Avian influenza

and avian paramyxoviruses

in the New Zealand bird population

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Abstract

A comprehensive study using virological and serological approaches was carried out to determine the occurrence of avian paramyxoviruses (APMVs) and avian influenza viruses (AIVs) in live healthy mallard ducks (*Anas platyrhynchos*) in addition to caged birds, wild birds (other than waterfowl), and poultry.

Thirty-three viruses were isolated from 321 tracheal and cloacal swabs from mallard ducks and were characterised as: 6 AIV (two H5N2 and four H4N6), 10 APMV-1, and 17 APMV-4. Of 335 serum samples tested for AIV antibodies, 109 (32.5%) sera were positive by nucleoprotein-blocking ELISA (NP-B-ELISA). Serum samples (315) were examined for antibody to APMV-1, -2, -3, -4, -6, -7, -8, and -9 by the haemagglutination inhibition (HI) test. The largest number of reactions, with titres up to ≥1/64, was to APMV-1 (93.1%), followed by APMV-6 (85.1%), APMV-8 (56%), APMV-4 (51.7%), APMV-7 (47%), APMV-9 (15.9%), APMV-2 (13.3%), and APMV-3 (6.0%). All of the H5N2 isolates of AIV and the APMV-1 isolates from this and earlier New Zealand studies had low pathogenicity indices when assessed by the Intravenous Pathogenicity Index (IVPI) with the result 0.00 and Intracerebral Pathogenicity Index (ICPI) with results 0.00–0.16. Partial genomic and antigenic analyses were also consistent with the isolates being non-pathogenic. Phylogenetic analysis of the 10 APMV-1 isolates showed nine to be most similar to the reference APMV-1 strain D26/76 originally isolated in Japan and also to the Que/66 strain, which was isolated in Australia. The other isolate was very similar to a virus (MC 110/77) obtained from a shelduck in France.

Antibodies to APMV-1, -2, and -3 were detected in 4.8, 1.7, and 2.6%, respectively, of caged bird samples. The majority of these caged birds were "exotic" or "fancy" poultry breeds. Amongst wild birds, 4.2% had titres to APMV-2 and over half of these were passerine birds; 1.7% of the samples had titres to APMV-1 and 0.8% to APMV-3 antigen. No APMVs or AIVs were isolated from any of the cloacal swabs collected

from these birds. Of the 1778 poultry serum samples tested only five reacted with APMV-3 antigen and these were later found to be cross-reactions to APMV-1. No reactions were detected with APMV-2 antigen. Although, we can be confident that APMV-1 is present in caged birds, wild birds, and poultry of New Zealand, there is no conclusive evidence of the presence of APMV-2 and APMV-3 in poultry or APMV-3 in wild birds. The results also do not provide conclusive evidence for the presence of APMV-2 in wild birds.

Despite New Zealand being free from ND and highly pathogenic avian influenza (HPAI) in commercial poultry and the lack of evidence of pathogenic APMV-1 and AIVs in other birds, a number of possibilities were suggested by which virulent strains of APMV-1 and HPAI viruses could emerge in New Zealand including: (1) introduction by migratory birds; (2) importation of live birds and avian products; and (3) mutation in endemic viruses of low virulence.

The findings from this study and elsewhere emphasise the importance of good biosecurity measures on poultry farms, to prevent the introduction of viruses of low virulence, as well as monitoring for the presence and type of APMV-1 and AIV in wild and domestic birds. The situation is likely to be dynamic with new strains emerging and the occurrence of clinically important introductions is a real possibility.

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Abbreviations

AGID	agar gel immunodifusion test
AIV	avian influenza virus
APMV	avian paramyxovirus
BLAST	basic local alignment search tool
bp	base pair
CPE	cytopathic effect
ELISA	enzyme linked immuno sorbent assay
EM	electron microscope/microscopy
F	fusion (glycoprotein)
FA	fluorescent antibody
HA	haemagglutinin
HI	haemagglutination inhibition test
HN	haemagglutinin neuramidase
HPAI	highly pathogenic avian influenza
ICPI	intracerebral pathogenicity index
IPX	immunoperoxidase
IVPI	intravenous pathogenicity index
L	large polymerase protein
LPAI	low pathogenic avian influenza
M	matrix protein
MAb	monoclonal antibodies
MDCK	canine kidney cell
MDT	mean death time
ML	maximum likelihood (method)
MP	maximum parsimony (method)
Mr	relative molar mass
mRNA	messenger RNA
NA	neuraminidase
ND	Newcastle disease
NDV	Newcastle disease virus

NIT	neuraminidase inhibition test
NJ	neighbour-joining (method)
NP	nucleocapsid protein
NP-B-ELISA	nucleoprotein-blocking ELISA
NS	non-structural proteins
OD	optical density
ORF	open reading frame
P	phosphoprotein
PA	acidic protein
PB	basic proteins
PBS	phosphate buffered saline
PCR	polymerase chain reaction
RBCs	red blood cells
RE	restriction enzyme
RNP	ribonucleoprotein
RT-PCR	reverse transcriptase-PCR
SDS	sodium dodecyl sulphate
SPF	specific pathogen free
ssRNA	single stranded RNA
UV	ultraviolet
Vero	African green monkey (cells)
VNT	virus neutralisation test
vRNA	viral RNA
VV	viscerotropic velogenic

Amino acids

Alanine	Ala, A
Arginine	Arg, R
Asparagine	Asn, N
Aspartic acid	Asp, D
Asparagine or aspartic acid	Asx, B
Cysteine	Cys, C

Glutamine	Gln, Q
Glutamic acid	Glu, E
Glutamine or glutamic acid	Glx, Z
Glycine	Gly, G
Histidine	His, H
Isoleucine	Ile, I
Leucine	Leu, L
Lysine	Lys, K
Methionine	Met, M
Phenyalanine	Phe, F
Proline	Pro, P
Serine	Ser, S
Threonine	Thr, T
Tryptophan	Trp, W
Tyrosine	Tyr, Y
Valine	Val, V

Chapter 1 Literature review

1.1 INTRODUCTION

Avian influenza viruses (AIVs) and avian paramyxoviruses (APMVs) have been of interest to veterinarians and researchers worldwide for decades because of the devastating diseases caused by highly pathogenic avian influenza (HPAI) and Newcastle disease virus (NDV). In addition to the immediate losses, up to 100% mortality within a flock, there is also an economic impact that can occur as an outcome of the restrictions and embargoes placed on countries with these infections (Alexander 1997, 2000a,b, 2001; Easterday et al. 1997; Swayne & Suarez 2000).

Epidemiologists have always had difficulty to provide information on how these viruses spread but most believe that wild birds, particularly waterfowl, have an important role. All possible combinations of influenza type A virus and APMV-1, -2, -4, -6, and -8 have been isolated from waterfowl to date reinforcing the important role of these birds as a reservoir for AIVs and some of the APMVs (Hinshaw et al. 1980b, 1985; Deibel et al. 1985).

1.2 AVIAN INFLUENZA

All influenza viruses belong to the family Orthomyxoviridae and are divided into four genera on the basis of antigenic differences between their nucleocapsid and matrix protein (Cox et al. 2000):

Genus Influenza virus A
Genus Influenza virus B
Genus Influenza virus C
Genus Thogotovirus

For influenza A viruses, a further 15 haemagglutinin (HA) and 9 neuraminidase (NA) subtypes are recognised to date on the basis of the antigenic differences of the glycoproteins. All isolates of AIV belong to type A although these viruses also infect swine, horses, seals, whales, and humans (Murphy & Webster 1996; Cox et al. 2000).

Influenza type B viruses infect only humans and influenza C infects humans and swine. "Thogoto" and "Dhori" are tick-borne viruses transmitted between vertebrates (Murphy & Webster 1996; Cox et al. 2000).

1.2.1 Virion properties of Genus influenza A

Morphology

The virion of influenza A observed under the electron microscope (EM) is spherical and 80–120 or 20–120 nm in diameter. Filamentous forms can also occur with a length of several micrometres. The virion is surrounded by a lipid envelope with very characteristic "spikes" or projections radiating outwards (10–14 nm). Two types of spikes can be recognised: (1) rod-shaped spikes of HA protein; and (2) mushroom-shaped spikes of NA. About 500 spikes project from the surface of a spherical virus in the ratio 4–5 HA:1 NA. The HA and NA proteins are closely associated with matrix protein (M_1) which is located inside the lipid envelope. Within the matrix shell are the ribonucleoprotein (RNP) structures, which have helical symmetry and these are easily observed in disrupted particles and contain eight separate segments of single-stranded (ss) RNA (Lamb & Krug 1996; Cox et al. 2000).

Virion and its gene

Eight ssRNA segments ranging from 2341 to 890 nucleotides comprise the viral genome. The three largest RNA segments (1, 2, and 3) encode the two basic proteins (PB1 and PB2) and the acidic protein (PA) (Lamb & Krug 1996).

The nucleocapsid protein (NP) encoded by RNA segment 5 is the major structural protein to form the RNP and also is one of the type-specific antigens that differentiate A, B, and C influenza viruses. The four proteins—PB1, PB2, PA, and NP—have a role in the assembly of the polymerase complex and subsequent viral transcription (Lamb & Krug 1996). Segment 4 of the RNA codes HA, the surface glycoprotein, which has three major roles in influenza virus replication (Lamb & Krug 1996):

(1) HA binds to a sialic acid-containing receptor on the cell plasma membrane to attach the virus to the cell and initiate the infection;
(2) HA plays a role in the fusion between the virus envelope and the endosomal membrane to consequently release the nucleocapsid to cytoplasm; and
(3) HA is a major viral antigen that provokes the production of neutralising antibody.

Neuraminidase is the second surface glycoprotein of the virus and is coded by RNA segment 6. NA is a minor surface antigen that undergoes antigenic variation. It is composed of a single polypeptide chain and its biological activity is to remove sialic acid from its own glycoprotein, from HA, and from the infected cell surface. This prevents self-aggregation and promotes release from the infected cell. Although the role of NA is still unclear it may be associated with assisting the virus to reach the epithelial cells through the mucin layer in the respiratory tract (Lamb & Krug 1996).

RNA segment 7 is responsible for coding two proteins, the matrix or membrane proteins M_1 = 252 amino acids and M_2 = 97 amino acids. M_1 is the most abundant virion protein and it provides rigidity to the membrane. It is believed to be a multifunctional protein by interacting with cytoplasmic tails of the NA, HA, and M_2 proteins, as well as with RNP structures. M_1 underlies the lipid bilayer and, as a membrane protein, is soluble in chloroform, methanol and 0.5*M* KCl. M_1 protein is type

specific and coding sequences for matrix proteins are highly conserved (Lamb & Krug 1996).

RNA segment 8 is 890 nucleotides in length and encodes for two non-structural (NS) proteins—NS1 = 237 amino acids and NS2 = 21 amino acids (Lamb & Krug 1996). The function of NS1 has been identified; it regulates the nuclear export of mRNA and inhibits pre-mRNA splicing (Krug 1998). NS proteins are abundant in influenza virus-infected cells and NS2 is thought to occur in virions, where they may be associated with M_1 but their function is still unknown (Lamb & Krug 1996; Krug 1998).

Physicochemical and physical properties

Virions are very sensitive to heat and at 56°C and pH <5 infectivity is reduced within minutes. Lipid solvents and detergent (anionic, cationic, and neutral) also substantially reduce infectivity. Exposure of the virus to UV waves, gamma irradiation, or treatment with formaldehyde or β-propiolactane inactivates the virus without affecting antigenicity (Easterday et al. 1997).

AIV replication

The replication of AIV begins from the moment of attachment of the HA molecule to sialic acid present on the cell surface glycoproteins or glycolipids. AIV enters cells by receptor-mediated endocytosis, into an endosomal vesicle. The uncoating of the influenza virion in endosomes is dependent on pH. The reduction of the pH to about 5 allows the transition of cleaved HA into its low pH form and the transcription complex is released into the cytoplasm (Lamb & Kolakofsky 1996; Hay 1998).

In infected cells the viral RNA is transcribed into messenger RNA (mRNA) initiated by a host-cell primer, specifically by 5′ capped RNA fragments derived from newly synthesised host cell RNA polymerase 2 transcripts by viral endonuclease activity associated with the viral PB2 protein. The mRNA chains are then elongated until 15–22 nucleotides before the 5′ ends of the viral RNA, where transcription is terminated and polyadenylate is added to the mRNA (Hay 1998).

Two steps are involved in replication of the viral RNA: (1) synthesis of template RNAs that are full copies of the viral RNA; and (2) copying of the template RNAs into viral RNA. Both replication steps occur without the primer or polyadenylation and all occur in the nucleus (Shapiro et al. 1987; Shapiro & Krug 1988).

After transcription and replication, RNPs are released back to the cytoplasm with the association of M_1 protein required for the transport of RNPs (Martin & Helenius 1991). New RNPs are assembled, with the association of HA, NA, and M_2 synthesised in the endoplasmic reticulum. After assembly RNPs are transported through the Golgi apparatus to a region of plasma membrane (Kornfeld & Kornfeld 1985). HA and NA associated with the plasma membrane as glycoprotein spikes incorporate matrix protein and RNPs from the cytoplasm and the fully assembled virion is released through budding of the cytoplasmic membrane with the association of NA activities (Lamb & Krug 1996).

1.2.2 Epizootiology

Wild birds—natural reservoirs

The occurrence of AIV in wild birds was initially documented in 1961 during an investigation of common terns (*Sterna hirundo*) in South Africa (Becker 1966). However, it was not until the mid 1970s when systematic investigations in wild birds revealed the enormous pool of AIV present in these bird populations.

Waterfowl, particularly wild ducks, are known to be infected with AIV without showing any evidence of disease and they shed the virus in their faeces (Slemons et al. 1974, 1991; Webster et al. 1976; Hinshaw et al. 1978, 1980, 1985; Devaux 1979; Deibel et al. 1985; Graves 1992). All of the nine different NA subtypes and the 15 HA subtypes of AIV have been isolated from wild ducks and other aquatic birds throughout the world including: Australia (MacKenzie et al. 1984), North America (Slemons et al.

1974; Hinshaw et al. 1980b), China (Shortridge et al. 1977; Shortridge 1982), Japan (Yamane et al. 1979), and Europe (Hannoun & Devaux 1980; Ottis & Bachmann 1980).

Potentially all orders of aquatic birds may serve as reservoirs of influenza viruses and they have been isolated from members of the orders: Procellariiformes (shearwaters), Peledaniformes (cormorants), Anseriformes (swans, geese, ducks), Galliformes (turkeys, quail), Ciconiiformes (herons, ibis), Gruiformes (rails, coots), Gaviformes, Passeriformes (starling, myna), Charadriiformes (gulls, turnstones), Columbiformes (pigeons), Podicepediformes, Procellariformes, Pelecaniformes, and Piciformes (Stallknecht & Shane 1988). No isolates have been obtained from Psittaciformes (parrots) or the other orders of birds. However, a wide variety of avian species in laboratory experiments, or investigations of captive birds in quarantine, have been found susceptible to influenza infection and a number of influenza isolates have been made (Alexander 1982b; Senne et al. 1983).

In wild ducks, which are the most extensively studied aquatic bird species for influenza, there is a considerable variation in the frequency of influenza virus isolation and a number of factors may influence this, including: species, age of the bird, time of year, and proximity to migration routes (Hinshaw et al. 1980b; Deibel et al. 1985; Graves 1992). Studies in the Northern Hemisphere have found that the highest prevalence of influenza viruses was in August and September and juvenile birds were the most commonly infected as they congregate in marshalling areas. For example, in Canada, before migration up to 30% of juvenile birds could shed the virus (Hinshaw et al. 1980b). The proportion of birds shedding decreases with time and the number of isolates fell to 1.6–2% in November in the lower Mississippi (Webster et al. 1976) and in December and January fell to 0.4% in Louisiana (Stallknecht et al. 1990c). None of the birds shedding AIVs showed any clinical signs associated with infection.

Caged birds

The first AIV isolates from caged birds were reported after 1975 when a number of countries imposed quarantine restrictions on imported birds including North America, Europe, and Japan (Alexander 1981). The majority of AIV, with H4 and H3 subtypes,

were from passerine species held in quarantine and only rarely from psittacine species (Alexander 1981; Rigby et al. 1981; Senne et al. 1983). The number of AIV isolates obtained from birds in quarantine between years varied, as observed by D. J. Alexander during the period 1975–98 in Great Britain, and there were years when no AIV isolations were made (Alexander 2000c).

