suddenly appeared in Northern Ireland, where the viscerotropic disease had not been seen in 3½ years and the two Irelands had been regarded as largely disease free for 30 years. It was successfully controlled with only 36 confirmed affected layer flocks, plus 10 more slaughtered as 'dangerous contacts'. Contemporary investigations failed to reveal the source of the Irish epidemic. Using archival virus samples from most of the affected flocks, RT-PCR was conducted with primers selected for all six NDV genes. Phylogenetic analyses of three genes, HN, M and F, confirmed vaccine as the cause of one of the outbreaks. The other six samples were identical and closely related to previous outbreaks in the United States and western Europe initiated by infected imported Latin American parrots. The probable cause of the epidemic followed from the importation from The Netherlands of bulk feed grains contaminated with infected pigeon faeces.

INTRODUCTION

Newcastle disease virus (NDV) causes Newcastle disease (ND), one of the most serious poultry diseases. ND infection can cause great economic losses and there is a constant danger of introduction into disease-free areas [1].

Prior to 1973, Northern Ireland had only suffered sporadic and easily contained outbreaks of ND over the past 30 years. A viscerotropic velogenic virus

assumed to have originated in seabirds caused a limited outbreak in small, free-range chicken flocks on the north-west coast in 1949; in 1964, an asymptomatic non-clinical Ulster 2C virus, probably introduced by wild birds, was isolated and the few affected flocks were slaughtered out; in 1970 a limited viscerotropic velogenic virus outbreak occurred in backyard flocks in the Lurgan area and was associated with an illegally imported parrot. The province had been ND-free for 3½ years when birds were first noted to be ill on 12 November 1973, in a 90 000-bird laying unit, at Andrews, 15 miles north-west of Belfast.

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A positive HI test was obtained the next day but it was not considered significant as *Mycoplasma synoviae* infection was widespread on the farm and it was considered to be a lentogenic 2C NDV strain. Soon, over 50% of the birds in the affected building were sick and developing respiratory symptoms. On the basis of embryonated egg mortality NDV was confirmed on 16 November. Seven days later it was confirmed in a 3700-bird mixed rearing and laying unit 2 miles north-east, and by the end of the month in a further seven establishments, four of which were in the direction of the prevailing wind and at a significant overall distance. Subsequent investigations precluded that the three could have resulted from an airborne infection [2] and failed to elicit any direct or indirect contacts. It was therefore held that there were at least four primary outbreaks in the first fortnight – the index outbreak plus the three ‘non-windborne’ outbreaks. By the time the epidemic finished on 29 December 1973, there had been 36 confirmed outbreaks, and a further 10 flocks slaughtered as ‘dangerous contacts’. It should be noted that this epidemic seemed to start with multiple primary outbreaks [3].

The purpose of this study was to investigate the source of the Antrim '73 outbreak using molecular genetic techniques, namely genomic sequence comparisons with other NDV epidemic isolates. As stated by Aldous and Alexander [4], these techniques have largely superseded antibody analysis as they enable rapid and accurate detection and characterization of NDV isolates in a single test.

The approximately 15 kb RNA genome encodes six viral proteins: 3'-NP-P-M-F-HN-L-5' [5]. The majority of published work has concentrated on the functionally important surface glycoprotein genes, HN (haemagglutinin–neuraminidase) and F (fusion protein), which are required to initiate viral infection [6, 7]. As a result, the phylogenetic grouping of NDV isolates has been ascertained by sequencing only those regions of its genome. Part of this work was to discover if the different NDV genes varied independently of each other, therefore several regions of the genome were sequenced to determine any similarities.

Previous studies [1, 8, 9] have shown that different NDV isolates can be differentiated into eight distinct groups (I–VIII) based on restriction-site analysis and partial sequencing of the F gene. These genotype groupings have since been used by other groups [10–12] and will be adopted in this paper.

The following hypotheses were explored:

Hypothesis 1

That the Antrim '73 NDV was part of the general parrot series out of Latin America and related cases; therefore similar to and within Group V, e.g. CA 1083/71 (1085), CA 1085/71, Fontana, Largo and NY 70181 [13, 14].

Hypothesis 2

That Antrim '73 was merely part of the Western Europe epidemic, therefore with similarities to Essex '70, Northants '72, H/10/72, and HR-270/84 [15–18].

Hypothesis 3

That Antrim '73 was not part of the eastern epidemic and its derivatives and therefore distant from the following members of Group VI (a, b, c, d) and VII – Iraq AG68, Kuwait 256, Lebanon '70, Israel '70, H-310/82, Greece '68, Warwick '66, 760/83, CH-1/95, and Taiwan '95 [19, 20].

MATERIALS AND METHODS

Virus isolates

The NDV isolates in this study were samples taken from infected chickens of the six earliest affected farms during the first days of the 1973 NDV outbreak in Co. Antrim, Northern Ireland, and from one flock that had clearly had a severe reaction to the LaSota/B1 vaccine administered a week later. Of the six naturally infected farms, three were considered to be ‘primaries’, two were downwind ‘windborne’ cases, and one, which was not downwind of the index farm (Andrews), represented either a possible human contact-spread by a farm labourer who might have unwittingly infected his own domestic bantams and geese or another, minor, primary outbreak. The viral samples have been preserved at Stormont since the outbreak. The study includes 14 different samples, representing 7 different isolates (numbered 1–7) and two different methods of RNA extraction (set a and set b).

