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Elizabeth Aldous, June K. Mynn, Chad Fuller, Richard Irvine, Dennis Alexander, et al.. A molecular epidemiological investigation of avian paramyxovirus type 1 viruses isolated from game birds of the Order Galliformes. *Avian Pathology*, 2010, 39 (06), pp.519-524. 10.1080/03079457.2010.530938 . hal-00650362

HAL Id: hal-00650362

<https://hal.science/hal-00650362>

Submitted on 10 Dec 2011

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Journal:	<i>Avian Pathology</i>
Manuscript ID:	CAVP-2010-0052.R1
Manuscript Type:	Original Research Paper
Date Submitted by the Author:	13-Jul-2010
Complete List of Authors:	Aldous, Elizabeth; VLA Weybridge, Virology Mynn, June; VLA Weybridge, Virology Fuller, Chad; Veterinary Laboratories Agency, Virology Irvine, Richard Alexander, Dennis; VLA Weybridge Brown, Ian; VLA Weybridge, Virology
Keywords:	APMV-1, Newcastle disease, game birds, phylogenetics

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Cavp-2010-0052.R1

A molecular epidemiological investigation of avian paramyxovirus type 1 viruses isolated from game birds of the Order *Galliformes*

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Received: 25 March 2010

Abstract

The partial (370 nucleotides) fusion gene sequences of 55 avian paramyxovirus type 1 (APMV-1) isolates were obtained. Included were 41 published sequences, of which sixteen were from strains of APMV-1 of previously determined lineages included as markers for the data analysed and 25 were from APMV-1 viruses isolated from game birds of the Order *Galliformes*. In addition we sequenced a further 14 game bird isolates obtained from the repository at the Veterinary Laboratories Agency. The game bird isolates had been obtained from 17 countries, and spanned four decades. Earlier studies have shown that Class II APMV-1 viruses can be divided into at least 15 lineages and sub-lineages. Phylogenetic analysis revealed that the 39 game bird isolates were distributed across 12 of these sub-lineages. We conclude that no single lineage of Newcastle disease viruses appears to be prevalent in game birds and the isolates obtained from these hosts reflected the prevailing, both geographically and temporally, viruses in poultry, pigeons or wild birds.

Introduction

Newcastle disease (ND) (or avian paramyxovirus type 1 (APMV-1) is regarded throughout the world as one of the two most important diseases of poultry and other birds, not only due to the serious disease and high flock mortality that may result from some ND virus (NDV) infections, but also the economic impact that may ensue due to trading restrictions and embargoes placed on areas and countries where outbreaks have occurred (Commission, 1992; Alexander, 2008).

Although the first isolations of NDV were from chickens, since that time the virus has been isolated from many domestically-reared flocks including turkeys (Alexander *et al.*, 1998; Alexander *et al.*, 1999; Roy *et al.*, 1999), ostriches (Huchzermeyer & Gerdes, 1993), pheasants, other game birds (Aldous & Alexander, 2007) and pigeons (Vindevogel & Duchatel, 1988). NDV can also infect many wild bird species including cormorants, wild ducks, starlings, penguins and wild waterfowl (Lipkind *et al.*, 1987; Takakuwa *et al.*, 1998; Kuiken, 1999). Kaleta & Baldauf, (1988) reviewed NDV isolations from all bird species and recorded that NDV infections have been established and detected in at least 241 species of birds, representing 27 of the 50 orders of the class *Aves*. It would seem probable that all birds are likely to be susceptible to infection with this virus (Alexander, 2009).

Although in its broadest definition the term 'game birds' includes any bird that is hunted for sport or for food (i.e. including ducks, geese etc), it is more usually applied to species of the families *Phasianidae* (pheasants and partridges), *Tetraonidae* (grouse) and *Numididae* (Guinea fowl) and in the present study we have considered isolates from birds in this narrower definition. Some game bird species may be semi-domesticated (e.g. pheasants, partridges *etc.*) or even intensively reared (quail), but generally rearing is extensive and/or the birds are released into the wild for hunting.