Poultry

Chickens and turkeys are not regarded as natural host species for AIVs and this was studied in two independent surveys of wild turkeys in which no serological evidence of AIV infection was found (Davidson et al. 1988; Hopkins et al. 1990). No similar study has been conducted on the ancestor of the modern chicken (red jungle fowl), but it is thought unlikely that this species has a role in maintaining AIV (Swayne & Suarez 2000).

Influenza in chickens has been relatively rare in comparison to the infection of domestic turkeys or ducks, but 12 of the 17 primary outbreaks of HPAI since 1955, when fowl plague was recognised as being caused by an influenza virus (Alexander et al. 1986), were in chickens (Swayne & Suarez 2000).

The most devastating outbreak in chickens was in Pennsylvania, United States, in 1983–84 caused by AIV H5N2 subtype, which resulted in the slaughtering of 17 million chickens (Eckroade & Silverman 1986; Fichtner 1986). The outbreaks began in April 1983 and were associated with low mortality and isolation of low pathogenic H5N2 (Eckroade & Silverman 1986), but it was not until October 1983 that the HPAI virus was isolated in association with clinical signs of classical HPAI and high mortality (Fichtner 1986). Similar H5N2 AIV to that which caused the Pennsylvania outbreak reappeared in five north-eastern states of the United States in 1986 (Garnet 1986) and all of which were connected to the movement of poultry from live bird markets to New York City.

In Pakistan, an epizootic affecting 2.2 million birds, began in December 1994, in a wintering area for migratory birds and spread to 156 of 286 farms in a 100 km radius

(Naeem 1998). HPAI virus subtype H7N3, which caused high mortality, was isolated from affected birds.

The outbreak in Mexico had a very similar epidemiology to the outbreak in Pennsylvania 1983 and began with the isolation of a low pathogenic AIV subtype H5N2 in May 1994 during an investigation of respiratory disease in chicken flocks (Villarearl & Flores 1998). The low pathogenic virus circulated in commercial chicken flocks of 11 Mexican states until January 1995 when HPAI virus of the H5N2 subtype was isolated from birds in association with high mortality and lesions typical for HPAI (Swayne et al. 1997; Villarearl & Flores 1998).

Australia has experienced five outbreaks of HPAI in commercial chickens between 1975 and 2001: two outbreaks caused by HPAI virus subtypes H7N7 in 1976 (Turner 1976) and 1985 (Barr et al. 1986) in Victoria; followed by an outbreak in 1992 (Selleck et al. 1997) also in Victoria; in 1995 in Queensland caused by the H7N3 subtype (Westbury 1998); and the last in 1997 in New South Wales caused by the H7N4 subtype. In each case the disease outbreak was limited (Westbury 1998).

Eight outbreaks caused by the HPAI virus subtype N5N2 in mixed backyard poultry flocks were reported in Italy in 1997–98 (Capua et al. 1999). There is no explanation of the first outbreak but the next five were linked to live bird purchases from the first or second outbreak and five of the eight outbreaks occurred in open areas accessible to migratory waterfowl (Capua et al. 1999).

In Italy, from 1999 to 2000 there was another outbreak in commercial poultry affecting chickens and turkeys caused by HPAI AIV virus subtype H7N1 (Capua et al. 2000). This outbreak had a similar epidemiology to the Pennsylvania 1983 (Ekroade & Silverman 1986) and Mexico 1995 (Villarearl & Flores 1998) outbreaks in which low pathogenic avian influenza (LPAI) virus circulating in the commercial poultry population of northern Italy beginning March through to December 1999 (Capua et al. 2000), became HPAI virus as confirmed on 17 December 1999. Infection spread to most commercial poultry causing the death of over 13 million birds (Capua et al. 2000).

During March–May 1997 in Hong Kong, HPAI virus subtype H5N1 caused outbreaks initially in three chicken farms, with mortality up to 100%, spreading to the live bird market in Hong Kong (Claas et al. 1998). On 21 May AIV subtype H5N1 was isolated from a 3-year-old boy (Claas et al. 1998) forcing the authorities in Hong Kong to depopulate the entire chicken population of c. 1 million.

Since 1963, when Wells (1963) isolated AIV from turkeys for the first time, most countries rearing turkeys have had problems due to AIV infection. In the United States at least two patterns of AIV infection have been observed. In the states where turkey farms are situated on migratory flyways, e.g., Minnesota, influenza infection has been recorded every year since 1966 (Pomeroy 1981; Halvorson et al. 1998). In other states the outbreaks have been very sporadic.

In Canada, between 1964 and 1971, 63 outbreaks were reported in Ontario in turkey farms as yearly events (Lang 1981). The situation changed when biosecurity in farms improved to prevent introduction of virus from wild birds and as a result of this only six outbreaks were recorded between 1971 and 1981 (Lang 1981). Most of the infections were due to LPAI with various AIV subtypes (H4, H5, H6, H8, and H9) involved and HPIA virus infections have been rarely reported. Of the 17 reported HPIA outbreaks in poultry since 1955 only five have been primarily from turkeys (Swayne & Suarez 2000), which includes three in United Kingdom, one in Ireland, and one in Canada with AIV H5 and H7 subtypes (Alexander 2000c). The latest reported outbreak in Italy in 1999–2000, killed 2.2 million turkeys, although the majority of birds affected were chickens (Capua et al. 2000).

No disease outbreaks have been recorded in commercial duck farms to date and the behaviour of influenza virus in these birds is not clear (Alexander 2000c). However, various AIV subtypes have been isolated in limited surveys either from live ducks or ducks at slaughter (Alexander 1981; Shortridge 1982) including HPAI virus subtype H5N8 from commercial ducks in Ireland (Alexander 2000c).

There have been a number of reports of AIV isolation from ratites and the first record of the isolation of AIV subtype H7N1 was in South Africa in 1991 from young

ostriches and was associated with high mortality (Allwright et al. 1993). In 1994, AIV subtype H5N9 was isolated from ostriches in South Africa and from emus and cassowaries in the Netherlands during routine export testing (Alexander 2000c). In 1995 and 1996 in Zimbabwe, the H5N2 AIV subtype was isolated from ostriches (Alexander 2000c) as well as from ostriches imported to Denmark and the Netherlands in 1996 (Jorgensen et al. 1998). In the United States, there were reports of the isolation of AIV from rheas and emus with various AIV subtypes between 1992 and 1996. These include: H3N2, H4N2, H5N2, H7N1, H7N3, H5N9, H10N4, and H10N7 (Panigrahy & Senne 1998). All these viruses were of low pathogenicity for chickens.

Other poultry from which AIV isolations have been made are muscovy ducks (*Carinia moschata*), pheasants (*Phasianus* spp.), Japanese quail (*Coturnix coturnix japonica*), chukars (*Alectories chukar*), guinea fowl (*Numida meleagris*), and various types of goose (Alexander 1993b, 2000c). In general, the viruses were isolated during epizootics or enzootics in other commercial poultry.

Transmission

The transmission of AIVs is not fully understood and may vary with the strain of the virus, the species of bird (age and health), and environmental factors. AIV is excreted from the respiratory tract, conjunctiva, and in faeces and is likely to be transmitted by direct contact between infected and susceptible birds and indirectly through exposure to aerosol or fomites contaminated with the AIV (Alexander 1993a; Ritchie 1995a; Easterday et al. 1997). A number of experiments were carried out (mainly in poultry) to assess the transmissibility of AIV and to observe the behaviours of different isolates of AIV with different pathogenicity on different bird species (Alexander et al. 1978, 1986).

AIV is transmitted horizontally and there is no evidence that the virus is transmitted vertically (Easterday et al. 1997). However, it should be noted that AIV was isolated from the surface of an egg laid by a hen that was exhibiting clinical signs (Cappucci et

al. 1985) and from most eggs laid 3 and 4 days after experimental infection with the Pennsylvania H5N2 strain (Beard et al.1984).

Persistence and perpetuation of influenza in nature

Influenza viruses replicate in wild ducks, predominantly in the cells lining the intestinal tract, without causing any signs of disease and are excreted with the faeces into lake water. The quantities of the excreted virus can be so high that the virus can be isolated from lake water (Hinshaw et al. 1979) for up to 4 days at 22°C and over 30 days at 0°C (Webster et al. 1978). Depending on the virus strain, pH, water salinity, and temperature, it has been estimated that infectivity of the virus could persist for up to 207 days at 17°C and 102 days at 28°C (Stallknecht et al. 1990b).

Therefore the virus may be passed to other susceptible birds through lake or drinking water via the oral or cloacal route. Transmission through the faeces also provides the possibility of spreading the viruses to wild and domestic birds during migration. It is not fully understood how influenza viruses are maintained in the duck population from year to year although there are a number of theories to explain this. The virus can be shed by a duck for up to 30 days after primary infection (Hinshaw et al. 1980a) and therefore the virus could be maintained through serial passages in the population over the winter. Consistent with this theory, Markwell & Shortridge (1982) isolated influenza virus of the same subtypes from domestic duck faeces or pond water in a duck farm every month for up to 2 years. In addition, antibodies to AIV are very weak and short-lived and ducks can be readily infected with the same virus within 2 months of the initial infection (Hinshaw et al. 1980a; Kida et. al. 1980). The virus may remain viable in the frozen lakes over winter until birds return even if the virus could not be isolated from the ice of lakes in the winter (Ito et al. 1995).

There have been an number of influenza isolates obtained from sea birds including gulls, terns, shearwaters, guilemonts, sandpipers, and ruddy turnstones, representing most of the different HA and NA subtypes (Becker 1966; Hinshaw & Webster 1982). The majority of the isolates were non-pathogenic in chickens and ducks but a H5N3

isolate from a South African tern (Becker 1996) was highly pathogenic for domestic poultry. The influenza gene pool in shorebirds overlaps to some extent but not completely with that in ducks (Kawaoka et al. 1988; Sharp et al. 1993), but studies have shown that half of the influenza isolates from shorebirds will infect ducks (Kawaoka et al. 1990).

1.2.3 Infection and disease

Clinical signs, gross lesions, and histological lesions vary depending on species, age of host, presence of secondary infection with other organisms, and environmental factors.

Strains of influenza virus can be grouped into three categories on the basis of the severity of the disease caused following infection in chickens and turkeys (the most commonly affected species) (Alexander 1993a):

(1) High pathogenicity—characterised by high mortality up to 100%;
(2) Moderate pathogenicity—with high morbidity and mortality occasionally as high as 50–70%; and
(3) Low pathogenicity—with inapparent disease or mild respiratory signs, depression, drop in egg production.

However, most isolates from the field practically fall into two groups after laboratory pathogenicity assessment: highly pathogenic or low virulent isolates which include those producing mild disease or none (Alexander 1993a).

Highly pathogenic avian influenza viruses

HPAI is the result of systemic replication of the virus and cell disruption in a variety of visceral organs, brain, and skin, whereas low virulent viruses in general replicate locally, predominately in the respiratory and alimentary tracts (Swayne & Suarez 2000).

Chickens and turkeys affected by HPAI die suddenly and generally more clinical signs can be observed in longer surviving birds. In those birds examined over more than 48 h, a series of clinical signs can be seen: sudden onset of high mortality; drop of egg production in breeders and laying chickens (to zero after 3–5 days); respiratory signs, sinusitis, excessive lachrymation; oedema of the head, face, and neck; cyanosis of unfeathered skin; and nervous signs (e.g., torticollis, paresis, paralysis, convulsions) (Barr et al. 1986; Alexander 1993a; Hooper et al. 1995; Kobayashi et al. 1996; Mo et al. 1997; Swayne & Suarez 2000).

Ducks are refractory to disease caused by HPAI viruses and usually no deaths or clinical signs are observed (Slemons & Easterday 1972; Alexander et al. 1986; Alexander 1993a). However, death and clinical signs such as swelling of the eyes, inflammation, depression, and partial paralysis has been observed in ducks after experimental inoculation of A/chicken/German/34 virus (Alexander et al. 1978).

There has been only one report of disease in wild birds associated with HPAI virus—in South Africa where AIV H5N3 virus was found to cause death in terns (Becker 1967). In experimental infection, Narayan et al. (1969), found geese and pigeons to be resistant to HPAI virus A/turkey/7732/66. Slemon & Easterday (1972), using the same virus, showed that pheasants were resistant to infection but 3/20 quails (*Coturnix coturnix japonica*) died and 1/19 pigeons had clinical signs. Torticollis was observed in quails and depression in pigeons in later experiments.

Influenza viruses other than HPAI

The majority of influenza viruses isolated from poultry are of low virulence. However, there have been a number of reports of disease with different clinical signs caused by some of these viruses.

Alexander & Spackman (1981) reported 2% white-shelled eggs as the only clinical sign. Johnson et al. (1977) reported an outbreak of disease in chickens in Alabama, United States, with up to 69% mortality. Other clinical signs associated with this outbreak were mild to severe respiratory disease, depression, anorexia, sinusitis, and a

drop in egg production with low fertility and hatchability. Complicating factors acting synergistically such as concurrent infection, use of live vaccine, environmental stress, and compromised immune system contribute to the severity of disease (Samadieh & Bankowski 1970; Newman et al. 1981). Similar conclusions were made by Homme & Easterday (1970) in their experimental inoculation of ducks, geese, and pheasants with a low virulence strain, suggesting that complicating factors are required to produce disease.

Before the outbreak in Pennsylvania in 1983, AIVs circulating in poultry caused disease varying from inapparent to respiratory disease, up to 15% mortality, and drop in egg production (Eckroade & Silverman 1986).

The majority of AIVs have been isolated from apparently healthy wild birds trapped or killed by hunters, mainly associated with surveillance programs, with no disease signs reported (Alexander 1993a).

AIVs obtained from captive psittacine birds were usually obtained after sudden death or following an acute onset of depression, green diarrhoea, and nervous signs. Other infected birds may develop a 2-week course of lethargy, upper respiratory disease, and neurological signs including ataxia and torticollis (Alexander 1993a; Ritchie 1995a).

Gross lesions

Gross lesions, as well as clinical signs, depend on a number of factors including: virus strain, species, age, and very importantly, the duration of infection (Allan et al. 1977; Easterday et al. 1997; Hooper et al. 1995) as well as whether they were from naturally occurring or experimental infection.

With HPAI viruses, the gross lesions can be absent if death occurs up to 2 days after infection (Hooper & Selleck 1998) but some strains such as the A/chicken/HongKong/220/97 (H5N2) and A/chicken/Italy/330/97 (H5N2) have caused lesions including severe lung congestion, haemorrhage, and oedema in chickens (Suarez et al. 1998; Capua et al. 1999). During the acute stage of infection, lasting 3–5

days, chickens may have ruffled feathers, congestion and or cyanosis of the comb and wattles and swollen heads, general congestion and haemorrhages (Acland et al. 1984; Kobayashi et al. 1996; Swayne 1997; Hooper & Selleck 1998). Primary lymphoid organs are usually severely atrophic and the pancreas may have blotchy light-yellow and dark-red areas (Hooper & Selleck 1998).

Gross lesions reported in chickens infected with low virulent AIV include respiratory lesions, sometimes with tracheal oedema and caseous tracheal exudate (Eckroade & Silverman 1986). Halvorson et al. (1980) reported that the most obvious lesions in infected chickens were swollen kidneys with visceral urate deposits (Halvorson et al. 1980).

Histopathology

Histopathology of AI infection has not played a major role in the diagnosis and/or study of the disease particularly in chickens and turkeys because of lack of consistency in changes caused by different HPAI viruses. Although there are some similarities, there are also differences, and the characteristic lesions are listed for different viruses: multifocal lymphoid necrosis was characteristic for infection with turkey/Ontario/7732/66; pancreatic necrosis for turkey/Ontario/6213/66 and chicken/Penn/83 viruses; myocarditis for tern/S.Africa/61, turkey/Ontario/7732/66, and chicken/Penn/83 viruses; skeletal muscle, brain, and comb lesions were observed in infection with chicken/Penn/83 and tern/S.Africa/61 but infection with tern/S.Africa/61 lacked pancreatic lesions (Easterday et al. 1997).