RNA extraction

RNA extraction of the Antrim '73 samples was carried out at Southeast Poultry Research Laboratory,

Table. Successful RT-PCR primers designed for this study

Primer	Sequence	Location	Product size (bp)
KR-HN1F	GAA TGA WGA AAG RGA RGC	123–143 ^a	300
KR-HN1R	GAG AGA GAY GTT ATT GCR	402–422 ^a	
KR-HN2F	GTC ACA TCA TTC TAT CCC TCT	532–553 ^a	
KR-HN2R	AGA YCC RCC CCC YAC	991–1008 ^a	487
KR-HN4F	ATA CAA GCG MTA CAA TGA CAC	1098–1119 ^a	312
KR-HN4R	CTA TGA AGA GTR GCT GTT	1389–1409 ^a	
KR-HN5F	ART TCT CAC AGT AGG GAC ATC	1299–1321 ^a	365
KR-HN5R	CTG CYT TGG TRC TGC	1646–1663 ^a	
KR-M1F	GAT CCA GCG YCT TGA CTC	135–153 ^b	523
KR-M1R	CGG GTC YAC CTC CAC ATY	639–657 ^b	
KR-M3F	GTG SCA AAY AAR TAC TCR	439–459 ^b	424
KR-M3R	CAC CTC TYG CYT TYA	845–863 ^b	
KR-F1F	ATT ATY GGC GGT GYR	407–424 ^c	325
KR-F1R	CAG GKG AAG TGA TTT GTG	713–731 ^c	
KR-F2F	CAG CAG GTT GGT GTA GAR	656–674 ^c	485
KR-F2R	CCG CTC AAR CAG GAA TAA	1122–1140 ^c	
KR-L1F	TGG GGT CAT CCT GGT CTA	474–492 ^d	389
KR-L1R	TGA ATG CGA AGA AAT GTC	977–995 ^d	
KR-L4F	CAG CGG AGA TGA ACA GTT	3538–3556 ^d	451
KR-L4R	CCA CAT TCC CCT CTT TGA	3970–3989 ^d	
KR-P4F	TCA TCA ACT TCC GAC YCA	512–532 ^e	533
KR-P4R	CAC CCC CTT GWG TCA CAT	1025–1044 ^e	

^a Consensus of sequences J03911, M19432, M19478–79, M22110, M24705–17, U37187–93, X85971. Location based on J03911 [24].

^b Consensus of U25828–38. Location based on U25828 [25].

^c Consensus of A03663 and D00243. Location based on D00243 [6].

^d Based on X05399 [26].

^e Consensus of M20302 and X60599. Location based on M20302 [27].

GA, USA (SEPRL). Genomic viral RNA to be used for cDNA synthesis was obtained by suspending infected cells in guanidium isothiocyanate solution [21]. Lysed infected cells were layered over a 1.2 ml cushion of 5.7 M CsCl (10 mM EDTA; pH 8.0) and centrifuged at 160 000 *g* for 16 h in a Beckman SW60 rotor and designated isolates la–7a [22]. Alternatively, viral RNA was purified by acid phenol extraction [23] of infective allantoic fluid for samples 1b–7b. The RNA from the virus strains Northants '72 and Essex '70 was isolated in Hungary by Lomniczi et al., using previously published method [8].

Primers

Published NDV sequences were obtained from GenBank and primer sets were designed for each gene using Oligo 5.0 (Molecular Biology Insights Inc., Cascade, CO, USA). Four different primer pairs were chosen for each of the following genes: NP, P, M, F, L, and the 3'-end of the genome, and six primer

pairs for the HN gene. The primer pairs that successfully produced amplicons of the expected sizes are shown in the Table. Other primers, K1, K2, MV1 and B2, were devised by Lomniczi et al. [9] for amplification of the first quarter of the coding region of the F gene. The oligonucleotides were prepared using an Applied Biosystems DNA synthesizer at the Nucleic Acid Chemistry Facility of Los Alamos National Laboratory, NM, USA (LANL).

RT-PCR

For the Antrim '73 samples with the KR primers, reverse transcription and subsequent PCR of the resulting cDNA was conducted using a PerkinElmer RT-PCR kit (PerkinElmer Inc., Boston, MA, USA), in accordance with the manufacturer's instructions. The reverse transcription reaction consisted of: 10 mM Tris-HCl (pH 8.3), 50 mM KCl, 5 mM MgCl₂, 1 mM each dATP, dCTP, dGTP and dTTP, 100 pmol selected R primer, 20 U RNase inhibitor, 50 U MuLV

reverse transcriptase, plus deionized H₂O (sterile/DEPC-treated) up to 20 μ l total volume, containing 100 ng RNA (concentration determined by UV spectroscopy). Thermal cycling conditions for the above reaction were as follows: 1 cycle of 42 °C for 15 min, 99 °C for 5 min, 50 °C for 5 min. The following reagents were used for the subsequent PCR reaction: 10 mM Tris-HCl (pH 8.3), 50 mM KCl, 1.5 mM MgCl₂, 50 pmol corresponding F primer, 2.5 U *Taq* polymerase, plus deionized H₂O (sterile/DEPC-treated) up to 80 μ l total volume, added to the reverse transcription reaction. Thermal cycling conditions for the PCR reaction were as follows: 1 cycle of 95 °C for 2 min, 35 cycles of 95 °C for 1 min, 60 °C for 1 min, 1 cycle of 72 °C for 7 min. RT-PCR of the Antrim '73 samples, together with the Essex '70 and Northants '72 RNA samples, using the primer pairs K1/K2, MV1/B2, was carried out as previously described [8]. The resulting RT-PCR products were visualized using agarose gel electrophoresis. The amplicons of the expected size were isolated from the PCR reaction mix and cleaned using Qiaquick mini columns (Qiagen, Valencia, CA, USA) according to the manufacturer's protocol, before sequencing.