The two most recent outbreaks of ND in the UK were in game bird species. In 2005 pheasants presenting with mild ataxia and blindness were confirmed to be infected with virulent NDV, lineage 5b (Aldous *et al.*, 2007), and in 2006 partridges showing clinical signs of loss of condition, diarrhoea, progressive neurological signs and mortality totalling about 30%, were also confirmed to be infected with virulent NDV. Infections of game birds with APMV-1 viruses are not uncommon, but these outbreaks, in the absence of infections in chickens or turkeys, were of considerable concern for control of this notifiable avian disease. In light of this new, elevated risk that may be presented by this group of birds, the viruses isolated from these host species that are held in the repository of the International Reference Laboratory for ND at the Veterinary Laboratories Agency (VLA) were obtained and sequenced. The data has been analysed to enable insights into any host restrictions that may occur within these species and the recognition of any potentially high risk strains.

The genetic and antigenic relationships between many different isolates of NDV have been investigated and discussed (Ballagi-Pordany *et al.*, 1996; Alexander *et al.*, 1997b; Lomniczi *et al.*, 1998; Aldous *et al.*, 2003; Czegledi *et al.*, 2006). In the present study, the phylogenetic relationships between 55 isolates, including 39 from game bird hosts were investigated. The analysis was based on the nucleotide sequence of a 370-nucleotide fragment at the 3' end of the fusion protein gene. As one of the major antigenic determinants of NDV, the fusion protein gene is likely to display greater genetic variation than the internal genes, an important characteristic for studying quite closely related virus populations, where a more conserved gene may show insufficient sequence variation to allow deduction of evolutionary hypotheses.

Materials and Methods

Isolates. Virus isolates were grown in the allantoic cavities of 9- to 10-day-old embryonated fowls' eggs originating from a commercial specific-pathogen-free flock and were supplied by the International Reference Laboratory at VLA-Weybridge. Details of the isolates, including their abbreviations and accession numbers are listed in Table 1. Intracerebral pathogenicity index (ICPI) values for the viruses (where available) were supplied by the International Reference Laboratory.

RT-PCR and PCR. Viral RNA was extracted from infective allantoic fluid using Qiagen QIAamp viral RNA kit. The preparation of cDNA was carried out as described (Collins *et al.*, 1993), using an alternative cDNA primer (#MSF1) 5' GACCGCTGACCACGAGGTTA. 'Reddy-mix' PCR master mix (AB gene) was used for the PCR, using the cDNA forward primer and reverse primer (#2) 5' AGTCGGAGGATGTTGGCAGC, to generate an amplicon of approximately 700bp. The final concentrations of components in the PCR mix were 1.25 units Taq DNA polymerase, 75mM Tris-HCL, 20mM (NH₄)₂ SO₄, 1.5mM MgCl₂, 0.01% (v/v) Tween 20, 1µM each primer and 0.2mM of dATP, dCTP, dGTP and dTTP. The cycling parameters were an initial denaturation step of 1 min at 95°C, followed by 30 cycles of: 1 min at 94°C, 1 min at 50°C, 3 min at 72°C, completed with a final extension step of 72°C for 10 min. The PCR products were separated by gel electrophoresis using 2% w/v agarose gel in Tris-acetate buffer, stained with ethidium bromide and visualised under UV light. The product was excised and purified using QiaQuick (Qiagen) gel extraction kit.

Nucleotide sequencing. The forward primer used was (#7) 5' TTAGAAAAACACGGGTAGAA (Collins *et al.*, 1996), the reverse primer was the same as for PCR (#2). Nucleotide sequencing was carried out using 'BigDye' DNA sequencing kits (V3.1) and a 3130 genetic analyser according to manufacturer's instructions.