1.2.4 Disease diagnosis

Clinical signs, gross and microscopic lesions have a role in the diagnosis of AI infection, although none of the signs/findings can be considered pathognomonic. Therefore, the diagnosis of the disease relies on virus isolation and a demonstration of the virulence of the isolate for an appropriate host (Alexander 2000a). However, there are a number of laboratory techniques available, which can be used in conjunction with

virus isolation in order to supplement diagnosis and these include serological (e.g., haemagglutination inhibition (HI), agar get immunodiffusion (AGID), enzyme linked immunosorbent assay (ELISA) and viral antigen/RNA detection (e.g., fluorescent antibody (FA), immunoperoxidase (IPX), ELISA, molecular based techniques).

Virus isolation

The preferred samples from dead birds, such as intestine/intestinal contents (faeces) or cloacal and oral-nasal swabs, should be collected. In addition, samples from trachea, lungs, air sacs, spleen, kidney, brain, liver, and heart may also be collected. From live birds, tracheal and cloacal swabs should collected. From small birds, fresh faeces may be collected instead so as not to harm the bird (Alexander 2000a).

Samples should be placed in phosphate buffered saline (PBS) at pH 7.0–7.4 (or similar buffer) with antibiotic mixture, to suppress bacterial growth, and after processing (which includes grinding/mincing and centrifugation of samples) can be inoculated into a variety of hosts including specific pathogen free (SPF) embryonated fowl eggs (9- to 11-day-old), primary chicken fibroblast and liver cell cultures, and cell lines such as canine kidney cell (MDCK). When using cells, host trypsin is required to cleave the HA glycoprotein for the production of the infectious virus (Beard 1989; Easterday et al. 1997; Alexander 2000a). Embryonated eggs are regarded as the most sensitive host for primary AIV isolation and the procedure involves incubation of inoculated embryonated eggs for 4–7 days at 35–37°C. Negative allantoic-amniotic fluid (as tested by HA) harvested from the first passage should be inoculated once more (Beard 1989; Easterday et al. 1997; Alexander 2000a).

Direct detection of antigen/RNA

Direct detection of AIV antigen is not routinely used particularly for initial diagnosis at present, however direct immunofluorescence as a screening test has been used successfully on impression smears of organs and tissues (McNulty & McFerran 1986). There are also reports of this test used in outbreaks of HPAI in Pennsylvania 1983 (Easterday et al. 1997) and in Australia (P. Selleck pers. comm.).

A variety of antigen detection ELISAs have been described (Siebinga & de Boer 1988; Kodihalli et al. 1993; Davison et al. 1998) and Stanislawek et al. (2002) used these for the diagnosis of AI. Although the results using these ELISAs can be obtained in a very short time most of such ELISAs lack sensitivity and do not detect the virus if present at low titre.

Immunoperoxidase (IPX)-based tests have a similar drawback (Campen et al. 1989; Hooper et al. 1995) and, although they can be very specific by using monoclonal antibodies, only tissues containing high titres of virus will provide conclusive diagnosis. Additionally, none of the above tests will differentiate AIV by HA or NA and will not provide information on virus virulence.

The detection of viral RNA in infected cells using *in situ* hybridisation has been performed with moderate sensitivity (Feldmann et al. 2000) but this is a cumbersome technique with no wide application.

In contrast, reverse transcriptase-polymerase chain reaction (RT-PCR) has been found to be very useful not only for detection of virus RNA but for subtyping and pathotyping the influenza virus infection in clinical samples or after growth in embryonated egg fluids (Starick et al. 2000; Lee et al. 2001; Munch et al. 2001).

Hofmann et al. (2001) recently developed a RT-PCR that amplifies all eight segments of the viral RNA of AIV by designing primers with complementary sequences to the conserved vRNA-termini of the 15 HA and 9 NA known AIV subtypes.

Serology

Serological tests provide important information for disease diagnosis and epidemiological investigations. The most common tests used to demonstrate the presence of antibodies in birds are AGID, HI, and various types of ELISAs. To a lesser degree other tests such as the virus neutralisation test (VNT), neuraminidase inhibition

test, and single radial haemolysis may be used in special circumstances (Easterday et al. 1997).

The AGID test is used to detect antibodies to both the nucleoprotein and matrix AI viral proteins because all influenza A viruses have antigenically similar NP and M antigens (Beard 1970). It is also relatively simple to perform and does not require sophisticated equipment. However, AGID is less sensitive than, e.g., ELISA (Swayne & Suarez 2000). Antibodies can be detected readily by the AGID test in chickens, turkeys, and pheasants (Easterday et al. 1997) to all known AIV subtypes but there are number of reports that such antibodies are absent or not detectable by AGID in ducks (Slemons & Easterday 1972; Hinshaw et al. 1980a; Alexander et al. 1981). The persistence of type A NP/M antibodies may vary between species and in turkeys could be detectable for several months (Alexander & Allan 1982).

The HI test is routinely used in circumstances when a known H subtype is circulating in the field or when there is interest to determine certain H subtype antibodies (e.g., H5 and H7) from a trade point of view. It is similar to the AGID test in that the HI test is able to detect antibodies in various avian species except wild ducks where antibodies cannot always be detected (Kida et al. 1980; Austin & Hinshaw 1984; Ritchie 1995a; Easterday et al. 1997). In a study of domestic Peking ducks used as sentinels, there was a serological response to natural infection to a number of AIV subtypes and a rise in antibodies was detected 4 weeks after virus isolation. The HI titres were low and declined to insignificant levels within 4–8 weeks (Suss et al. 1994).

A number of different types of ELISAs have been developed including direct ELISAs for chickens (Snyder et al. 1985; Meulemans et al. 1987) and competitive ELISAs for all bird species (Boer et al. 1990; Zhou et al. 1998; Stanislawek et al. 2002). Both tests detect antibodies against nucleoprotein and, in general, ELISAs have greater sensitivity than AGID and HI tests. A further advantage of the competitive ELISAs is that antibodies to any AIV type A subtypes can be detected and sera from almost any bird or mammal species can be tested.

1.2.5 Assessment of pathogenicity

The most widely accepted criteria for classifying AIVs as highly pathogenic or not pathogenic are defined by the Office International des Epizooties (OIE) in its manual (Alexander 2000a) and contain biological and *in vivo* procedures for AIV assessment.

In biological tests, an AIV isolate that is lethal for six or more of eight, 4- to 8-week-old susceptible chickens within 10 days following intravenous inoculation of infective allantoic fluid, is regarded as HPAI.

For those isolates that kill from 1 to 5 chickens and are not H5 or H7 subtypes, inoculation of primary chicken embryo cells or MDCK cells is required to assess them for cytopathic effect (CPE) or plaque formation. Only HPAI viruses grow without trypsin, producing CPE or plaques whereas avirulent viruses require trypsin to be added to the culture to produce CPE or plaques (Bosch et al. 1979). For these AIVs that grow in tissue culture without trypsin and for all H5 and H7 isolates of low virulence, the amino acid sequences of the cleavage site of the HA gene must be determined.

The European Union Directive is similar and states that HPAI is: "an infection of poultry caused by an influenza A virus that has an intravenous pathogenicity index in 6-week-old chickens >1.2 or any infection with influenza A viruses of H5N7 subtype for which nucleotide sequencing has demonstrated the presence of multiple basic amino acids at the cleavage site of the haemagglutinin" (OIE 2000).

HA cleavage site and pathogenicity

The pathogenicity of AIVs has been shown to be associated on a molecular level with the presence of multiple basic amino acids at the cleavage site of the HA glycoprotein (Bosch et al. 1981). The minimum sequence motif for highly pathogenic viruses is B-X-B-R, where B = basic amino acids arginine or lysine, X = non-basic amino acid, and R = arginine (Vey et al. 1992; Horimoto et al. 1994; Senne et al. 1996).

HA, which is synthesised as a precursor molecule HA0, is activated by post-translational cleavage by host proteases into the subunits HA1 and HA2 to obtain its full biological properties (Rott 1992).

The cleavage activation of HA glycoprotein is controlled by specific cellular proteases (Bosch et al. 1979), which for non-pathogenic AIVs with only a single basic amino acid (arginine) at the HA cleavage site, is recognised by trypsin-like protease presumably present in cells of the respiratory and intestinal tracts. In HPAI viruses that contain a series of basic amino acids at the cleavage sites of the HA, cleavage is activated by intracellular subtilisin-like endoproteases (e.g., furin) so the virus can replicate widely in many tissues throughout the infected host resulting in severe disease and death (Rott 1992; Stieneke-Grober et al. 1992; Walker et al. 1992). Therefore, sequences of multiple basic amino acids at the cleavage site of HA determine pathogenic properties of the virus.

In addition to the proteolytic cleavage site of the HA, structural features such as the presence of carbohydrate near the receptor-binding site has been shown to affect virulence (Perdue et al. 1995).

The role of the other seven genes in AIV virulence is not fully understood, however AIVs, like other viruses, must exhibit a genome constellation that permits optimal virus reproduction in a host (Rott 1992). Therefore, we should be cautious with assessing pathogenicity exclusively on the changes in HA and the idea of the "constellation hypothesis" specifying pathogenicity (Rott 1992; Perdue et al. 2000) inspires us to also look at other AIV genes to have a full picture.

1.2.6 Economic losses

In the last few decades AIV has caused considerable economic losses in domestic poultry. The best example to illustrate this is the HPAI H5N2 virus outbreak in Pennsylvania, United States in 1983–84. The United States Federal Government spent US$60 million to eradicate AI, which included US$40 million for indemnities and US$20 million for other costs (Lasley 1987). In addition to the cost to the Government,

individual producers suffered direct losses of US$55 million, but the consumers paid an estimated bill of US$349 million to cover the increased cost of eggs because of production lost in the quarantine area (Lasley 1987). In Minnesota, United States, in 1978 more than 140 turkey flocks were infected with LPAI virus resulting in estimated losses of c. US$4 million (Poss et al. 1981). The estimated eradication cost of the outbreak of HPAI in 1985, Bendigo, Australia was over AU$2 million. Additionally, estimated losses of interstate orders and consumer resistance (such as perceived human disease risk and/or reaction against slaughtering the poultry) was c. AU$600,000 per week (Cross 1987).

1.2.7 Prevention, control, and eradication

International trade agreements and standardisation of sanitary health requirements impose on most countries (members of OIE) procedures involving the movement of birds and avian products in event of AIV infection (OIE 2001). The approach to AIV infection depends on the pathogenicity of the AIVs, types of birds infected, distribution of infected birds, requirements of domestic and international markets, and economic status of the country (Swayne & Suarez 2000). When moderately pathogenic AIV is involved, the control programme usually deals with the reduction in incidence to an economically manageable level. However, to manage the incidence of HPAI, eradication is the only option to comply with the OIE List A diseases and trading partner satisfaction (Easterday et al. 1997; Swayne & Suarez 2000).

In a prevention programme, good management practices and biosecurity are the most important factors to stop AIV entering susceptible birds (Easterday et al. 1997; Swayne & Suarez 2000). Of course, for people making decisions or working directly on farms this cannot be done without understanding how the virus is transmitted and what is the potential source of the virus. Both of these topics have been discussed in paragraphs above.

For effective control and eradication of moderate AIV and HPAI, in addition to good management practices and biosecurity, the program should also incorporate other

elements including: diagnostics, surveillance, education, quarantine, depopulation, and vaccines (Swayne & Suarez 2000).

Diagnosis and surveillance are the first actions in an outbreak situation because rapid diagnosis and characterisation of the virus is crucial in decision-making leading to elimination of the disease before the virus spreads into susceptible birds in other areas. However, the spread of the virus has to be determined through comprehensive surveillance in commercial and backyard poultry, migratory and other wild birds providing data for the response and building confidence of the international trading partners. A good example of well managed HPAI outbreaks are five limited outbreaks in Australia (Westbury 1998), where rapid diagnosis and quick action of the Government resulted in eradication of the disease early in the outbreak and a prompt return to the country's disease classification that existed before the outbreak.

Biosecurity plays an important role in the prevention and control of outbreaks and we can distinguish two types: containment of AIV on the infected farm; and prevention of the introduction of AIV to susceptible birds (Swayne & Suarez 2000). This must be followed at all levels with the poultry industry and other groups involved in the outbreak and include proper disinfection of poultry waste and equipment and changes of clothes by people working on the farm to avoid them spreading the virus on fomites.

Information concerning the disease and the control program must be provided to everyone involved including farm workers, veterinarians, government regulatory authorities, and the media. The media have an additional role in the control of AIV by providing information to the public concerning the low potential for transmission of AIV to people through consumption of poultry products (Poss et al. 1987; Swayne & Suarez 2000).

Quarantine imposed in a farm or a region by the controlled movement of people, equipment, and poultry is essential to prevent the spread of AIV to new farms or regions. Dead birds should be incinerated, composted or disposed of in other ways to comply with biosecurity and environmental standards. Equipment should be disinfected before removal from the farm. Cleaning and disinfection of the buildings after

depopulation is critical and a rest period of at least 3-4 weeks is required before re-population (Poss et al. 1987; Alexander 1993a; Swayne & Suarez 2000).

"Stamping-out" or a slaughter policy is the most commonly used procedure in the event of an outbreak of HPAI and it is also the recommended procedure by the OIE (Alexander 1998; Westbury 1998; OIE 2001).

Various vaccines were available and tested in chickens and turkeys in the past (Brugh et al. 1979; Brugh & Stone 1986). However, vaccination was not used for the control and eradication of HPAI until 1995 in Mexico and Pakistan (Swayne & Suarez 2000). The most common vaccine tested was inactivated vaccine administered in oil emulsion by injection. Live non-pathogenic AIV strains with appropriate HA also have been considered in the past (Beard & Easterday 1973; Butterfield & Campbell 1978) because they have great economic and application advantages over inactivated vaccine. However, the possibility of reassortment with AIVs present in poultry and the creation of highly pathogenic strains make such vaccines unsatisfactory. AI inactivated oil emulsion vaccines from field studies have shown that these vaccines do not provide full protection, although they reduce disease signs and virus shedding (Halvorson et al. 1986; McCapes & Bankowski 1986). Additionally, inactivated vaccines can interfere with serological surveillance, because it is not possible to distinguish between antibodies produced by the vaccine or field AIVs. This problem has to some degree been overcome by genetically engineered vaccines, where the HA gene was expressed in vaccinia virus (Sutter et al. 1994), fowl poxvirus (Taylor et al. 1988; Beard et al. 1991; Boyle et al. 2000) or retroviruses (Hunt et al. 1988). Robinson et al. (1993) directly inoculated H7 HA-expressing DNA and achieved some protection in vaccinated birds (Robinson et al. 1993). This technology, although it can overcome some of the problems associated with AI inactivated vaccines, does not provide a product with characteristics of an "ideal" vaccine.

In general vaccines have a role in the control and eradication of HPAI as one of the response elements but not as a stand-alone practice.

1.2.8 Antigenic drift and shift

Antigenic variation of influenza viruses can occur in two ways—as a drift and as a shift (Easterday et al. 1997).

Antigenic drift is a minor antigenic change in the HA and/or NA protein due to accumulation of point mutations in the viral proteins. AIVs have the lowest evolutionary rate of these changes in comparison to human influenza viruses, which have the highest and swine and equine influenza A viruses, which have a medium rate of evolutionary change (Webster et al. 1992; Murphy & Webster 1996). Bean et al. (1992) in their study found that the human pandemic strain of 1968 had diverged from the progenitor accumulating approximately 7.9 nucleotides and 3.4 amino acid substitutions per year. In the equine 2 influenza A virus, mutation has been estimated to be 3.1 nucleotides and 0.8 amino acids per year in contrast to avian viruses, which have much less variation and some have not changed for 50 years (Kida et al. 1987; Bean et al. 1992; Murphy & Webster 1996). A variety of reasons could contribute to this situation including the lack of immunological pressure and the short life of birds (Murphy & Webster 1996; Easterday et al. 1997).

Simultaneous infection of a host animal with two or more influenza viruses creates the possibility of genetic reassortment and the generation of new viruses with very distinct biological properties (Webster et al. 1992; Easterday et al. 1997). This is possible because of the segmented nature of the genome yielding potentially 256 genetically different viruses (Easterday et al. 1997). Mixed infection with reassortment in birds particular in waterfowl (ducks) occurs readily (Desselberger et al. 1978; Scholtissek et al. 1978; Hinshaw et al. 1980a) and those involving exchanges of HA and NA proteins detectable by antigenic analysis are regarded as antigenic shift (Easterday et al. 1997). This also could explain how viruses with any possible combination of antigenic subtypes have been isolated from waterfowl.