Sequencing analysis

Direct nucleotide sequencing of both forward and reverse strands of the RT-PCR amplicons was conducted using fluorescently labelled dideoxynucleotides (ABI Prism, PerkinElmer) and an automated nucleic acid sequencer (ABI 373), with the corresponding primers used for RT-PCR. Subsequent sequence editing and amino-acid predictions were carried out using the EditSeq function of DNA Star (Lasergene, Madison, WI, USA). The DNA Star MegAlign programme was used for sequence alignments and initial cluster analyses.

Phylogenetic analysis

Nucleotide sequences were aligned manually. Each nucleotide in the alignment was considered as homologous to the aligned nucleotides from other taxa. Due to the conserved nature of the gene regions used in this study, no gaps were required to maintain homology. Aligned sequences were analysed by maximum parsimony analysis using PAUP* software [28]. Characters (aligned nucleotide bases) were treated as unweighted, and ancestral character states were not inferred nor designated. Heuristic searches with tree-bisection-reconnection branch swapping were

performed. Trees were treated as unrooted, and input order of taxa was randomized with ten additional sequence replications. The resulting phylogram is shown in Figure 3 (branch lengths correspond to the number of changes).

RESULTS

Initial primer sets

Primers were selected to amplify regions of each gene and the 3'-end of the NDV genome, based on the consensus of published sequences. There are sequences for all regions of the genome available in GenBank, but often a region has only been sequenced for a single or a few isolates. Previous studies have noted the necessity of using more than one primer pair or degenerate primers [25, 29]. Thus, for this study it was deemed essential to design at least four different pairs of primers, some of which were degenerate, for each region of the genome studied.

Each set of primers was used in the initial RT-PCR reactions with each of the 14 Antrim isolates; 7 different isolates (samples 1-7), 2 different extraction methods (sets a and b). Several primer pairs were unsuccessful, the majority at the PCR stage, the rest at the sequencing stage. The primers that produced amplicons were comparable for both sets of samples, although more primers failed with set b, possibly reflecting different RNA qualities due to the two extraction methods. The primers that allowed repeated successful amplification of the expected size product and gave unambiguous sequence data are listed in the Table.

Overlapping sequences produced from different primer pairs along the same gene were consolidated into a single contig before sequence alignment and analysis. In addition to repeat sequencing of the isolates, the overlapping sequences of the RT-PCR products increased the confidence in the final sequence assignment. The results showed that, for each gene region analysed, Antrim '73 isolates 2-7 were essentially identical, only differing by a few isolated nucleotides. For example, at nucleotide position 129 of the HN gene contig Antrim 4b has an adenine instead of a guanine, and at position 331 Antrim 7b has a thymine instead of a cytosine (see GenBank entries for all sequence data). Antrim '73 sample 1, however, differed considerably in sequence. This implied that samples 2-7 originated from a single NDV strain.