Sequence analysis. Nucleotide sequence analysis and alignment was done using Lasergene DNASTAR software version 8. The sequences were aligned to begin at the start codon (ATG at position 47) of the fusion protein gene and finish just downstream from the cleavage activation site. The sequences were aligned using the Clustal V method in Megalign. Subsequent phylogenetic analysis was done using MEGA, v4.1. The results are presented as phylogenetic trees in which the branch lengths are proportional to the predicted number of substitutions.

Results & Discussion

The results obtained from the phylogenetic analysis are shown as a phylogenetic tree in Figure 1, which was generated from a data set based on the partial fusion gene nucleotide sequences of 56 APMV-1 isolates. Included in this dataset are 41 published sequences, of which 16 represent previously determined lineages of APMV-1 as markers for the data analysed and presented in this study (Ballagi-Pordany *et al.*, 1996; Aldous *et al.*, 2003); the remaining 25 published sequences are APMV-1 isolates from game birds. In addition to this we sequenced a further 14 game bird isolates obtained from the NDV repository at VLA. The predicted virulence of these isolates is presented in Table 2.

Only Class II viruses (Czegledi *et al.*, 2006), which correspond to viruses of lineages 1-5 defined by Aldous *et al.*, (2003), were included in this study as there was no available data on isolations of Class I (Lineage 6 Aldous *et al.*, (2003)) viruses from game bird hosts. The five genetic lineages defined for Class II viruses can be further divided into at least 15 sub-lineages. As can be seen (Figure 1, Table 1) the 39 isolates from game birds analysed in this study are distributed across 12 of these sub-

lineages. Of the main lineages for virulent NDVs (3, 4, 5) the four sub-lineages with the greatest numbers of game bird viruses are 3b, 4b, 5b and 5d, although 3a, 3c, 4c, 4d and 5a are also represented. Nine isolates fall into lineages 1 and 2, the majority of which are likely to be of low virulence and could be the result of spread from wild birds, deliberate infection with live vaccines or accidental infection with live vaccines as a result of spread from poultry.

Four game bird isolates were placed in sub-lineage 4b, one from Italian pheasants, the other three from pheasants (2) and partridges in the UK. These isolations covered a period from 1995-2006 and all appeared to be the result of spread from pigeons as there were no outbreaks in other poultry at the time they occurred (Alexander *et al.*, 1997a; Irvine *et al.*, 2009).

Ten of the isolates were placed in lineage 3 in sub-lineages 3a (1 isolate), 3c (2) and 3b (7). Nine of these viruses were isolated between 1960 and 1977 and reflect the prevailing ND strains in poultry at that time. The tenth virus placed in sub-lineage 3b was isolated from quail in Italy in 1987 and is consistent with the finding that viruses with specific genetic characteristics do not necessarily disappear as other variants arise and may continue to be isolated many years after their first appearance (Lomniczi *et al.*, 1998).

The main conclusion from the present study is that no single lineage of ND viruses appears to be prevalent in game birds. This is unlike the situation in racing, farmed and feral pigeons in which a panzootic caused exclusively by ND viruses of lineage 4b has continued for about 30 years (Vindevogel & Duchatel, 1988; Aldous *et al.*, 2004; Alexander, 2009). The viruses isolated from game birds appear generally to represent geographically and temporally contemporaneous viruses affecting chickens, turkeys and/or other poultry. There is no way of knowing whether chickens and other

poultry affected game birds or the game birds were responsible for spread to chickens and other poultry. Presumably transmission has occurred in both directions. However, infections of game birds, especially pheasants, with viruses virulent for chickens do not always result in severe disease (Aldous & Alexander, 2008; Aldous et al., 2007). There is a real concern, therefore, that because of the international nature of trade in such birds and, when they are to be hunted, their release into the wild, they may represent a reservoir host for the spread and dissemination of virulent ND viruses.

Acknowledgements

This work was supported financially by a Department of Environment, Food and Rural Affairs research grant (SE0779). The authors thank Ruth Manvell for supplying the virus isolates.