There is evidence that AIVs play an important role in the creation of human pandemic strains through reassortment, including Asian H2N21957 and Hong Kong H3N2 strain 1968 (Gething et al. 1980; Kawaoka et al. 1989). The Asian virus obtained HA, NA,

and PB1 genes from an avian virus and the remaining five from the preceding human H1N1 (Gething et al. 1980; Kawaoka et al. 1989), whereas the Hong Kong virus obtained HA and PB1 from an avian donor and other genes from Asian H2N2 (Fang et al. 1981; Kawaoka et al. 1989).

1.2.9 Avian influenza infection in humans and other mammals

Humans

Natural AIV transfer of infection to humans was not reported until 1996, although a number of reports as a result of laboratory accidents have been made (Alexander & Brown 2000).

Human infection with H7N7 viruses causing hepatitis, but with no seroconversion to this virus was reported in 1970 by Campbell et al. (1970). A laboratory technician developed kerato-conjuctivitis, a self-limiting infection over 2 weeks, after accidentally splashing infectious allantoic fluid containing A/chicken/Vic/76 H7N7 virus, onto her face. The virus was re-isolated from her conjunctiva but no antibody was detected to this virus (Taylor & Turner 1977).

In 1996 Kurtz et al. reported the isolation of influenza virus from the eye of a woman with conjunctivitis. The virus, A/England/269/96, after partial sequencing of seven genes showed close homology to avian viruses and full sequences of the HA gene of the 268/96 virus had 98.2% nucleotide identity with H7N7 AIV isolated from turkeys in Ireland in 1995 (Banks et al. 1998). No H7 antibody was detected in the woman by 5 weeks after infection. Ducks were the most likely source of the virus as the woman was involved in breeding different types of ducks, all of which had access to a pond inhabited by wild waterfowl (Kurtz et al. 1996).

In May 1997, a 3-year-old child died from viral pneumonia in a Hong Kong hospital (Yuen et al. 1998) followed by another 17 reported infections of humans with five deaths (Shortridge et al. 1998). An influenza H5N1 subtype was isolated from patients

and/or infection of the virus was confirmed by serology (Shortridge et al. 1998). Nucleotide sequences of the virus from the index case showed 99% identity of all eight genes to the H5N1 virus isolated from the outbreak of HPAI in poultry in Hong Kong in March 1997 (Claas et al. 1998; Suarez et al. 1998). Molecular analysis of HA and NA genes of the 16 viruses isolated between May and December 1997 from humans were essentially similar with no cumulative changes that would have indicated evolution/adaptation to humans (Bender et al. 1999). Therefore, direct spread of the HPAI H5N1 virus from poultry to humans is most likely scenario.

In March 1999, two young girls aged 1 and 4 years, were hospitalised in Hong Kong with influenza-like symptoms. Influenza avian-like virus subtype H9N2 was isolated from the respiratory tract of both girls (Peiris et al. 1999a). Similar to the situation with the H5N1 human virus, genetic analysis showed the H9N2 virus was of avian origin and both girls appeared to be infected directly from poultry (Peiris et al. 1999b).

Pigs

Pigs have an important role as an intermediate host for reassortment of influenza A viruses of avian and human origin as they are the only domesticated mammalian species that is susceptible to infection with, and that allows productive replication of, both avian and human influenza viruses (Brown 2000). In 1979, epidemics of influenza in swine in Belgium found that the isolated strain of influenza H1N1 virus was antigenically closely related to H1N1 strains previously isolated from ducks in North America and the Federal Republic of Germany (Pensaert et al. 1981). All the genes to the prototype H1N1viruses, dominant in European pigs, were of avian origin (Schultz et al. 1991). North American classical swine H1N1 influenza virus is antigenically and genetically distinguishable from European H1N1 but closely related to H1N1 virus isolated from wild ducks (Pensaert et al. 1981). This virus was re-introduced into birds causing economic losses. For example in 1991, a number of outbreaks was recorded in several turkey breeding farms in Germany, France, and the Netherlands (Ludwig et al. 1994).

There was also a report on the introduction of H1N1 AIVs into pigs in China. Genetic analysis of the viruses showed that each of the eight gene segments was of avian origin (Guan et al. 1996). Phylogenetic analysis indicated that these viruses were an independent introduction into pigs in Asia from an Asian sub-lineage of the Eurasian avian lineage (Guan et al. 1996). Recently, H9N2 viruses have been introduced to pigs in Southeast Asia most likely from poultry, although it will be difficult to predict the behaviour of these viruses in pigs in the future (Brown 2000).

Horses

Influenza infection in horses has mainly been caused by two subtypes (H7N7 type 1 and H3N8 type 2), a phylogenetically distinct lineage from other influenza A viruses (Murphy & Webster 1996). However, there have also been records of the isolation of subtypes other than these two, such as H1N1, H2N2, and H3N2, from horses in the past (Tumova 1980). In March 1989 an outbreak of respiratory disease occurred in horses in northeast China that caused up to 20% mortality in some herds (Guo et al. 1992). An influenza virus subtype H3N8, isolated from the infected horses was antigenically and molecularly distinguishable from the equine 2 (H3N8) viruses currently circulating in the world and after molecular analysis, six of eight gene segments (including HA and NA) were found to be closely related to the recent AIVs and were probably introduced directly to horses from avian species without reassortment (Guo et al. 1992).

Marine mammals

In 1979–80, influenza A virus subtype H7N7 was isolated repeatedly from lung and brain tissues taken from harbour seals with pneumonia and with up to 20% mortality on the Cape Cod Peninsula, United States (Lang et al. 1981). Antigenic and genetic analysis of the seal virus isolates showed them to be closely related to avian viruses and with most likely, direct transmission without reassortment (Webster et al. 1981).

In 1983 a similar case was recorded on the New England coast and influenza A virus H4N5 subtype was recovered from harbour seals with 2–4% mortality (Hinshaw et al. 1984), dying of viral pneumonia from June 1982 through to March 1983. The virus was

antigenically and genetically closely related to recent avian virus strains but differed from mammalian viruses, including H7N7 isolates recovered from seals in 1980 (Hinshaw et al. 1984). There were further influenza virus isolations of H4N6 and H3N3 in 1991–92 from the seals in Cape Cod Peninsula, United States, as part of surveillance and all were shown to be avian viruses that directly entered the seal population (Callan et al. 1995).

Two influenza A viruses of the H13N2 and H13N9 subtypes were isolated from tissues from a beached pilot whale (Hinshaw et al. 1986). Serological, molecular, and biological analyses indicated that the whale isolates were closely related to the recent H13 influenza viruses circulating in gulls (Hinshaw et al. 1986; Chambers et al. 1989).

Mink

During October 1984 an outbreak of respiratory disease occurred on 33 mink farms, in a coastal region of southern Sweden, with 100% morbidity and 3% mortality (Klingeborn et al. 1985). Influenza A H10N4 subtype was isolated from the mink and was confirmed to be closely related to isolates from chickens and wild ducks in England in 1985 (Berg et al. 1990). Direct infection of AIV from mallard ducks and/or chickens was suggested by Berg et al. (1990) to cause this outbreak.

1.3 AVIAN PARAMYXOVIRUSES

Avian paramyxoviruses (APMVs) are enveloped, negative-stranded RNA viruses, which belong to the family *Paramyxoviridae*. The name "paramyxo" is derived from the Greek words para "by the side of" and myxa "mucos" (relating to activity of haemagglutinin and neuraminidase). In 2000 the family *Paramyxoviridae* was reclassified by the International Committee on the Taxonomy of Viruses into two subfamilies, *Paramyxovirinae* and *Pneumovirinae*, based on morphological criteria, the organisation of the genome, the biological activities of the proteins, and the genome sequence relationship of the encoded proteins (Seventh Report of the International

Committee on Taxonomy of Viruses 2000). Examples of the members of the family *Paramyxoviridae* are listed below:

Order *Mononegavirales*

Family *Paramyxoviridae*

Subfamily *Paramyxovirinae*

Genus *Respirovirus*

Species *Bovine parainfluenza virus* 3 (BPIV-3)

Human parainfluenza virus 1 (HPIV-1)

Human parainfluenza virus 3 (HPIV-3)

Sendai virus (Murine parainfluenza virus 1)

Simian virus 10 (SV-10)

Genus *Rubulavirus*

Species *Avian paramyxovirus* 2 (Yucaipa) (APMV-2)

Avian paramyxovirus 3 (APMV-3)

Avian paramyxovirus 4 (APMV-4)

Avian paramyxovirus 5 (Kunitachi) (APMV-5)

Avian paramyxovirus 6 (APMV-6)

Avian paramyxovirus 7 (APMV-7)

Avian paramyxovirus 8 (APMV-8)

Avian paramyxovirus 9 (APMV-9)

Human parainfluenza 2 (HPIV-2)

Human parainfluenza 4 4a and 4b (HPIV-4)

Mapuera virus (MPRV)

Mumps virus (MUV)

Newcastle disease virus (Avian parainfluenza virus 1) (NDV and APMV-1)

Porcine rubulavirus (La-Piedad-Michoacan-Mexico virus) (PoRV)

Simian virus 5 and 41 (SV-5 & SV-41)

Genus *Morbillivirus*

Species *Canine distemper virus* (CDV)

Cetacean morbillivirus virus (CEMV)

Measles virus (Edmonston virus) (MEM)

Peste-des-petits-ruminants virus (PPRV)

Phocine distemper virus (PDV) (seal distemper virus)

Rinderpest virus (RPV)

Subfamily *Pneumovirinae*

Genus *Pneumovirus*

Species *Bovine respiratory syncytial virus* (BRSV)

Human respiratory syncytial virus (HRSV) (Human respiratory syncytial virus A2, B1, S2)

Murine pneumonia virus (MPV)

Genus *Metapneumovirus*

Species *Turkey rhinotracheitis virus* (TRTV)

There is new evidence, obtained by sequencing the whole genome of APMV-1, that all APMVs may be sufficiently different from other members of the *Rubulavirus* genus to be placed in a separate genus (de Leeuw & Peeters 1999).

1.3.1 Virion properties

The virion is about 150 nm or more in diameter. It is pleomorphic and although it is usually spherical in shape, filamentous forms can also be seen. The virion is surrounded by a lipid bilayer envelope, which is derived from the host cell membrane lipids. There are 8–12 nm glycoprotein spikes projecting from the surface of the envelope, which can be readily seen under the electron microscope. Inside the envelope is the nucleocapsid core (ribonucleoprotein), which consists of a single molecule of linear, non-infectious, negative sense ssRNA genome. The size of the RNA is fairly uniform in *Paramyxoviridae* and varies between 15,156 for NDV and 15,892 for measles virus. Some virions may contain positive sense RNA, so self-annealing of extracted RNA may occur. The Mr of the genome is 5–7 × 10^6. The nucleocapsid has helical symmetry, is 13–18 nm in diameter, and has a 5.5–7 nm pitch according to the subfamily (Rima et al. 1995).

Members of the subfamily *Paramyxovirinae* contain 6–7 transcriptional elements that encode 10–12 proteins (Mr 5–250 × 10^3) of which 4–5 or more are derived from the 2–3 overlapping ORFs in the P locus. Pneumoviruses have 10 ORFs encoding 10 proteins of Mr 4.8–250 × 10^3. Virion proteins common to all genera include: three nucleocapsid-associated proteins, namely RNA-binding proteins (N or NP), a phosphoprotein (P), and a large putative polymerase protein (L). The envelope contains an unglycosylated matrix protein (M) and two glycosylated envelope proteins, comprising a fusion protein (F) and an attachment protein (G, H, or HN). Variable proteins include the non-structural proteins (C, 1C/NS1 and IB or NS2), cysteine-rich protein (V), a small

integral membrane protein (SH or 1A), and a second inner envelope unglycosylated protein (M2 or 22 kDa protein). Enzymes of the virion (variously represented among the genera) include a transcriptase and adenylate transferase, mRNA guanylyl and methyl transferases, protein kinase, and neuraminidase (Rima et al. 1995).

APMV-1 genome, its encoded proteins and their functions

The genome of NDV—the most studied APMV virus-—codes for six proteins (Millar & Emmerson 1988) and the gene order 3′- NP-P- M-F-HN-L-5′ is identical to that of other members of rubulaviruses and paramyxoviruses. However, there are differences in proteins such as the presence of an additional gene encoding the SH protein in some rubulaviruses or presence of a C protein in paramyxoviruses, which makes the P protein in APMV viruses smaller. NDV infected cells contain, in addition, two virus-coded non-structural V and W proteins that arc encoded by the same mRNA as the P protein. The length of individual NDV genes is 3′-NP(1746)-P(1451)-M(1241)-F(1792)-NH(2031)-L(6704)-5′ nucleotides (Millar & Emmerson 1988).

The third genus, *Morbillivirus*, although related to other members of the subfamily on the basis of gene map, comprises viruses that are distinct antigenically and differ in the diseases they cause (Lamb & Kolakofsky 1996). There is a proposal to reclassify the rubulaviruses and separate NDV and other APMVs to form an additional genus in the subfamily *Paramyxovirinae* (de Leeuw & Peeters 1999). Data from the two NDV strains D26 and BeaudetteC reveals that 98.8% of the viral genome is transcribed into the six monocistronic polyadenylated mRNAs and that 90.7% of the genome corresponds to regions that are translated into proteins (Millar & Emmerson 1988).

The NP, P, and L proteins in association with the genomic RNA, form the viral nucleocapsid (Millar & Emmerson 1988), which can be seen under the EM as a "herring bone", either free or emerging from disrupted virus particles (Alexander 1988c). These proteins have not been studied in as much detail as other F, HN, or M proteins and their functions are assumed by analogy with similar proteins such as those of vesicular stomatitis virus (VSV) (Lamb & Kolakofsky 1996). The NP serves several

functions in virus replication, including encapsidation of the genome RNA into RNAse-resistant nucleocapsid (the template for RNA synthesis), and association with the P-L polymerase during transcription and replication. There is also evidence that the NP controls the rates of transcription and replication from the genome template (Lamb & Kolakofsky 1996). The P protein is a modular protein that plays an important role in all RNA synthesis. Together with the L protein it forms the viral polymerase P-L, and with unassembled NP (NP^o) forms a complex ($P\text{-}NP^o$), which is probably the active form in RNA encapsidation (Lamb & Kolakofsky 1996). The L protein is a required part of the viral transcriptase, as mentioned above and probably provides the polymerase function of the transcriptase complex, and is involved in polyadenylation (Millar & Emmerson 1988; Lamb & Kolakofsky 1996).

The M (matrix or membrane) protein of APMV-1 forms a shell on the inner surface of the viral lipid envelope and plays an important role in paramyxovirus assembly. It is considered to be the central organiser of viral morphogenesis, making interactions with cytoplasmic tails of the integral membrane proteins (NH and F), the lipid bilayer, and nucleocapsids (Millar & Emmerson 1988; Lamb & Kolakofsky 1996).

All *Paramyxoviridae*, including APMVs, possess two encoded glycoproteins (HN and F), which are embedded in the lipid membrane and together with the M protein form the viral envelope (Lamb & Kolakofsky 1996).

The haemagglutinin-neuraminidase (HN) glycoprotein contains both the haemagglutinating and neuraminidase activities. These are responsible for the initial attachment of the virus particle to its cellular receptor and receptor-destroying activity to prevent self-aggregation of viral particles during budding at the plasma membrane, respectively (Millar & Emmerson 1988; Samson 1988; Lamb & Kolakofsky 1996). Additionally, HN is the major antigenic determinant of the paramyxovirus. The ability to agglutinate red blood cells (RBCs) together with the specific inhibition of agglutination by antisera is used in the diagnosis of the diseases caused by these viruses.

The fusion (F) protein is responsible for fusion of the virus particle with, and penetration through, the host cell membrane and uncoating of the viral nucleocapsid in one step (Lamb & Kolakofsky 1996). The fusion protein is synthesised as an inactive precursor (F0), which requires proteolytic cleavage to generate the disulphide-linked fragments F1 and F2 for viral infectivity (Millar & Emmerson 1988; Lamb & Kolakofsky 1996).