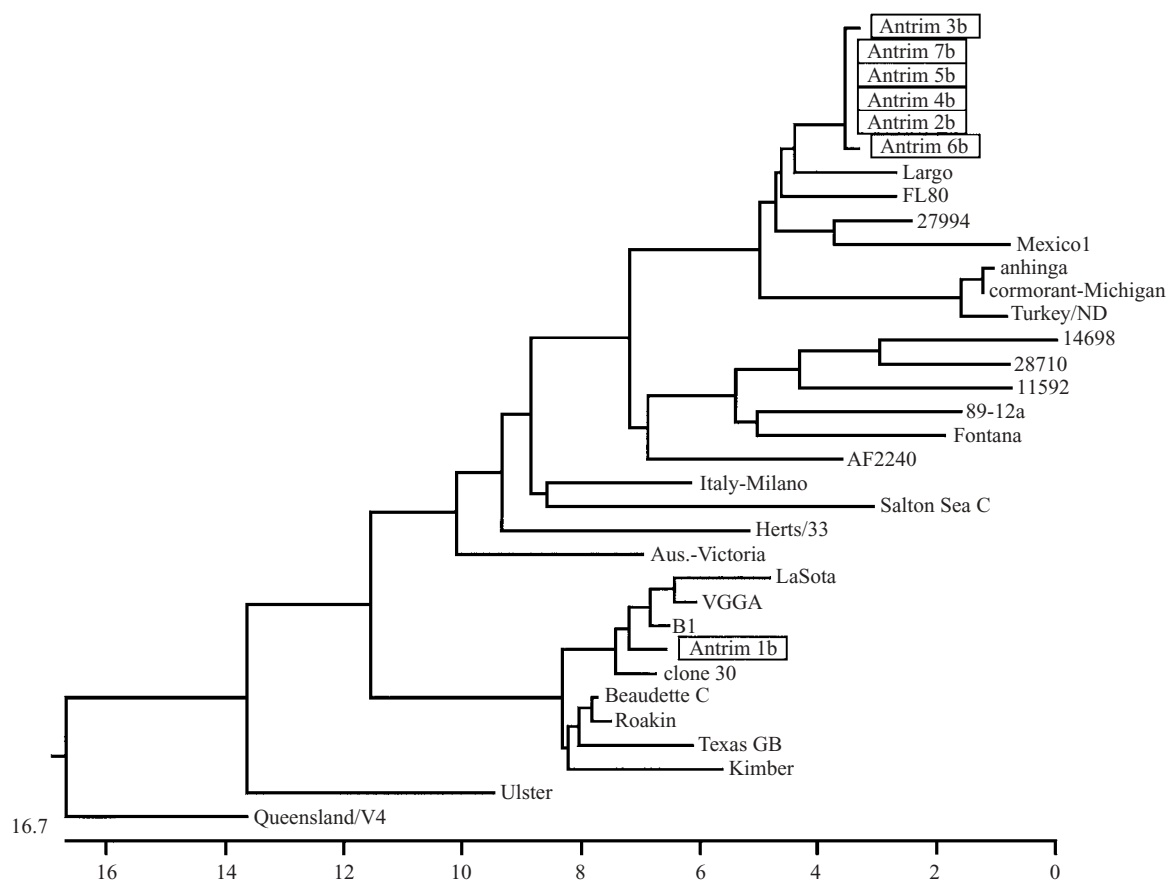


Fig. 1. Cluster analysis of M gene region of various NDV isolates. The x axis represents the number of substitutions. Antrim '73 samples represented by Antrim 1b–7b (boxed). GenBank accession numbers are as follows: Largo (U25832), FL80 (AF124452), 27994 (AF124447), Mexico 1 (AF124453), anhinga (AF124450), cormorant-Michigan (AF124451), Turkey/ND (U25836), 14698 (AF124446), 28710 (AF124448), 11592 (AF124445), 89-12a (AF124449), Fontana (U25829), AF2240 (AF060563), Italy-Milano (AF124442), Salton Sea C (AF124444), Herts/33 (U25830), Aus.-Victoria (M16622), LaSota (U25833), VGGA (U25838), B1 (U25828), Beaudette C (X04687), Roakin (AF124443), Texas GB (U25835), Kimber (U25831), Ulster (U25837), Queensland/V4 (U25834).

Cluster analyses of the F, HN and M gene data were performed, together with a representative number of corresponding sequences for each particular gene from GenBank. The results from the F gene analysis showed that the successful KR primers gave sequences upstream of the majority of published sequences. As a result the Antrim sequences did not overlap sufficiently to allow an unambiguous phylogenetic analysis. Sequences from genes L, P and NP could not be studied by cluster analysis due to lack of sequence variants accessible in GenBank.

The analysis for the HN gene region (results not shown) showed the Antrim '73 samples 2–7 clustering with the isolate AF2240. The latter strain was isolated in Malaysia and characterized as thermostable viscerotropic-velogenic (GenBank submission X79092, T. Wen Siang). This isolate has been shown to belong to genetic subgroup VIII [1]. However, the branch

lengths were long between AF2240 and the Antrim samples, indicating that these two groups are not closely related. Antrim '73 sample 1 clustered with all the vaccine strains (vaccine isolates 1–6 and B1) strongly indicating that the vaccine used during the outbreak is the origin of this sample, rather than a minor primary outbreak. Vaccine strains of NDV have also been identified during screening of isolates from other countries [30, 31].

The analysis for the M gene region is shown in Figure 1. (As the sequences obtained for both sets of isolates were the same, only the results for samples 1b–7b are shown.) This analysis showed the Antrim '73 samples 2–7 clustering with the Largo isolate. The Malaysian isolate AF2240 does not cluster with the Antrim '73 isolates and has Fontana as a nearest neighbour. As the branch lengths observed for this set of sequences were much shorter than those for the