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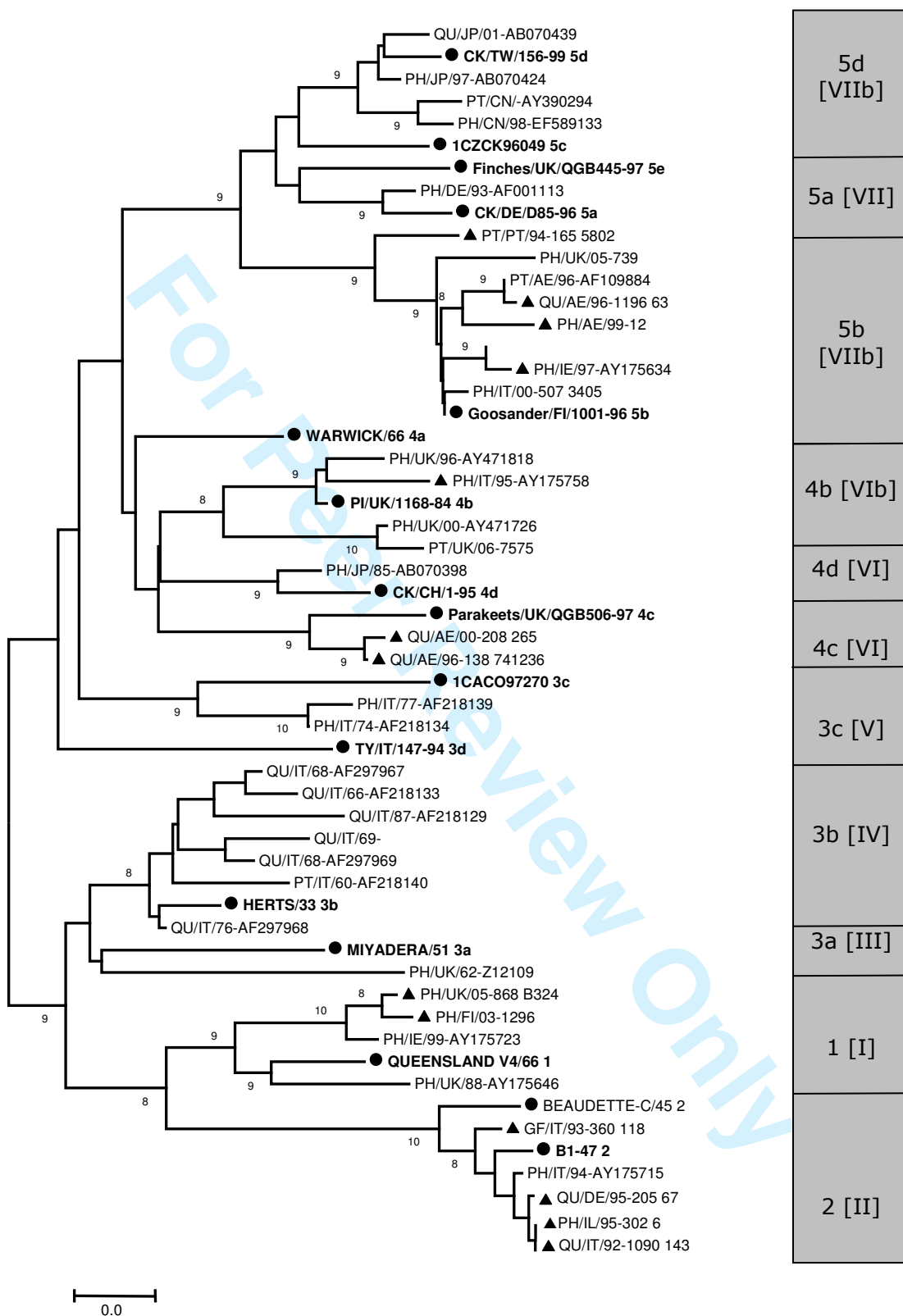
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Figure 1.