Virus replication

Avian paramyxovirus replication is according to the general pattern as described by Peeples (1988). The virus is attached to a target cell receptor through the HN glycoprotein and neuraminic acid—the likely cell receptor for APMV-1. The late function of HN in infection is to cause enzymatic cleavage of neuraminic acid residues on the virus to prevent self-aggregation of the virus after release from infected cells (Lamb & Kolakofsky 1996). The second surface glycoprotein, F glycoprotein, provides the penetration function of the viral envelope to the plasma membrane (Lamb & Kolakofsky 1996) but the mechanisms of interaction with the host cell membrane are not fully understood (Peeples 1988; Lamb & Kolakofsky 1996). There is evidence that the virus penetrates the cell membrane by endocytosis but direct fusion of the viral envelope with the cell membrane has also been seen under the electron microscope (Peeples 1988). Once the viral nucleocapsid complex enters the cytoplasm it releases the NP-RNA. The other two proteins, P and L, associated with the nucleocapsid, act as the transcriptase complex to produce a complementary transcript of six species of mRNAs corresponding to six APMV-1 genes. This is further converted to positive sense RNA, which acts as a messenger RNA (Peeples 1988). The messenger RNA utilises the cell's mechanisms enabling translation into proteins and virus genomes. The F protein is synthesised as a non-functional precursor F0, which requires cleavage to F1 and F2 by host proteases. The HN of some strains of APMV-1 may also be required to be cleaved to produce active HN (Millar & Emmerson 1988). The virus assembly and release, like other events of the life cycle of paramyxovirus replication, takes place in the cytoplasm. The nucleocapsid is assembled to form the helical RNA structure by association of free NP subunits with the genome or template and the P-L protein complex. The viral membrane glycoproteins are synthesised in the endoplasmic

reticulum and, after changes that occur in the Golgi network, they are transported to the plasma membrane (Lamb & Kolakofsky 1996). The assembly mechanisms of the virus particle at the plasma membrane are incompletely understood, but the viral M proteins are thought to play an important role in directing the ribonucleoprotein core to join the glycoprotein in the membrane and form a budding virion (Lamb & Kolakofsky 1996).

1.3.2 Antigenic relationships within and between serotypes

A number of serological tests have been applied to study APMVs: HI test (Alexander 1982a; Alexander et al. 1983), AGID test (Kida & Yanagawa 1981), VNT (Tumova et al. 1979), and neuraminidase inhibition test (NIT) (Kessler et al. 1979) have been used to type the APMVs. All the tests provide similar results. Nine types of APMVs have been confirmed to date and these were named PMV-1 to PMV-9 as suggested by Tumova et al. (1979) and further defined (WHO Expert Committee 1980) using the rules recommended for influenza A viruses. The new isolates were designated: serotype/species or type of bird from which it was isolated/geographical location of isolation-country or state/reference number or name/year of isolation. The use of avian PMV (APMV) has been recommended (Rima et al. 1995) so, after the changes, the nominated prototype strains for APMVs (Alexander 1993b) are:

APMV-1 Newcastle disease virus
APMV-2 chicken/California/Yucaipa/56
APMV-3 turkey/Wisconsin/68
APMV-4 duck/Hong Kong/D3/75
APMV-5 budgerigar/Japan/Kunitachi/75
APMV-6 duck/Hong Kong/199/77
APMV-7 dove/Tennessee/4/75
APMV-8 goose/Delaware/1053/76
APMV-9 domestic duck/New York/22/78

APMV-1 viruses, regardless of their virulence and source, had been considered to be an antigenically homogeneous group when using the HI test although some researchers (Arias Ibarrondo et al. 1978; Alexander & Collins 1984) showed some serological

variation. The variation also was confirmed by the VNT in certain strains and isolates (Schloer et al. 1975; Pennington 1978). Using nine monoclonal antibodies (mAb) prepared against APMV-1 strain Ulster 2C (Russell & Alexander 1983; Alexander et al. 1997) eight antigenic groups of viruses were identified on the basis of variation in binding of these mAb. However, when a larger panel of 26 mAbs was used (Alexander et al. 1997) 39 distinct patterns were recognised. Viruses placed in each group shared both biological and epizootiological properties. On the basis of restriction site analysis of the F gene region, six major groups of APMV-1 isolates have been established, and these were further expanded to seven and eight groups by other workers (Ballagi-Pordany et al. 1996; Lomniczi et al. 1998). Generally there is some correlation of clusters of viruses in groups created by mAbs (Ballagi-Pordany et al. 1996) and restriction site analysis, although some differences exist.

APMV-2 viruses, which have been isolated from captive or free-ranging Passeriformes, parrots, mynahs, lovebirds, chickens, turkeys, ducks, rails, and budgerigars (Ritchie 1995b) may differ serologically in the HI test and there were reports of ≥4-fold differences in cross HI titres with APMV-2 strains Bangor and Yucaipa (McFerran et al. 1974). APMV-2 isolates have been divided into four groups using three mouse MAbs in the HI test (Ozdemir et al. 1990).

APMV-3 isolates were mainly obtained from turkeys and psittacine birds (mainly in quarantine) and there are distinct differences between isolates of these viruses when tested (Anderson et al. 1987) using six mAbs in the HI test. Psittacine isolates from Europe and the United States seem to be very similar, whereas turkey isolates could be divided with those from England and France as one group of isolates and those from Germany and the United States as another group by examining the patterns of mAb binding. The United States and German turkey isolates appeared to be more similar to the psittacine bird isolates than those from turkeys in other countries. Isolates from the United States have also been studied (Tumova et al. 1979) and a close relationship was confirmed between all United States APMV-3 isolates.

APMV-4 viruses from different countries and species (free-ranging waterfowl including ducks, geese, rails, and pheasants) have been found to be closely related and no

antigenic variation has been detected when tested by HI or neuraminidase inhibition tests (Shortridge & Alexander 1978; Alexander et al. 1979). However, they can be distinguished using oligonucleotide mapping (Nerome et al. 1983).

APMV-5 isolates (obtained only from budgerigars) form an antigenically very homogeneous group and no major antigenic variation has been reported (Nerome et al. 1978).

For APMV-6, there are no major serological variations between isolates from different countries (Shortridge et al. 1980) and similar observations were reported (Nerome et al. 1984) although for some strains antigenic differences have been noted using oligonucleotide mapping techniques (Shortridge et al. 1980).

APMV-7 isolates from doves showed homogeneity in the HI test (Alexander et al. 1981) but the relationships of other isolates obtained in other countries from the family Columbidae (Alexander et al. 1991) showed variation in reactivity in the HI and AGID test amongst themselves and with the prototype strain.

No antigenic variation in the HI test was observed in AMPV-8 viruses isolated from ducks or geese from different countries and only one isolate has been reported to belong to APMV-9. It was isolated from domestic ducks (Alexander 1993b).

Avian paramyxoviruses generally are grouped into serotypes on the basis of closest similarity within strains in serological tests that also distinguish them from other similar groups. A number of tests have been used to divide APMVs into serotypes including HI, AGID, VNT, PAGE (Alexander 1993b), but the HI test is recognised as the method of choice and is still the most common test for serotyping APMV isolates (Alexander 1993b). Although distinct APMV serogroups have been identified, there is some cross-reactivity with viruses from other serogroups. The most important cross-reaction is between APMV-1 and APMV-3 viruses (Smit & Rondhuis 1976; Alexander et al. 1983; Box et al. 1988; Stanislawek et al. 2001), which may interfere in ND diagnosis or epidemiological investigations if this is not considered during testing. There are also cross-reactions recorded between APMV-1 and APMV-4 (Kessler et al. 1979), APMV-

2 and APMV-6 (Shortridge et al. 1980), APMV-1, APMV-3, and APMV-8 and APMV-1, APMV-3, and APMV-9 (Alexander et al. 1983) in the HI test. Using antibody forming cell assays following immunisation of chickens, representatives of APMV serotypes (excluding APMV-5) have been divided into two super-serogroups: APMV-1, -3, -4, -7, and -9, and APMV-2, -6, and -8 respectively (Russell 1989).

1.3.3 Epidemiology

The epidemiology of paramyxoviruses is difficult to understand precisely because of the lack of systematic surveillance and other complicating factors such as transportation by humans of infected birds around the world. However, our knowledge of APMVs is expanding through ongoing surveillance and other ecological studies undertaken in a number of countries.

APMV-1 (NDV)

APMV-1 has been reported to infect animals ranging from reptiles to humans (Lancaster 1966). All birds probably are susceptible to infection and 241 species of birds representing 27 of 50 orders of the class have been shown to be susceptible to infection with APMV-1 (Kaleta & Baldauf 1988).

Wild birds

The most frequently obtained isolates of APMV-1 are from migratory feral waterfowl or other aquatic birds (Alexander 2000b) and usually these viruses have been isolated during extensive surveillance primarily concerned with the ecology of influenza viruses (Alexander 1993b). Most of these viruses are of low virulence for chickens, although pathogenic strains also have been isolated. The best examples of ND outbreaks in wild birds were reported in double-crested cormorants (*Phalacrocorax auritus*) in North America at the time that there were outbreaks of ND reported in 1990 in Canada (Alberta, Saskatchewan, and Manitoba) (Wobeser et al. 1993). In 1992 ND reappeared

in cormorants in western Canada, around the Great Lakes and in the northern midwest United States, spreading later to domestic turkeys (Heckert 1993; Heckert et al. 1996).

Caged birds

APMV-1 isolates have often been isolated from caged birds and most of these were isolated as a result of quarantine investigations (Ashton & Alexander 1980; Senne et al. 1983). The isolates, are often very virulent, unlikely reflecting the true epizootic situation in the country of origin. It is thought that they are probably the result of enzootic APMV-1 in the quarantine station or spread from local backyard poultry (Kaleta & Baldauf 1988). Illegal importation was assumed to be responsible for the 1991 outbreak of ND in caged birds in six states of the United States (Panigrahy et al. 1993).

Domestic poultry

All types of commercial poultry, including chickens, turkeys, pheasants, guinea fowl, ducks, geese, pigeon, and ostriches, have been infected with APMV-1 although the geographical distribution of natural infection with APMV-1 is difficult to define because of the live vaccines used in most countries (Kouwenhoven 1993; Alexander 1995, 2000b). Disease incidence and severity vary greatly in relation to the pathogenicity of the virus, intensity of the poultry industry in the country, geographical isolation, chicken population density, biosecurity, and vaccination strategy (Kouwenhoven 1993). One example is the large number of outbreaks in Europe, from 1991 to the present, in backyard flocks rather than in well managed commercial poultry (Alexander 2000b). Additionally, the definition of "freedom" is concerned mainly with the absence of pathogenic strains of APMV-1. Non-pathogenic viruses are frequently isolated from commercial poultry in countries considered free of ND (Alexander 2000b) including New Zealand (Stanislawek et al. 2002).

Other avian paramyxoviruses

Wild birds

APMV-2, -4, -5, -7, and -8 have been isolated from feral birds (Alexander 1993b, 2000b; Ritchie, 1995b). APMV-2 has been primarily isolated from small perching birds of the order Passeriformes, either free ranging or captive, in Europe, Indonesia, Senegal, Kenya, Israel, Japan, India, and Costa Rica (Alexander 1993b; Ritchie 1995b). Other species from which isolates have been obtained include captive psittacines (Alexander et al. 1982; Senne et al. 1983) (which usually have been in contact with caged passerines (Alexander 2000b)), mallard ducks (Lipkind et al. 1982b), and cattle egret (Lipkind et al. 1982a).

Viruses of the APMV-4 and APMV-6 serotypes have been frequently isolated from migratory wild ducks and geese and appear to have a worldwide distribution (Alexander 2000b).

In the United States and Japan, APMV-8 has been isolated from migratory ducks and geese and it is likely that these viruses also have a worldwide distribution (Alexander 2000b).

APMV-7 serotype viruses have been isolated only from feral and/or captive pigeons and doves in Japan, United States, and England (Alexander et al. 1991) although a worldwide distribution has been suggested (Alexander 2000b).

No viruses of APMV-3, -5, and -9 serotypes have been isolated from wild birds to date (Alexander 1993b, 2000b).

Caged birds

There are records of isolates of APMV-2, -3, and -5 serotypes from caged birds (Alexander 1993b, 2000b). As for APMV-1, the majority of the isolates have been obtained from birds dying in quarantine after being imported from various countries (Europe, Middle East, Southeast Asia, Africa, and Central America). APMV-2 and -3 serotypes predominated (Alexander et al. 1982, 2000b; Senne et al. 1983) and APMV-2

viruses were mainly isolated from passerines and APMV-3 mainly from psittacines. However, APMV-2 viruses can infect psittacines and passerines are susceptible to infection with APMV-3 viruses if there is contact between these species (Alexander 1993b). The APMV-5 serotype was isolated in Japan in the mid 1970s from budgerigars experiencing acute infection (Nerome et al. 1978). APMV-5 was also recovered from the spleen and liver of dying budgerigars in a European aviary (Gough et al. 1993) and there is no other well documented case of isolation of APMV-5 from birds any where else in the world.

Poultry

APMV-2, -3, -4, -6, -7, and -9 have been recorded to infect poultry. APMV-2 has been primarily associated with disease or inapparent infection in turkeys (Lang et al. 1975; Lipkind et al. 1979; Alexander 2000b), but there are also a number of reports of APMV-2 infection in chickens (Lipkind et al. 1982a; Shihmanter et al. 1995) in North and Central America, Europe, Middle East, and Southeast Asia. APMV-3 has only been detected in turkeys in Europe and North America (Tumova et al. 1979; Macpherson et al. 1983; Alexander 2000b). APMV-4, -6, and -9 have been mainly isolated from ducks and geese in North America, and most likely were introduced to farms by wild waterfowl (Shortridge 1980; Alexander 1993b, 1997). APMV-6 has also been isolated from turkeys with respiratory and egg production problems (Alexander 1997). Although APMV-7 is mainly associated with pigeons and doves, APMV-7 has been isolated from turkeys (Saif et al. 1997) and ostriches (Woolcock et al. 1996) in North America.

Transmission

Most of the information regarding transmission and spread was obtained because of APMV-1 infection, but the same general pattern is believed to apply to other paramyxoviruses because of their simila replication and mode of infection (Lancaster & Alexander 1975; Alexander 2000b).

Natural infection between birds (excluding predatory birds) occurs by respiratory or intestinal routes as the result of either inhalation of excreted droplets or ingestion of infectious material/faeces (Lancaster & Alexander 1975; Alexander 2000b). A number

of factors will influence the success of transmission including: temperature, humidity, density, and behaviour of birds and this is particularly important in infections by the respiratory route. In cases where no respiratory signs are associated with APMV-1 infection, faeces will be the main source of the virus, including the pigeon variant virus (Alexander et al. 1984; Alexander & Parsons 1984). Vertical transmission of paramyxoviruses is controversial and there are not enough convincing data to equivocally support this (Alexander 1997).

Spread

Introduction of APMVs into the bird population of a country can occur by a number of means including: movement of live birds, movement of people and equipment, movement of poultry products, contaminated poultry food or water, airborne spread, contaminated vaccines, and non-avian hosts (Lancaster & Alexander 1975; Alexander 1997, 2000b).

Migratory passerines and in particular waterfowl, play an important role in the introduction of paramyxoviruses into the bird population of a country. An APMV-1 epizootic in cormorants and pelicans in 1990 and 1992, as discussed above, is a very good example (Wobeser et al. 1993; Banerjee et al. 1994). The congregation of migratory birds after breeding creates an excellent opportunity for the viruses to be maintained in the bird population (Hinshaw et al. 1980b) because there are a lot of susceptible birds. The APMV-2 which caused disease in turkeys in Israel was most likely introduced from migratory ducks from which virus was also isolated (Lipkind et al. 1982b).