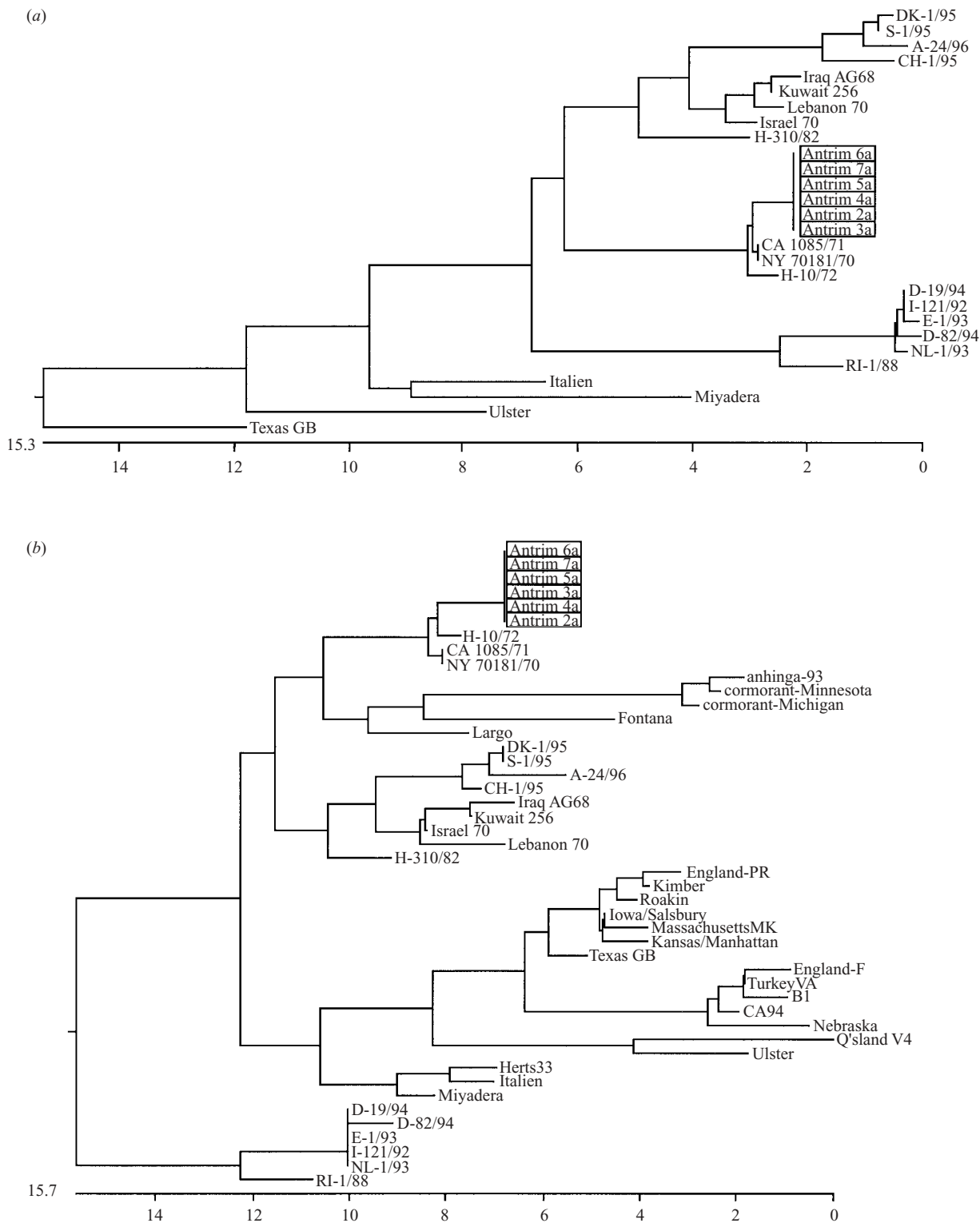


Fig. 2. (a) Cluster analysis of 389 (47–435) nucleotide region of F gene. The x axis represents the number of substitutions. GenBank accession numbers are as follows: DK-1/95 (AF001129), S-1/95 (AF001131), A-24/96 (AF001133), CH-1/95 (AF001132), Iraq AG68 (AF001108), Kuwait 256 (AF001109), Lebanon 70 (AF001110), Israel 70 (AF001111), H-310/82 (AF001112), CA 1085/71 (AF001106), NY 70181/70 (AF001105), H-10/72 (AF001107), D-19/94 (AF001114), I-121/92 (AF001127), E-1/93 (AF00113126), D-82/94 (AF001117), NL-1/93 (AD001124), RI-1/88 (AF001134), Italien (M17710), Miyadera (M18456), Ulster (D00243), Texas GB (M23407). (b) Cluster analysis of 108 (328–435) nucleotide region of the F gene. The x axis represents the number of substitutions. GenBank accession numbers are as follows: anHINGA-93 (U22265),

HN gene analysis, these results were more convincing. The Largo virus belongs to genetic subgroup V (mAb group A virus) [8, 32] indicating that the Antrim '73 samples also fall into this subgroup. Thus, for the HN gene cluster analysis, the AF2240 isolate would appear to be related to the Antrim '73 samples simply due to the absence of any genotype V viruses in the sequence set used. The M gene analysis had B1 and LaSota as the nearest neighbours to Antrim sample 1, again indicative of the fact the Antrim 1 is the vaccine strain.

The cluster analyses showed that the phylogenetic grouping of the Antrim samples was most likely genotype V. However, the analysis was dependent upon those subsets of published sequences available for comparison. The lack of accessible variants for genes other than the HN or F genes severely limits the study of individual gene variation and evolution.

F gene cluster analysis

The F gene of the NDV genome was chosen for subsequent detailed analysis, for a number of reasons. This gene is important for virulence and so numerous other studies have been conducted on the F gene of different NDV outbreaks leading to many GenBank sequences. This enables a more accurate phylogenetic analysis. This region of the NDV genome also allows for detailed phylogenetic analysis due to the presence of a variable region (residues 47–420) in the first quarter of the gene [1]. Additionally, the deduced amino-acid sequence of the F0 protein (residues 1–130) makes possible the unambiguous allocation of genotype [9], regardless of host species, year of isolation or location [33].

The primers selected were those previously reported by Lomniczi et al. [9]. The K1/K2 primer pair successfully amplified a 1008-bp product, and using this as a template, a second reaction with MV1/B2 primers produced a 557-bp amplicon for Antrim '73 samples 2–7 (only set a was tested). Antrim '73 sample 1, however, did not yield a RT–PCR product, undoubtedly due to the greater sequence diversity of this sample. Sequencing of the RT–PCR products showed that the remaining Antrim '73 samples (2–7) were identical

for that region, supporting the sequencing results obtained using the KR primers.