Figure_1. Evolutionary relationships of 56 APMV-1 isolates. The evolutionary history was inferred using the Neighbor-Joining method (Saitou & Nei, 1987). The percentages of replicate trees in which the associated taxa clustered together in the bootstrap test (1000 replicates) are shown next to the branches (Felsenstein, 1985). 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 (Tamura et al., 2004) and are in the units of the number of base substitutions per site. Codon positions included were 1st+2nd+3rd. There were a total of 370 positions in the final dataset. Phylogenetic analyses were conducted in MEGA4 (Tamura et al., 2007). ▲ indicates isolates sequenced in this study and ● indicates the 'reference strains' to indicate genetic lineages according to (Aldous et al., 2003). Host abbreviations are PH: pheasant, QU: quail, PT: partridge, GL: gull, CK: chicken, PI: pigeon, DK: duck. Country abbreviations are according to their ISO 3166-1 two-letter code (http://www.iso.org/iso/english_country_names_and_code_elements). The grey box indicates the NDV lineages (Aldous et al., 2003) and genotypes (Lomniczi et al., 1998) of the isolates in this study.

Table 1. *Isolate details*

Study Reference	Isolate details	Year isolated	Lineage ^a	GenBank Accession Number
Reference isolates				
Queensland/V4	Australia-Queensland V4/66	1966	1 (I)	M24693
B1/47	U.S. Hitchner B1/47	1947	2 (II)	M24695
Beaudette C/45	Beaudette C/45	1945	2 (II)	M24697
Miyadera/51	Miyadera/51	1951	3a (III)	M24701
Herts/33	Herts/33	1933	3b (IV)	M24702
CA/GL/97-AY135756	1CAGL97270	1997	3c (V)	AY135756
TY/IT/147-94 3d	TY/IT/147-94	1994	3d (VIII)	AF218132
Warwick/66 4a	Warwick/66	1966	4a (VI)	U52457
PI/UK/1168-84 4b	GB 1168/84	1984	4b (VIb)	AF109885
Parakeet/UK/QGB506-97 4c	QGB 506/97	1997	4c (VI)	AF109887
CK/CH/ 1-95 4d	CH 1/95	1995	4d (VI)	AF001132
CK/DE/D85-96 5a	D85-96	1996	5a (VII)	AF001119
Goosander/FI/ 1001-96 5b	FI 1001/96	1996	5b (VIIb)	AF091623
CK/CZ/96- AF109883	1CZCK96049	1996	5c (VIIa)	AF109883
CK/TW 156-99 5d	TW 156/99	1999	5d (VII)	AF234031
Finches/UK/QGB 445-97	QGB 445/97	1997	5e (VII)	AF109886
Published game bird isolates				
PH/UK/88- AY175646	AV 1491/88 2-9-88 NDV	1988	1	AY175646
PH/IE/99-AY175723	AV 740/99 39/99	1999	1	AY175723
PH/IT/94- AY175715	AV 567/94 19/94	1994	2	AY175715
PH/UK/62-Z12109	Field Pheasant	1962	3a	Z12109
PT/IT/60-AF218140	IT 7/60	1960	3b	AF218140
QU/IT/66-AF218133	IT 3/66	1966	3b	AF218133
QU/IT/68-AF297967	IT 5/68	1968	3b	AF297967
QU/IT/68-AF297969	IT 48/68	1968	3b	AF297969
QU/IT/69-AF218137	IT 52/69	1969	3b	AF218137
QU/IT/76-AF297968	IT 66/76	1976	3b	AF297968
QU/IT/87-AF218129	IT 126/87	1987	3b	AF218129
PH/IT/74-AF218134	IT 47/74	1974	3c	AF218134
PH/IT/77-AF218139	IT 64/77	1977	3c	AF218139
PH/UK/96-AY471818	494/96	1996	4b	AY471818
PH/IT/95- AY175758	AV 3/96 364-95	1996	4b	AY175758
UK/PH/00-AY471726	691/00 L/K	2000	4b	AY471726
PH/JP/85-AB070398	Tochigi/1985	1985	4d	AB070398
PH-DE/93-AF001113	DE 16/93	1993	5a	AF001113
PT/AE/96-AF109884	AE2321/96	1996	5b	AF109884
PH/IE/97-AY175634	AV 1220/97 VF-97-4291 Tr	1997	5b	AY175634
PH/IE/97-AY175635	AV1220/97 4246	1997	5b	AY175635
PH/JP/97-AB070424	Ibaraki/1997	1997	5d	AB070424
QU/JP/01-AB070439	Chiba/2001	2001	5d	AB070439
PT/CN/-AY390294	ZG	Pre 2003	5d	AY390294
PH/CN/98-EF589133	ND98	Pre 2007	5d	EF589133
Isolates sequenced in this study				
PH/FI/1296-03	AV 1296/03	2003	1	-
PH/UK/868-05 B324	AV868/05 24/B3247/5/2005	2005	1	-
PH/IL/302-95 6-85	AV302/95 6/85	1985	2	-
QU/IT/1090-92 143	AV1090/92 143/71/F0/1971	1992	2	-
Guineafowl/IT/93-360 118	AV 360/93 118/90/1993	1993	2	-
QU/DE/95-205 67	AV 205/95 67/94/1994	1995	2	-
DK/BG/07-1113 1113	AV1113/07 1113/2007	2007	2	-
PT/UK/06-7575	AV7575/06 10B0016/09/06	2006	4b	-
QU/AE/96-138 741236	AV138/96 1236/1996	1996	4c	-
QU/AE/00-208 265	AV208/00 265/2000	2000	4c	-
PT/PT/94-165 5802	AV 165/94 5802/94/1994	1994	5b	-
QU/AE/96-1196 63	AV1196/96 63/1996	1996	5b	-
PH/AE/99-12 211	AV12/99 211/1999	1999	5b	-