The trade of caged birds also significantly contributes to the spread of paramyxoviruses, because these birds are derived from countries where they are trapped from the wild. The volume of the trade is extremely large, for example up to 250,000 exotic birds were imported to England in the late 1970s and c. 3 million birds were imported to the United States between 1973 and 1981 (Alexander 1988b). Various paramyxoviruses have been isolated from imported birds while in quarantine in Europe and the United States (Alexander et al. 1982; Senne et al. 1983). Although quarantine procedures are

followed during the importation of birds, prematurely removed or smuggled birds pose a threat to introduce exotic viruses to the bird population of the country (Alexander 1997). Racing pigeons also have to be considered as an excellent vehicle for spreading APMVs because birds are expected to cover long distances and they have ample opportunity to mix with other pigeons and feral birds (Alexander 1988b). As an example of this, pigeons were blamed for an outbreak of ND in poultry in the United Kingdom in 1984 (Alexander et al. 1985). Movement of people and equipment is still regarded as a significant method for the spread of APMVs, most likely by the personal transfer of infective faeces from one farm to another on hair, clothing, footwear, equipment, and vehicles (Alexander 2000b). In the past, poultry and poultry products were considered as a major way to introduce NDV, although latest legislation has significantly reduced such spread (Alexander 2000b). Airborne spread of APMVs is very controversial and is generally not regarded as a significant means of spread (Alexander 2000b). Hugh-Jones et al. (1973) detected NDV in their investigation only 64 m downwind of an infected farm (Hugh-Jones et al. 1973). However, in situations where poultry farms are sufficiently concentrated and the climatic conditions are favourable, airborne spread could play a role (Alexander 2000b). In general, contamination of avian vaccine is not an issue as good manufactory practices make this a very unlikely event. However, NDV contamination of other avian vaccines has been recorded in Denmark leaving this way of spread still open (Jorgensen et al. 2000).

1.3.4 Disease caused by APMV-1

The disease in birds caused by APMV-1 varies widely and a number of factors such as virus, host, age of species, route of infection, health status of birds (infection with other organisms, immune status), and environmental stress will influence the clinical signs observed (Alexander 2000b). No clinical signs can be considered as pathognomonic, however certain signs do appear to be associated with particular viruses and therefore APMV-1 viruses have been divided into five pathotypes on the basis of the predominant signs observed in infected chickens. The APMV-1 virus pathotypes are as follows—

(1) Viscerotropic velogenic (VV): characterised by acute lethal infection, usually with haemorrhagic lesions in the intestines of dead bird.

(2) Neurotropic velogenic (NV): characterised by high mortality, which follows respiratory and neurological disease, but gut lesions are not usually observed.

(3) Mesogenic: characterised by respiratory and neurological signs with low mortality.

(4) Lentogenic: viruses causing mild infection of the respiratory tract.

(5) Asymptomatic enteric: inapparent infection in which viruses replicate mainly in the gut.

This grouping is not consistent for birds other than chickens and some overlapping of categories with particular isolates does occur (Alexander 2000b).

The incubation period varies from 2 to 15 days (5 days in average) and the symptoms observed in general include depression, anorexia, drop in egg production, ocular and nasal discharge, conjunctivitis, rhinitis, sneezing, coughing, dyspnoea, bluish discoloration of facial appendages, yellow-green diarrhoea, ataxia, torticollis, opisthotonos, convulsion, circling, tremors and paralysis of the wings and legs (Kouwenhoven 1993; Ritchie 1995b; Alexander 1997). No pathognomonic gross or microscopic lesions can be considered in infection with any APMV-1 viruses and the lesions that are observed in particular outbreaks depend on the factors mentioned above. Virulent viruses typically cause haemorrhagic lesions of the intestinal tract most commonly in the proventriculus, the posterior parts of the duodenum, jejunum, and ileum, which later can develop into diphtheritic inflammation and necrosis (Kouwenhoven 1993). In severe cases haemorrhages can also be found in the subcutis, muscles, larynx, peritracheal/oesophagal tissues, serous membranes, trachea, lungs, airsacs, pericardium, and myocardium. Pneumonic changes are less frequently observed and are usually complicated by bacterial infection in longer standing cases. Breast muscles are dark-red and dry due to dehydration (Kouwenhoven 1993). Microscopic lesions consist of hyperaemia, necrosis, cellular infiltration, and oedema. Lesions seen in the central nervous system are those of nonpurulent encephalomyelitis (Alexander 1997).

1.3.5 Disease caused by other avian paramyxoviruses

Wild birds

There is no evidence that APMVs other than APMV-1 cause disease in wild birds (Alexander 1993b). All isolates obtained were from either apparently healthy trapped birds, hunter-killed birds, or birds found to be dead.

Caged birds

The majority of isolates obtained from caged birds are APMV-2 and -3 as a result of testing procedures in quarantine either from live healthy or dying birds and usually no record of disease other than death is available. In one report an African grey parrot became dull with white pasty droppings and died within 8 days of showing clinical signs (Collings et al. 1975). APMV-2 virus was isolated from tissue and congestion of lung and excess watery mucus in the upper respiratory tract were observed. In experimental infection with APMV-2, a significant decrease in the activity of finches (*Amadina fasciata*) over a 3-week period has been observed (Goodman et al. 1990).

There are also a number of reports on disease caused by APMV-3 viruses. APMV3/parakeet/Netherlands/449/75 was isolated from a flock of *Neophema* sp. experiencing high flock morbidity. Signs of central nervous system infection resembling ND were observed in psittacine species and passerines in aviaries in The Netherlands (Smit & Rondhuis 1976). The disease was reproduced in *Neophema* and red-rump parakeets (*Psephotus haematonotus*) but budgerigars (*Melopsittacus undulatus*) and cockatiels (*Nymphicus hollandicus*) were not susceptible to the virus. APMV-3 virus was also isolated from exotic finches (*Ortygospiza atricollis* and *Poephila cincta*) showing signs of lethargy, yellowish diarrhoea, conjunctivitis and dysphagia. Some of the affected birds died within a few days of exhibiting clinical signs, others recovered (Schemera et al. 1987).

The first report of disease caused by APMV-5 viruses was associated with an epizootic amongst budgerigars (*Melopsittacus undulatus*) in Japan in 1974–76 that was characterised by depression, diarrhoea, and high mortality (up to 100%) (Yoshida et al. 1977). In experimental infection, birds developed depression, dyspnoea, diarrhoea, occasionally torticollis, and death occurred within 2 weeks (Nerome et al. 1978). A report of APMV-5 infection in a European aviary also involved budgerigars and 20% of the birds died over a 2-year period. The predominant clinical signs were diarrhoea and vomiting (Gough et al. 1993).

Poultry

Only APMV-2 and APMV-3 viruses have been consistently shown to infect and cause disease in poultry but there have been cases where APMV-6 and APMV-7 have been associated with clinical disease in turkeys (Alexander 2000b). APMV-2 virus has been associated with respiratory disease and egg production problems in chickens (Lipkind et al. 1982a; Shihmanter et al. 1995) and turkeys (Lang et al. 1975; Bradshaw & Jensen 1979; Lipkind et al. 1979). The clinical signs vary from mild to severe respiratory disease with sinusitis, conjunctivitis, and pneumonia. Turkeys usually have been more severely affected than chickens and the severity of the disease may be influenced by infection with other organisms (Alexander 1993b). The morbidity in a turkey flock affected with APMV-2 could be up to 100% with mortality varying from 5 to 90% (Lipkind et al. 1979).

APMV-3 has been associated with respiratory and egg production problems only in turkeys (Tumova et al. 1979; Macpherson et al. 1983), although under experimental conditions chickens were demonstrated to be susceptible to infection with APMV-3 (Alexander 2000b).

APMV-6 virus was isolated on one occasion from a turkey flock with respiratory and egg production problems (Alexander 1997).

There is also a report of the isolation of APMV-7 from a turkey flock with mainly respiratory problems and increased mortality (Saif et al. 1997). The disease was

reproduced in SPF poults indicating that the virus was a primary pathogen of turkey (Saif et al. 1997).

1.3.6 Disease diagnosis

The laboratory diagnostic procedures for virus isolation and serology are essentially similar for all APMVs and are primarily those recommended by the OIE for ND (OIE 2000). None of the clinical signs or lesions are pathognomonic for ND as other viral diseases have to be considered, and therefore virus isolation/detection and characterisation is essential for definitive diagnosis (Alexander 1997, 2000b; OIE 2000). Whereas serological testing can detect the presence of antibody it does not give information required with regard to pathogenicity of the virus and therefore has limited diagnostic value (Alexander 1997, 2000b). For other APMVs, serology provides some information regarding infection with specific APMVs, although interpretation of the results, in particular when low titres are involved, can be very difficult (Stanislawek et al. 2001) and virus isolation/detection would have unquestioned advantages for disease diagnosis.

Virus isolation

Samples from live birds should include tracheal and cloacal swabs but from small birds faeces could be collected alternatively to avoid harm (OIE 2000). Samples from dead birds should include, in addition to oral-nasal swabs, tissues such as lung, kidneys, intestine, spleen, brain, liver, and heart and should be collected in the early stages of disease (OIE 2000).

Samples are placed in PBS at pH 7.0–7.4 with antibiotic mixture and prepared for virus isolation which includes grinding/mincing and centrifugation of tissue samples and centrifugation only for swabs (OIE 2000). Similar to diagnosis of influenza, a number of choices with regard to hosts for primary virus isolation are available including primary cell culture and cell lines. However, embryonated fowl eggs are regarded as the most sensitive and practical host for most APMV isolations (Alexander 1997).

Supernatant is inoculated into the allantoic cavity of 9–11-day-old SPF embryonated fowl eggs and incubated for 4–7 days at 35–37°C. Dying eggs and all remaining at the end of incubation are chilled at 4°C and allantoic/amniotic fluid harvested and tested for HA activity. Fluid that gives negative results should be inoculated at least once more (OIE 2000). Most types of APMVs will grow in the allantoic cavity with one exception, APMV-5, which requires the amniotic or yolk sac route of inoculation, using 9–10- and 6–7-day-old embryonated fowl eggs respectively (Nerome et al. 1978). As the amniotic/allantoic fluid infected with APMV-5 does not haemagglutinate RBC or haemagglutinates to a lesser degree, purification of the virus is required (Nerome et al. 1978; Gough et al. 1993).

Differential diagnosis

Differential diagnosis is basically the some as described for avian influenza (see section 1.2.4).

Direct detection of antigen/RNA

Most of the detection systems developed and used are mainly for APMV-1 and include the immunofluorescence test on tracheal sections or impression smears from other organs (Alexander 1988a) or IPX tests on thin sections prepared form various organs (Hooper et al. 1999c; Kuiken et al. 1999). However, all of these tests detect NDV antigen only, without providing information on the pathogenicity of the virus and this problem is omitted by using RT-PCR to determine the F0 cleavage site sequences either on isolates or tissues and faeces from infected birds (Jestin & Jestin 1991; Seal et al. 1995; Kant et al. 1997; Oberdorfer & Werner 1998; Gohm et al. 2000). Oligonucleotide probes targeting the F0 cleavage site also have been used to detect NDV and differentiate pathogenic and non-pathogenic strains by hybridisation techniques (Jarecki-Black et al. 1992).

Serology

Antibodies to APMV-1 can be detected in birds by a number of tests including single radial immunodiffusion, single radial haemolysis, AGID, and VNT (Alexander 1997). However, the most commonly used tests at present are ELISA and HI tests.

A variety of ELISAs have been developed and used (Miers et al. 1983; Snyder et al. 1983; Wilson et al. 1984) and have been found to be particularly useful for monitoring post-vaccination flock screening procedures because of the possibility of automation (Snyder et al. 1984). Sensitivity and specificity varies between ELISAs but generally a good correlation has been achieved in comparison to the HI test (Adair et al. 1989; Brown et al. 1990).

Despite some technical advantageous of ELISAs, the HI test is the most widely used test at present and is regarded by some as the test of choice for all APMVs (Kouwenhoven 1993; Alexander 2000b). In chickens the antibodies can be detected about 7 days after the onset of symptoms and they rarely give non-specific reactions (Kouwenhoven 1993). Sera from other avian species occasionally give non-specific agglutination and absorption using chicken RBCs is required (Alexander 2000b). Cross-reactions with other APMVs, particularly to APMV-3 have to be considered when reaching a definitive diagnosis (OIE 2000; Stanislawek et al. 2001).

Serological tests for other APMVs are basically the same as used for APMV-1 with the exception of APMV-5, where differences in agglutination of chicken RBCs has been reported (Nerome et al. 1978; Gough et al. 1993).

1.3.7 Assessment of pathogenicity

The variations in virulence of APMV-1 circulating in bird populations requires further assessment of the pathogenicity to confirm that the virus isolated from birds showing signs of disease is the one responsible (OIE 2000). Several *in vivo* and *in vitro* tests are available to assess pathogenicity and include: mean death time in eggs (MDT),

intracerebral pathogenicity index (ICPI), and intravenous pathogenicity index (IVPI) as *in vivo* tests, and *in vitro* tests using molecular techniques or monoclonal antibodies binding patterns are also used (OIE 2000).

In vivo assessment of pathogenicity

MDT involves inoculation of APMV-1 infectious allantoic fluid into 9–10-day-old embryonated SPF eggs and incubation at 37°C for 7 days. All velogenic strains kill embryos in less than 60 h, mesogenic strains take 60–90 h to kill and lentogenic take more than 90 h (OIE 2000).

In the ICPI assessment fresh infectious allantoic fluid is inoculated intracerebrally into ten 1-day-old SPF chickens which are observed daily for any clinical signs or deaths. The most pathogenic viruses give a score close to 2.0 whereas lentogenic viruses give a value close to 0.0 (OIE 2000).

In the IVPI procedure, ten 6-week-old SPF chickens are inoculated intravenously with fresh infectious allantoic fluid and birds are examined for 10 days for any clinical signs or deaths. In pathogenic strains the index is close to 3.0 but for lentogenic and mesogenic is close to 0.0 (OIE 2000).

In vitro assessment of pathogenicity

In the spirit of reducing the use of living animals in research/diagnostics, other procedures have been developed including the assessment of amino acid sequences at the F0 cleavage site and the C-terminus of the HN glycoproteins (Alexander 1997).

For the F0 precursor glycoprotein cleavage site the mechanism controlling the pathogenicity is very similar to that described for influenza HA (see Chapter 1.2.5) and non-pathogenic APMV-1. Virus spread is restricted to the cells infected and in instances where the cells are unable to activate the F0 glycoprotein, only local infection results (Rott & Klenk 1988). In pathogenic APMV-1 strains the F0 glycoprotein can be

cleaved by host proteases found in a wide range of tissues, permitting the production of infectious virus and resulting in a fatal systemic infection (Rott & Klenk 1988).

The deduced amino acid sequences of pathogenic APMV-1 strains have motif ^{112}R/K-R-Q-K/R-R^{116} at the C-terminus of the F2 protein and F (phenylalanine) at residue 117 of the N-terminus of the F1 protein (OIE 2000). In non-pathogenic strains the motif is ^{112}G/E-K/R-Q-G/E-R^{116} and L (leucine) at residue 117. The conclusion in pathogenicity assessment of APMV-1 in general, after analysing some of the pigeon variant viruses (PPMV-1) with sequences ^{112}G-R-Q-K-R^{116} and F at residue 117 was, that at least three amino acids (either lysine or arginine) at the residue between 113 and 116 in addition to phenylalanine at residue 117 is required for APMV-1 to be pathogenic for chickens (OIE 2000). This assessment was officially recognised by the OIE as an alternative way to determine pathogenicity of APMV-1 (OIE 2000).

Monoclonal antibodies (mAbs)

A panel of mAbs has been developed and used for various purposes, including the identification of APMV-1 in the HI test, to overcome the cross-reaction problem (Alexander 2000b) or differentiate vaccine strains from epizootic viruses in geographical areas of interest (Srinivasappa et al. 1986).

A panel of mAbs has been used to group APMV-1 isolates on the basis that some monoclonal antibodies react only with certain isolates in cell cultures infected with the isolate (Alexander et al. 1997). Viruses, which have similar binding patterns, share similar properties providing information to understand the epizootiology of outbreaks (Alexander et al. 1997).

Phylogenetic analysis

Improved sequencing techniques and the availability of sequences in computer databases provides the opportunity to compare the sequences in question to those in the database. It was demonstrated that even short sequences can provide meaningful results and viruses sharing temporal, antigenic, geographical, and epidemiological similarities

usually fall into specific lineage or clades providing very useful information on local and global epidemiology (Seal et al. 1995; Lomniczi et al. 1998; Takakuwa et al. 1998; Alexander et al. 1999; Stanislawek et al. 2002).

1.3.8 Prevention and control

APMV-1 viruses with an ICPI 0.7 or greater and/or with multiple basic amino acids at the C-terminus of the F2 protein and phenylalanine at residue 117, cause infection in poultry recognised as ND (OIE 2000). This definition of ND is recognised by the European Union and some other countries including New Zealand as an important direction in control of ND worldwide (OIE 2000). To achieve this, all countries are expected to report outbreaks of ND within their borders to the OIE to prevent the spread of NDV through live bird trade and poultry products.