Subsequent cluster analysis of the region between nucleotides 47–435 of the F gene of different NDV strains showed that the nearest neighbours to the Antrim samples were CA 1085/71 and NY 70181/70, with H-10/72 on the next branch (Fig. 2*a*). A second analysis was conducted with 108 nucleotides (position 328–435) of this sequence to allow for the inclusion of more GenBank sequences to the analysis (e.g. particularly those submitted by Seal et al. [34]). This showed the Antrim samples to be most related to the H-10/72 isolate, and then the CA 1085/71 and NY 70181/70 isolates (Fig. 2*b*). These three isolates have previously been shown to belong to NDV genetic group V [9]. The fact that the branch lengths obtained for both analyses were much shorter than observed for the HN and M genes (Fig. 1), together with the larger number of sequence variants included in the analysis, allowed for greater confidence in the result. Deduction of the corresponding amino-acid sequence (between residues 1–130) also allowed for the identification of genetic group [9]. The amino-acid sequence for the Antrim '73 samples 2–7 was shown to be ¹¹²RRQKR/FVG¹¹⁹. The samples also had an alanine residue at position 106, thereby confirming the genetic grouping of these samples as genotype V.

Previous restriction-site mapping and sequencing analyses of different group V NDV viruses have led to the further division of the group into four subgroups: a, b, e, and i (B. Lomniczi, unpublished results). The above analyses of the different F gene regions (389 and 108 bp) resulted in different patterns of nearest neighbours to Antrim '73, not permitting the allocation of a particular subgroup. Thus, it was necessary to analyse other genotype V isolates in order to place the Antrim '73 samples in a particular subgroup.

RT–PCR and sequencing analysis was performed on Essex '70 and Northants '72 samples, using the K1/K2 and MV1/B2 primers, as previously described. In addition the sequence data for Hungarian group V isolates HR-270/84 and HR-40/95, were kindly supplied by Lomniczi et al.

cormorant-Minnesota (U22269), cormorant-Michigan (U22270), Fontana (U22274), Largo (U22279), England-PR (U22272), Kimber (U22278), Roakin (U22284), Iowa/Salsbury (U22276), MassachusettsMK (U22280), Kansas/Manhattan (U22277), England-F (U22271), Turkey VA (U22285), B1 (U22266), CA94 (U22267), Nebraska (U22282), Queensland V4 (U22283), Herts33 (M24702). Others as in part (a).