PH/IT/00-507 3405	AV507/00 3405	2000	5b	-
PH/UK/05-739	AV739-05 MCF	2005	5b	-

^aGenetic lineages according to Aldous *et al.*, (2003), for the reference viruses the lineages according to Lomniczi *et al.*, (1998) are shown in brackets.

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Table 2. *The deduced F0 cleavage site motif and predicted virulence for chickens of game bird isolates sequenced in the present study.*

Isolate	Cleavage site motif ^a	ICPI	Virulence ^b
PH/FI/1296-03	GKQGRL	0.5	Avirulent
PH/UK/868-05 B324	GKQGRL	0.0	Avirulent
PH/IL/302-95 6-85	GRQGRL	-	Avirulent
QU/IT/1090-92 143	GRQGRL	-	Avirulent
Guineafowl/IT/93-360 118	RRQRRF	1.75	Virulent
QU/DE/95-205 67	GRQGRL	0.15	Avirulent
DK/BG/07-1113 1113	GRQGRL	-	Avirulent
PT/UK/06-7575	RRQKRF	0.59	Virulent
QU/AE/96-138 741236	RRQRRF	-	Virulent
QU/AE/00-208 265	RRQRRF	-	Virulent
PT/PT/94-165 5802	RRQKRF	-	Virulent
QU/AE/96-1196 63	KRQRRF	-	Virulent
PH/AE/99-12 211	RRQRRF	-	Virulent
PH/IT/00-507 3405	RRQRRF	-	Virulent
PH/UK/05-739	RRQRRF	1.6	Virulent

^a cleavage site motif corresponds to amino acids 112-117 of the fusion protein.

^bThe OIE manual (Alexander, 2008) defines virulent ND viruses as those with a phenylalanine residue at position 117 and at least three basic amino acids (R or K) between 113-116; APMV-1 viruses that do not meet this criterion are virulent if they have an intracerebral pathogenicity index (ICPI) >0.7. The virulence prediction is based on this definition.

- ICPI data not available.