At the national level in countries that are free of ND, the primary aim is to prevent the introduction of these viruses through the restriction of trade in poultry products and live birds (poultry and caged birds) and this may vary between countries (Alexander 1997). Quarantine is usually established for live birds coming to countries including New Zealand and negative testing is required for these birds to be released (Alexander 1997) (W. L. Stanislawek unpubl. data). In some countries, e.g., Ireland, poultry feed is heat-treated to reduce the possibility of NDV introduction by this route (Alexander 2000b). The racing of pigeons was also banned/restricted or vaccination was imposed in some countries after the pigeon panzootic of PPMV-1 (with mean ICPI 1.44) in the 1980s in Europe (Vindevogel & Duchatel 1988).

To be prepared for an ND outbreak, many countries have stamping-out policies where infected birds and birds in contact have restrictions on their movement or marketing (Alexander 1997) whereas others are satisfied with vaccination policies as a prevention measure, and/or "ring vaccination" in the outbreak situation (Alexander 1997).

At the farm level, a well designed farm with biosecurity in mind such as the separation of different types of species and ages of birds and a good water supply source to prevent

viruses from infecting the flock (Alexander 2000b). In addition to this a number of points important to maintain the biosecurity include: bird-proofed houses, feed and water; minimised movement on and off the farm of people and equipment; disinfection of all equipment and vehicles entering the farm; and regimens of clothing changes and basic hygiene (Alexander 2000b).

Vaccination must be regarded as complementary to good management practices, biosecurity, and hygiene and should never be considered as an alternative approach (Alexander 2000b). The use of NDV vaccines differs between countries because of national and international legislation and/or ND epidemiology. For example, in The Netherlands vaccination is compulsory but in Finland, Sweden, and Norway vaccination is banned. In countries were ND is enzootic, usually backyard poultry and commercial poultry are vaccinated routinely to prevent losses associated with infection caused by a field virus (Alexander 2000b).

To consider what vaccine would be most suitable to use, at least three issues should be considered including: immunogenicity of the vaccine; live or killed vaccine; and virulence of the live vaccine if chosen (Alexander 2000b). In general antigenic APMV-1 variation does not influence protection against field isolates (Alexander & Parsons 1986; Alexander et al. 1992) although virulence of the virus used in vaccine will influence the immune response because the immune response increases as the pathogenicity of the vaccine virus increases (Reeve et al. 1974). For example, the La Sota strain gives better protection over the B1 strain, but more often will cause severe respiratory reactions particularly when administered by spray or aerosols (Alexander 2000b).

A vaccination program is usually developed to maintain the required bird protection using live vaccine containing a strain of lower virulence, e.g., Hichner B1 and following vaccination with higher virulent vaccine strain, e.g., La Sota and the use of inactivated vaccine, which is particularly useful if complicating pathogens are present and/or high levels of protective antibodies of long duration are needed (e.g., breeding stock at or near the point of lay) (Alexander 1997).

Most of these vaccines were developed for chickens but turkeys, guinea fowl, and partridges have been successfully vaccinated with La Sota live and or/oil-emulsion vaccines (Kelleher et al. 1988; Alexander 1997) Pigeons were also vaccinated using chicken live or inactivated oil-emulsion vaccine with a positive outcome (Vindevogel & Duchatel 1988).

For the control of paramyxoviruses other than APMV-1, all preventive measures used for NDV will also apply, even policies that are usually not developed specifically for this (Alexander 1997). Vaccines for APMV-2 and APMV-3 for turkeys and APMV-3 for parakeets have been developed and used (Alexander 2000b).

1.3.9 Avian paramyxoviruses in other species

There have been reports that APMV-1 can infect species other than avian species such as snakes, lizards, and geckos (Kouwenhoven 1993). Human infection caused by APMV-1 has also been recorded, mainly associated with laboratory staff propagating the virus or workers involved in the vaccination of poultry (Khan 1994). Infection in humans mostly appears as a self-limiting conjunctivitis, however systemic infections have also been reported with symptoms similar to influenza (Khan 1994).

1.4 AIM AND SCOPE OF THE THESIS

Knowledge of the current epidemiological situation with regard to AIVs and APMVs in the New Zealand bird population is required for various groups including the Ministry of Agriculture and Forestry, Poultry Industry, Department of Conservation, ornithological societies, and also for human epidemiologists (mainly with respect to avian influenza).

Therefore, this study first aimed to determine the presence of these viruses using virological and serological methods. The design of the study is described in Chapter 2 and virus isolation is reported in Chapter 3. Serological testing for the presence of

antibodies against APMVs except APMV-5 and influenza A, are reported in Chapters 3 and 4 respectively.

The second aim was to characterise the most important viruses including APMV-1 isolates and influenza H5N2, mainly to determine their pathogenicity by genetic and molecular methods. Additionally, partial genomic and antigenic analysis to determine the relationship of the New Zealand APMV-1 isolates to each other and to reference isolates are presented in Chapters 5–7.

In conclusion, the present New Zealand situation with regard to APMV-1 and some of the influenza viruses (H5N2 in particular) and the possible pathogenic changes due to mutation/reassortment of these isolates and their potential impact on bird populations are discussed in Chapter 8.

Chapter 2 Survey design and collection of samples

2.1 INTRODUCTION

Knowledge of the APMV and AIV status of New Zealand birds is of international interest to allow confident decision-making regarding the importation of birds, for the development of importation protocols, and for other trade considerations. In view of the current theories that virulent AI and ND viruses may emerge by mutation from viruses of low virulence (Alexander 2001), this information is also important for the New Zealand poultry industry as part of the exotic disease preparedness programme and also for bird conservationists for successful management of native and endangered species.

The geographical isolation of New Zealand limits the movement of birds between New Zealand and other countries and there is no large-scale migration of birds (waterfowl in particular) as occurs in Europe or North America. However, there are records of movement of birds in New Zealand, which include the migration of shorebirds of the family Scolopacidae (sandpipers and allies) within the order Charadriiformes. These birds breed in the low Arctic regions of Europe, Asia, and North America, and they migrate south for the boreal winter. The species that migrate to New Zealand in the highest numbers are bar-tailed godwit (*Limosa laponica*), lesser knot (*Calidris canutus*), ruddy turnstone (*Arenaria interpres*), curlew sandpiper (*Calidris ferruginea*), red-necked stint (*Calidris ruficollis*), and Pacific golden plover (*Pluvialis fulva*) (Heather & Robertson 1996).

In addition to large-scale migration, a number of Australian vagrants have been recorded in New Zealand including: Australian Gannet, little egret, cuckoos, brown owl, martins, satin flycatcher, swallow, pelican, heron, ibis, kite, falcon (Heather & Robertson 1996).

Limited numbers of duck and geese visitors, e.g., from Australia and Norfolk Island, also reach New Zealand (R. O. Cossee pers. comm.).

The consequence of these bird movements is the introduction of AIVs and APMVs to the New Zealand bird population and it is possible that some of these introduced strains of virus are pathogenic for domestic poultry.

No disease outbreaks associated primarily with APMV or AIV infections have ever been confirmed in New Zealand. However, in previous limited studies several viruses have been isolated including: AIV subtypes H1N3, H4N6, H6N4, and H11N3 from wild ducks; APMV-1 from poultry, wild ducks, and parrot; and APMV-4 from wild ducks (Durham et al. 1980; Austin & Hinshaw 1984; Stanislawek 1992).

Therefore, the aim of this study was to extend our knowledge of AIVs and APMVs in the New Zealand duck population as a important reservoir of these viruses and to include other species representing caged birds, wild birds (other than waterfowl), and poultry.

2.2 GENERAL CONSIDERATIONS

A study involving wild birds, in particular, always has to balance the potential information obtained and the harm that could be done to the birds in the process. This together with the additional costs in trapping had to be taken into consideration when planning the survey. Therefore, most of the duck trapping and some of the other wild bird trapping and sampling were combined with banding programs carried out by Regional Fish and Game Councils, Department of Conservation, or ornithological society personnel.

Within New Zealand, the most common duck, the mallard, does not migrate but stays more or less in one area for the duration of its life (T. Caithness pers. comm.). However, some movement of shovelers, grey teals, and black swans between the

islands of New Zealand occurs on a small scale in certain seasons (M. Williams pers. comm.).

Congregation provides optimal conditions for AIVs and APMVs to be spread to susceptible birds. In New Zealand, congregation seems to occur only in the most favourable feeding areas for waterfowl (T. Caithness pers. comm.). Congregation also occurs to different extents depending on region and season. For example, the East Coast in summer and autumn is generally dry and the few permanent lakes that exist accommodate a large number of waterfowl compared to the wetter West Coast areas where waterfowl are more dispersed (M. Williams pers. comm.). Although congregation in New Zealand can be considered to be significant, it does not occur on the some scale as occurs before migration, for example, in North America (Hinshaw et al. 1980b, 1985; Stallknecht et al. 1990c).

In New Zealand, February–May is likely to be the optimal period for sample collection because at this time juvenile ducks, the most susceptible to these viruses, congregate with their female parent in favourable feeding areas and as a consequence can be readily infected. Studies carried out in North America support these findings because AIV and APMV were isolated more frequently from juvenile ducks than from adult ducks and, in addition, most isolations were made in the late summer and autumn when congregation occurs before migration (Hinshaw et al. 1980b; Stallknecht et al. 1990c).

2.3 TIME AND LOCATION OF SAMPLE COLLECTION

The time and location of sampling is an important factor in the isolation of AIV and APMV and both of these factors were influenced to a certain degree for the reasons outlined above. Fortunately for all parties, a good representation of birds (including juveniles) in different locations and covering the whole of New Zealand was an important issue when trapping birds. In a situation when trapping was carried out by contractors, such as AgriQuality (formerly MAFQual) livestock officers, or a private person, the same principles as far as time, location, and bird species of concern were applied.

Table 2.1 Location and number of mallard ducks sampled in January and March 1997 in New Zealand. The ratio of juvenile to adult and female to male ducks is shown for each location.

Sites	No. of ducks sampled	Juvenile/adult ratio	Female/male ratio
North Island			
Kaituna (Bay of Plenty)	70	40/30	33/37
Feilding (Manawatu)	68	6/62	52/16
Carterton (Wairarapa)	70	40/30	35/35
South Island			
Temuka (Milford Lagoon)	43	4/39	22/21
Invercargill (Lake Murihiku)	95	20.75	48/47
Total	346	110/236	190/156

A total of 346 mallard ducks (Table 2.1) were trapped in standard wire mesh-type traps (Plates 2.1 and 2.2) from February to March 1997 in five regions in New Zealand (Fig. 2.1) with the assistance of Regional Fish and Game Council personnel (Plate 2.3). Blood samples, tracheal and cloacal swabs were collected.

A total of 522 wild birds representing 24 species from 13 various parts of New Zealand (Fig. 2.2) were captured from December 1997 to February 1999 in mist or cannon nets and/or were sedated using grain treated with 0.5% alphachloralose. Blood samples (522 samples) and cloacal swabs (175 samples) were collected (Table 2.2).

A total of 231 caged birds representing 25 species from 14 locations of New Zealand including pet shops, aviaries, zoos, and private collections around New Zealand were sampled from December 1997 to April 1999 (Fig. 2.3, Table 2.3). Blood samples (231 samples) and cloacal swabs (116 samples) were collected. A small number of blood and cloacal samples were also submitted from veterinary practitioners.

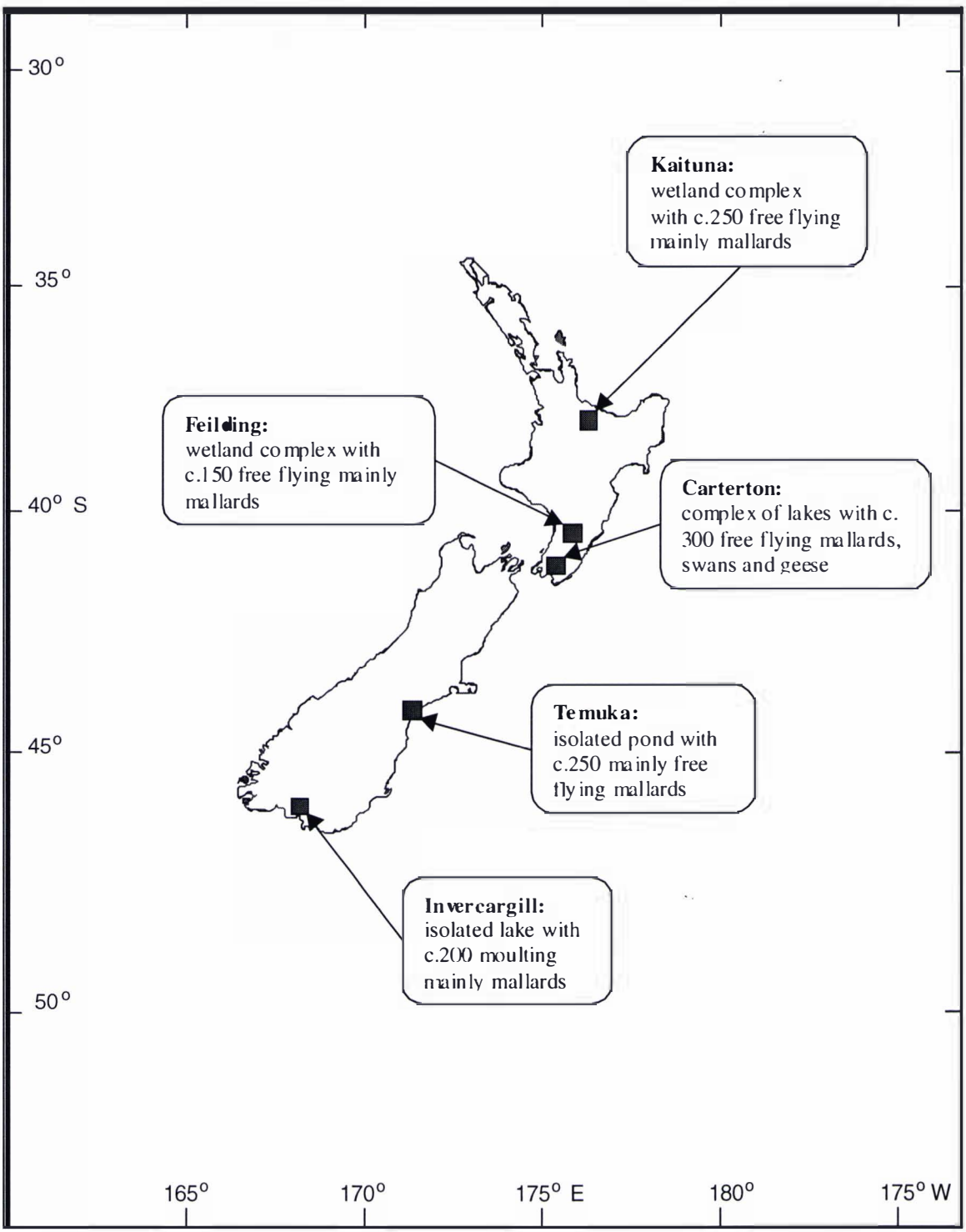


Fig. 2.1 Map of New Zealand showing mallard duck sites sampled during February–March 1997.

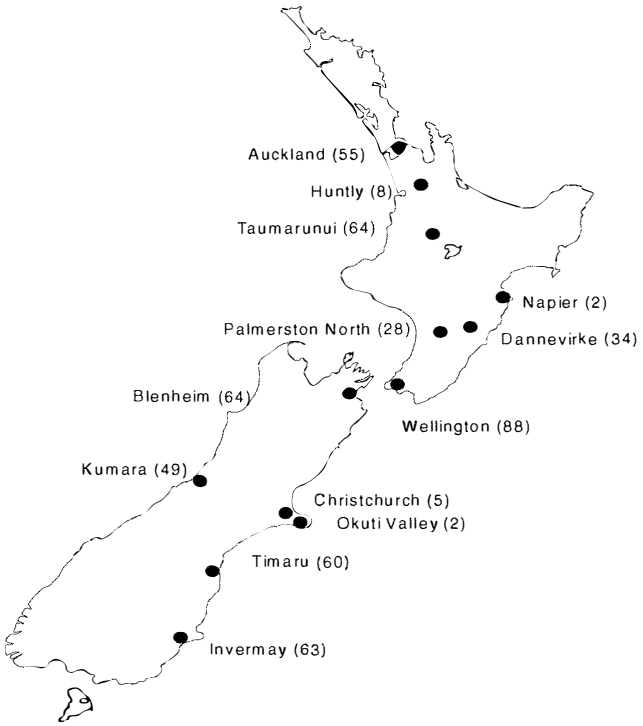


Fig. 2.2 Map of New Zealand showing locations of sampling sites and number of wild birds sampled from December 1997 to April 1999.