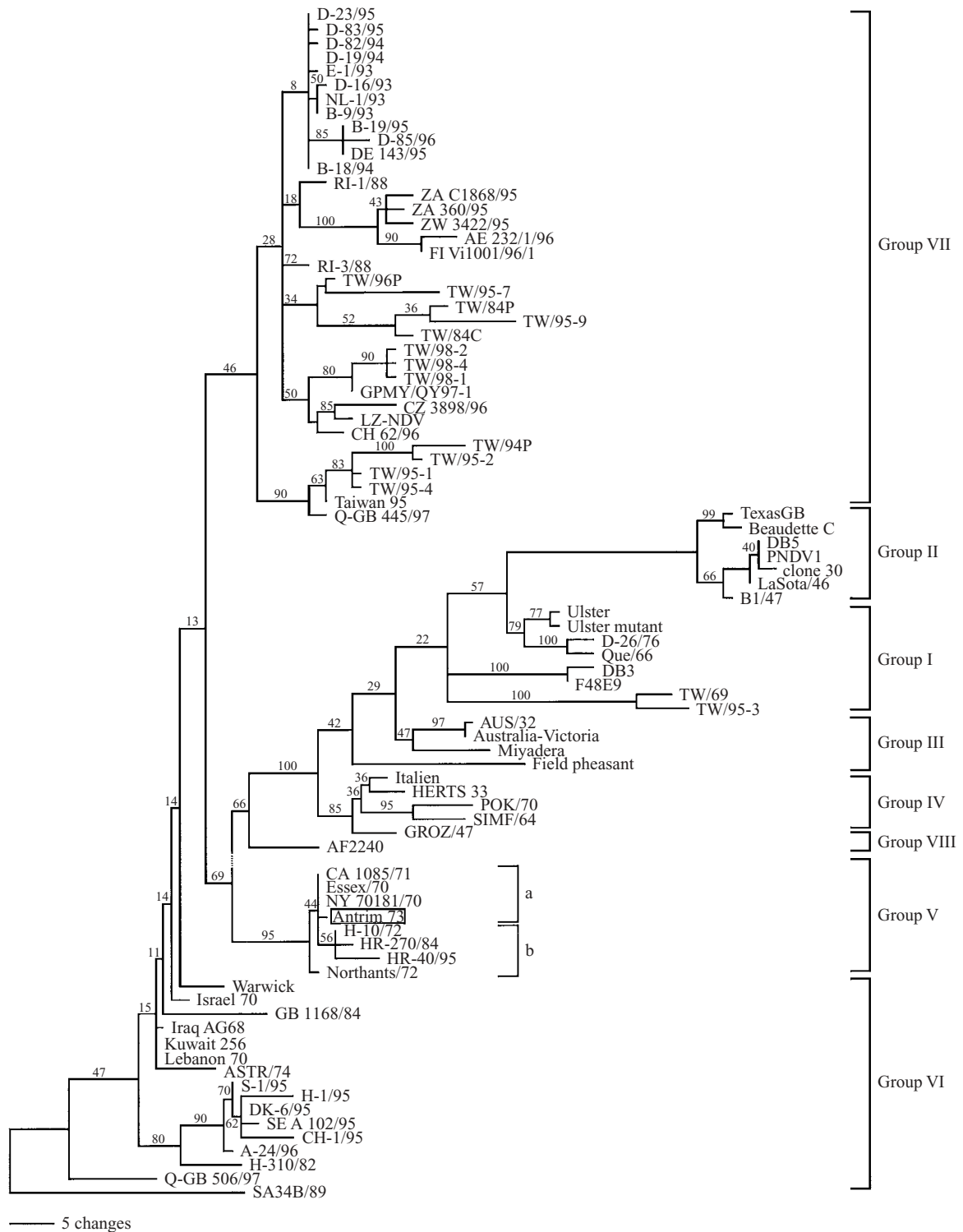


Fig. 3. PAUP phylogenetic analysis of 389 (47–435) nucleotide region of the F gene. GenBank accession numbers are as follows: D-23/95 (AF001115), D-83/95 (AF001118), D-16/93 (AF001113), B-9/93 (AF001120), B-19/95 (AF001123), D-85/96 (AF001119), DE 143/95 (AF109881), B-18/94 (AF001122), ZA C1868/95 (AF109882), ZA 360/95 (AF109876), ZW 3422/95 (AF109877), AE 232/1/96 (AF109884), FI Vi1001/96/1 (AF091623), RI-3/88 (AF001135), TW/96P (AF083971), TW/95-7 (AF083968), TW/84P (AF083967), TW/95-9 (AF083966), TW/84C (AF083965), TW/98-2 (AF083973), TW/98-4 (AF083964), TW/84P (AF083967), GPMY/QY97-1 (AF162714), CZ 3898/96 (AF109883), LZ-NDV (AF140343), CH 62/96 (AF109880), TW/94P (AF083961), TW/95-2 (AF083972), TW/95-1 (AF083960), TW/95-4 (AF083969), Taiwan 95 (U62620), Q-GB 445/97 (AF109886), Beaudette C (X04719), DB5 (AF079323), PNDV1 (AF079324), clone 30 (AF099661),

Phylogenetic analysis of F gene

In order to be confident in our assignment of subgroup, PAUP phylogenetic analysis [28] was conducted with the F gene sequences. This analysis concentrated on the 389-bp sequence of the F gene rather than the internal 108 bp. The previous F gene cluster analysis showed that the 108-bp sequences of Essex '70 and Northants '72 were identical, whereas the 389-bp sequence showed three differences between them and the 557-bp product showed four differences (results not shown). Essex '70 and Northants '72 have previously been shown to belong to different subgroups, a and b respectively (B. Lomniczi, unpublished results). Thus, the 108-bp sequence was deemed unsuitable for detailed phylogenetic analysis.

Figure 3 shows the results of the maximum parsimony PAUP analysis of the 389-bp region of the F gene. The different genotypes groups, and subgroups where appropriate, are shown beside the phylogenetic tree. The NDV sequences taken from GenBank were those available at the time for the F gene region studied. The different groupings of the GenBank sequences are in agreement with previously published results, with the exception of TW/69 and TW/95-3. Yang et al. [11, 35] previously showed these isolates to be similar to group III viruses. In contrast, all isolates from 1984 and seven out of eight isolates from 1995 were classified as group VII, as shown by this study. The Antrim '73 samples 2–7 are most closely related to group V isolates as previously observed for the F gene cluster analysis. More importantly, the group V subgroups, a and b, are clearly separate on this phylogenetic tree. The Antrim '73 samples belong to genotype group V subgroup a; most closely related to CA 1085/71, Essex '70 and NY 70181/70. A neighbour-joining PAUP analysis supports this result (results not shown).

GenBank accession numbers for all Antrim '73 sequences generated using the KR primers are as follows: P gene region, AY142246–AY142252; L gene regions, AY142253–AY142266 and AY142267–AY142280; F gene region, AY142281–AY142294; HN gene region, AY142295–AY142308; M gene region, AY142309–AY142322.

GenBank accession numbers for the Antrim '73 sequence, plus Northants '72 and Essex '70, generated using the MV1/B2 primers are AY142243–AY142245.

DISCUSSION

Phylogenetic analysis of the F gene region of the Antrim '73 samples showed that this outbreak is located within genotype group V subgroup a. The most closely related isolates are CA 1085/71, NY 70181/70 and Essex '70. Thus, both Hypotheses 1 and 2 appear to be correct – the Antrim outbreak was due to a spread of NDV from western Europe, which in turn seems to have originated from imported Latin American parrots. Group V viruses are native only in South and Middle America and there is a constant threat of infection from imported birds and free-flying cormorants [36–38]. In the 1970s most EU countries had or must have had group V outbreaks; those confirmed by Lomniczi et al. include England, Germany, Czechoslovakia, Hungary, Yugoslavia, Bulgaria and Italy. The aim of the phylogenetic studies is to discover the source of the outbreak and/or the founder strain.

In November 1971, Paraguayan parrots that had escaped from an importer's premises infected nearby commercial egg-laying flocks in southern California. By the time the last case was diagnosed in June 1973, the epidemic had cost \$56 million [13]. The situation had begun in March, 1970 when the first reported US case was in 14 psittacines in Brooklyn, NY; later in August some 4000 birds newly imported from Paraguay were affected. During 1970 three shippers exported 69% (over 12000) of their birds to the United States and the numbers of imported Paraguayan birds were even greater in 1971, totalling some 21000 in just the first 8 months of that year. At that time the birds did not undergo quarantine. The major targeted states were Florida, Oklahoma, California and New York [14].

In Switzerland there were widely dispersed outbreaks of ND associated in all cases with the recent purchase of parrots from one shipment from South

LaSota/46 (AF077761), B1/47 (AF309418), Ulster mutant (Z12110), D-26/76 (M24692), Que/66 (M24693), DB3 (AF079322), F48E9 (AF079172), TW/69 (AF083969), TW/95-3 (AF083970), AUS/32 (M24700), Australia-Victoria (M21881), Field pheasant (Z12109), POK/70 (Y19014), SIMF/64 (Y19017), GROZ/47 (Y18727), AF2240 (AF048763), Warwick (Z12111), GB 1168/84 (AF109885), ASTR/74 (Y19012), DK-6/95 (AF001130), SE A 102/95 (AF109879), Q-GB 506/97 (AF109887), SA34B/89 (AF083962). Others as for Figure 2. Bootstrap values are shown.

America [39]. Between January and September, 1970, velogenic NDV was isolated on ten separate occasions in The Netherlands from sick imported psittacine birds from Colombia and Paraguay. Some of these imported birds were found to be sick and infected when they reached South Africa [14]. Starting in May 1970, velogenic NDV was 'frequently' isolated in Germany from newly imported psittacines, the majority of which were from Paraguay; it was also recovered from Colombian birds [14]. In Hungary, group V was present between 1972 and 1982, with the introduction of at least two independent variants during that time (B. Lomniczi, unpublished results).

Deducing the founder strain of the Antrim samples, however, is not enough to show how the outbreak was caused. The following possible modes of entry of the NDV into Northern Ireland were investigated at the time but without success. No common or obvious source was found and this conclusion was supported by the following observations [40].

Direct, via quarantined viraemic birds. No quarantine records supporting this route.

Legal imports of exotic birds and such into semi-intensive quarantine. Enquiries did not yield any support for this route of entry.

Illegal or unrecorded exotic bird importations. By its nature this is difficult to investigate but the investigations carried out failed to reveal any persons on affected farms were so involved.

Movements of people or vehicles. Extensive enquiries concentrated on 'hard-to-explain' outbreaks failed to yield any positive information. For example, no contact was admitted by persons that might have explained the spread from Andrews (initial disease noted on 12 November) to Eves, a distant farm to the east-southeast and first noted with sick birds on 24 November. There was no forthcoming evidence that industrial movements were involved in the initial stages of the outbreak. There were six very small domestic flocks of layers, geese, and decorative fowl where the owners worked on an infected farm and might have brought the virus home. These would have contributed nothing to the epidemic except outbreak numbers.

Feed stuffs. None were indicated at the time. All protein meals were derived from poultry products that originated in Northern Ireland; similarly with meal and bone meals. Only grains were imported. (In retrospect bulk feed at the continental docks, and especially in Rotterdam, were at risk from local pigeons. While the initial outbreaks in Ulster were

on farms with a variety of feed sources, there were only two importers, using small coastal vessels with 10–20 ton lots supplying the 15 feed mills. The Netherlands had an active vaccination programme, which prevented disease but not viral infection. In addition the initial outbreaks were all in layers that had mash, not pelleted, complex rations with many components; broilers without outbreaks had simple rations.)

Vaccines. No vaccines were used at Andrews. At Eves infectious bronchitis vaccine was used in various units but not all were affected with ND. ND vaccines were not used in the province.

Human (kitchen) wastefood. No outbreak was found to be associated with the feeding of kitchen waste nor with such materials from Aldergrove Airport.

Imported eggs. There was no commercial incentive to import eggs. Boxes with the names of Scottish or English retail distributors contained domestic eggs that had been diverted for local trade still in their export packs.

Windborne virus. While winds with high humidity and adequate force were in the right direction to carry the virus from unreported or unrealized infected flocks in England, the distance was considered to be too great.

Wild birds. While there were historical precedents, no detailed investigations were carried out.

Sabotage. This was considered at the time both within and without the Northern Ireland Department of Agriculture. Enquiries at each infected premise covered this possibility. The security at many farms was poor. While the involvement of the IRA could not be ruled out at that time there was no evidence to support such a hypothesis beyond mere speculation and suspicion. And, following the outbreak of this poultry disease in early November, no coded or uncoded claims were made by either the IRA or the Loyalist paramilitaries, nor via Sinn Féin or the plethora of loyalist societies and political associations. The outbreak was large enough to have been a claimable success. And the farmers are largely Protestant. Since 1973, there have also been no retrospective claims. The belief was that sabotage had not occurred but at the time many features of the epidemic were unexplained.

The favoured theory is that infected pigeon faeces may have contaminated the chicken feed. Previous studies have stated that domestic fowl could possibly become infected after contact with infected pigeons or contaminated pigeon droppings [41]. Others have

reported that infected birds shed virus with the faeces that can survive for approximately 3 months at 20–30 °C and longer at cooler temperatures [42]. A more recent study showed that NDV isolates of pigeon origin exhibited virulent characteristics when inoculated into chickens [43].

More work is necessary to characterize the Antrim '73 outbreak further. Samples from the end of the epidemic could be studied to see if the virus differs from the isolates at the start. The panel of primers developed in this study can be utilized to analyse all genes for comprehensive results. Because of the short time-scale of the outbreak, if the sequences differed it would undoubtedly be due to the introduction of two different strains of ND virus. Isolates from some wild birds are also available. These could be sequenced to see whether the ND viruses isolated are coincidentals or overflow from main epidemic.

Two important issues are raised by this study. First, the necessity of continued storage of past outbreak samples so that they may be retrospectively analysed by future, new and emerging technologies. It is more productive to have complete collections to hand, even when the outbreak is of past decades, than to wait for new outbreaks to occur which may be sampled. Secondly, in forensic analyses of this kind, one cannot depend on the analyses of one gene. It is the holistic results that support a confident conclusion, even if they raise new questions.

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