Table 2.2 Serum samples and cloacal swabs collected from wild birds between December 1997 and February 1999 from throughout New Zealand.

Bird species	No. of samples
House sparrow (*Passer domesticus*)	171
Greenfinch (*Corduelis chloris*)	127
Chaffinch (*Fringilla coelebs*)	60
Goldfinch (*Carduelis carduelis*)	24
Yellowhammer (*Emberiza citrinella*)	30
Redpoll (*Carduelis flammea*)	15
Blackbird (*Turdus melura*)	12
Silvereye (*Zosterops laterlis*)	13
Lesser knot (*Carlidris canutus*)	26
Rainbow lorikeet (*Trichoglossus haematodus*)	17
Rock pigeon (*Columba livia*)	4
New Zealand pigeon (*Hemiphaga novaeseelandiae*)	2
Australian magpie (*Gymnorhina tibicen*)	4
Australasian harrier (*Circus approximans*)	3
Pukeko (*Porphyrio porphyrio*)	3
Red billed gull (*Larus novaehollandiae*)	3
Black billed gull (*Larus bulleri*)	1
Australasian gannet (*Morus serrator*)	1
Bar-tailed godwit (*Limosa lapponica*)	1
Kingfisher (*Halcyon sancta*)	1
Shag (*Phalacrocorax* sp.)	1
Swan (*Cygnus* sp.)	1
Pheasant (*Phasianus colchicus*)	1
Australian coot (*Fulica atra*)	1
Total	522

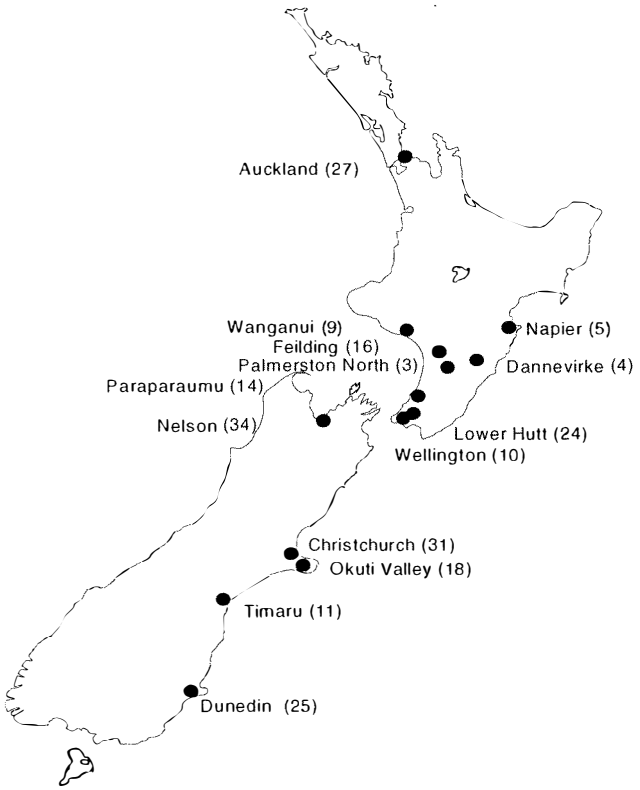


Fig. 2.3 Map of New Zealand showing locations of sampling sites and number of caged birds sampled from December 1997 to April 1999.

Table 2.3 Serum samples and cloacal swabs collected from caged birds between December 1997 and April 1999 from throughout New Zealand.

Species	No. of samples
Budgerigar (*Melopsittacus undulatus*)	48
Cockatiel (*Nymphicus hollandicus*)	34
Bourke's parrot (*Neophema bourkii*)	15
Turquoise parrots (*Neophema pulchella*)	5
Rosella (*Platycercus* spp.)	2
Lovebird (*Agapornis* spp.)	7
Elegant parrot (*Neophema elegans*)	4
Red-crowned parakeet (*Cyanoramphus novaezelandiae*)	1
Ring neck parrakeet (*Psittacus* spp.)	3
Red rump parrot (*Psephotus haematonotus*)	3
Lorikeet (*Glossopsitta* sp.)	1
Cockatoo (*Calyptorhynchus* spp.)	3
Domestic canary (*Serinus canarius domesticus*)	26
Zebra finch (*Poephila guttata*)	15
Java finch (*Lonchura oryzivora*)	6
Bengalese finch (*Lonchura striata domestica*)	4
Peafowl (*Pavo cristatus*)	3
Turkey (*Meleagris gallopavo*)	3
Helmeted guinea fowl (*Numida meleagris*)	10
Bantam (*Gallus* spp.)	3
Chinese silkey (*Gallus* spp.)	2
Rhode Island red X (*Gallus* spp.)	2
Chinese quail (*Coturnix chinensis*)	12
Dove (*Sreptopelia* spp.)	12
Pigeon (*Columba* spp.)	7
Total	231

Table 2.4 Poultry sera sampled from farms in different regions of New Zealand from 1996 to 1999. (-, no sera collected.)

	No. of birds/no. of farms				
Region	Broilers	Broiler-breeders	Laying hens	Turkeys	Ducks
Northland/ Auckland	98/8	73/5	142/15	-	76/2
Central North Island	214/13	195/11	110/12	7/1	15/1
Lower North Island	117/8	110/6	134/15	-	-
South Island	110/8	109/7	8/1	237/3	23/1
Total	539/37	487/29	394/43	244/4	114/4

2.4 PROCESSING OF SAMPLES

Whole blood samples for serology were collected from the wing or jugular vein using a 21-gauge needle for ducks and 25–27-gauge needles for small birds (Plates 2.4 and 2.5). Sera were to allowed to clot at 37°C for 1–2 h or overnight at room temperature. The serum was separated from blood clots by centrifugation at 2000*g* for 10 min and stored at –20°C until tested.

Tracheal and cloacal swabs for virus isolation were collected by inserting one cotton swab into the trachea (Plate 2.6) and a second swab into the cloaca (Plates 2.7 and 2.8), and rotating to obtain visible contamination. For small birds paediatric swabs were used to prevent injury. Tracheal swabs were only collected from mallard ducks because collection of these swabs from smaller birds could be harmful.

Swabs were placed into transport medium containing 10,000 units/ml penicillin, 10 mg/ml streptomycin, 250 μg/ml gentamycin, 5000 units/ml mycostatin, and 1% bovine serum albumin in isotonic phosphate buffered saline (PBS) (pH 7.4). The swabs were swirled around in the medium and then discarded. Samples were stored at –70°C until tested. Samples from the field may be stored up to 24 h at 4°C and than transferred to –70°C freezer.

A total of 1778 poultry serum samples were obtained from 117 different poultry farms throughout New Zealand. Samples were collected from broilers, broiler-breeders, and laying hens between October 1997 and April 1999, and from turkeys and ducks in 1996, 1998, and 1999 (Table 2.4).

2.5 DISCUSSION

During the design process of this project it was realised that it would be difficult to achieve all goals without compromise, including the number of samples collected from different species, the duration of sampling, and locations. This was particularly true for the samples collected from caged and from wild birds. From caged birds, the majority of samples were collected from aviaries and pet shops only from certain species (cost factor) and the number was also limited and very often regulated by the owner, preventing the use of any statistical approaches. The prevalence of AIVs and APMVs in caged birds is difficult to predict as no appropriate studies have been carried out in New Zealand in the past but we can estimate that the prevalence would be low. Using the epidemiological computer program "Win Episcope 2.0" we could determine the number of samples required for different size populations with different prevalence of disease and/or virus infection. For example, in an aviary with a population of 100 birds at 1% of virus infection prevalence, 96 samples would be required to detect at least one positive with 95% confidence. The number of samples would decrease in the same population if the prevalence rose and at 10% or 50% prevalence, the required samples would be 25 and 5 respectively. On average 10 samples were collected from one aviary. A similar situation was for the collection of samples from wild birds. Using the same epidemiological program with c. 5000 birds/flock of, e.g., lesser knots which we sampled in the Auckland area, with 1% prevalence of the disease/virus infection, 290 samples would be required to detect at least one positive with 95% confidence. The number of samples required would decrease when the prevalence increases and 29 and 5 samples would be required for 10% and 50% prevalence respectively. We collected only 26 samples, which means that at least one positive could be detected only, if the prevalence was 11% or above.

Although samples from caged and wild birds were collected from all over New Zealand, the design was based on convenience (e.g., associated with bird banding operations or access to big aviaries) rather that random sampling to guarantee that the samples would be representative.

However, sampling of mallard ducks was probably an exception, because the estimated prevalence of APMV and AIV in the duck population could be predicted more easily from a number of studies overseas. In addition, knowledge of other important factors also could influence the results, such as time of the year and/or ratio of susceptible birds (e.g., juvenile) and duration of virus shedding in ducks (Hinshaw et al. 1980b, 1985; Stallknecht et al. 1990b). Taking into consideration all these facts, sampling was planned in the New Zealand summer, early autumn when ducks congregate in lakes and ponds after the breeding season. All duck samples were collected from five locations within 3 weeks and an analysis of two of the sampling sites, Kaituna and Invercargill, can be used as a very good example of the importance of knowledge of the ecology of viruses. Using simply the same epidemiological program without other considerations, it would be possible to isolate a virus if the prevalence was not lower than 3% for Invercargill and 3.6% for Kaituna using data from Table 2.1 and Fig 2.1. Considering that the program estimates a very similar prevalence for both sampling sites the results obtained in this study were very different for these locations and both the results and the reason for this will be discussed in detail in the following chapters.

In poultry, nine samples would be required to detect at least one positive bird with antibodies with 95% confidence at a prevalence of 30% in 10 K or 100 K or 1 million bird population or farm. On average the number of samples collected from one poultry farm was higher than 9.

Thus for most of the sampling conducted in this study, nothing more can be claimed other than the actual results obtained on the specific samples that were tested. Virus either was isolated or it was not from the particular sample. Similarly antibody was either detected or not. It is not possible to extend from these results to make predictions about the whole population of particular species or classes of birds in New Zealand. The exceptions to this are clearly the mallard ducks and domestic poultry.

2.5 SUMMARY

Blood and tracheal and/or cloacal swabs were collected from 231 caged birds (representing 25 species), 522 wild birds (representing 24 species other than waterfowl), and 346 mallard ducks through out New Zealand. For caged and wild birds, samples were collected on the basis of convenience (e.g., associated with bird banding operations or access to big aviaries) rather that random sampling to guarantee that the samples would be representative. Samples from mallard ducks were collected in a more controlled manner to accommodate the ecology of APMVs and AIVs in ducks. Sera from poultry were collected during routine sampling procedures as a part of disease control programs.

Plate 2.1 Mallard ducks trapped in a standard wire mesh-type trap.

Plate 2.2 The author on location with trapped mallard ducks.

Plate 2.3 Regional Fish and Game Council personnel taking a break from setting up the traps.

Plate 2.4 The author collecting blood from the wing of a mallard duck.

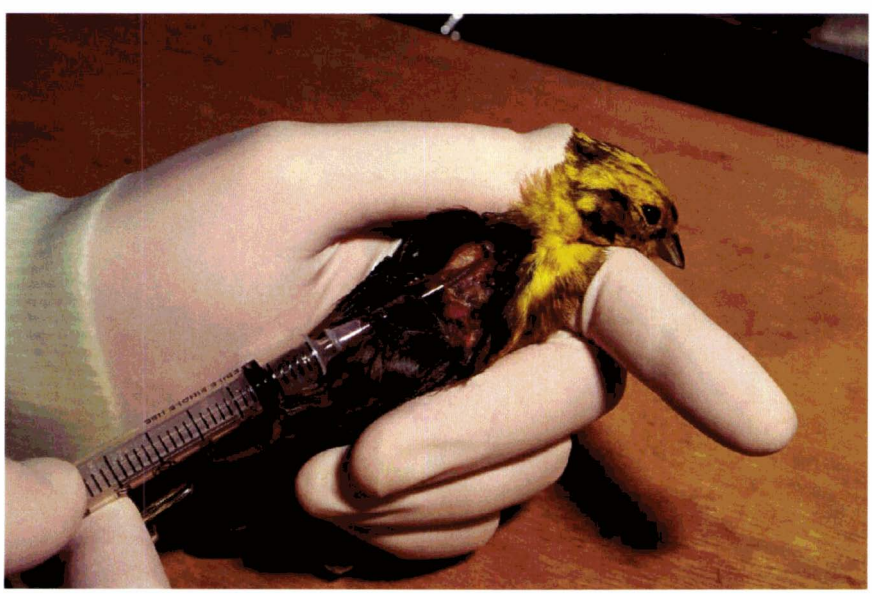


Plate 2.5 Blood collection from the jugular vein of a yellowhammer.

Plate 2.6 The author collecting a tracheal swab sample from a mallard duck.

Plate 2.7 Collection of a cloacal swab sample from a mallard duck.

Plate 2.8 Collection of a cloacal swab sample from a yellowhammer.

Chapter 3 Isolation of avian paramyxoviruses and avian influenza viruses from caged birds, wild birds, and mallard ducks

3.1 INTRODUCTION

Several factors influence successful virus isolation from clinical specimens and the most critical include: a suitable sample (site, size), the time of collection, an appropriate transport medium and transport condition, and adequate laboratory techniques.

The most commonly used host system, and probably the most sensitive for AIVs and the initial isolation of most APMVs, is embryonated fowl eggs either derived from a flock free of antibodies to these viruses or preferably from an SPF flock. Other host systems including primary chicken fibroblast cells or cell lines such as Vero and MDCK cells are used occasionally, particularly for AIV, but generally these cells are not recommended for primary isolation of AIV and APMV.

A number of techniques are used to confirm the presence of the virus including detection of HA, haemadsorbtion, ELISA, EM, immunostaining, and the RT-PCR test for viral RNA detection. Most of these techniques can be used directly to confirm the presence of the virus in a sample or the amount of virus can be amplified by passage through embryonated eggs. The choice of test will depend on the type of sample and laboratory capabilities.

The application of molecular diagnostics has improved dramatically during the last decade and revolutionised the way that laboratories provide virological services. This is particularly so in the field of human diagnostics where sensitivity and speed is critical and similar but less extensive application is made in veterinary laboratories. The high cost of equipment is always a consideration and it is necessary, as with any tests, to

remain conscious of the possible occurrence of false positive and false negative reactions.

3.2 MATERIALS AND METHODS

3.2.1 Embryonated fowl eggs

Fertile chicken SPF eggs were incubated for 9–10 days at 37–38°C and humidity 50–60% and were candled to identify live embryonated fowl eggs. Eggs were marked with a pencil to note the area of the major blood vessel near the membrane boundary. They were then swabbed with 2.5% potassium iodine solution in 95% ethanol to disinfect and a small hole was made in the egg shell using a metal "probe". Embryonated eggs prepared in this manner were used immediately for inoculation.

3.2.2 Processing and inoculation of tracheal and cloacal swabs

Tracheal and cloacal swabs samples stored at –70°C were thawed and centrifuged at 1000*g* for 10 min to clarify and the supernatant was removed and kept for inoculation. A 1 ml tuberculin syringe fitted with a 1.25 cm, 26-gauge needle, was used to inoculate 0.2 ml of sample into the allantoic cavity of an egg. The hole of inoculation was then sealed with nail polish and the egg was incubated at 35°C for 4 days. A minimum of 3–5 embryonated eggs were inoculated per sample. Eggs were candled daily to remove dead embryos and only those deaths that occurred more that 24 h after inoculation were examined to determine the cause of death.

3.2.3 Harvesting of allantoic and amniotic fluid

After 4 days all eggs were chilled at 4°C overnight to kill the embryo and to allow the blood to clot before harvesting. The presence of erythrocytes in the allantoic or amniotic fluids can significantly reduce the titre of AIV and APMV, which agglutinate erythrocytes. The surface of the egg shell was disinfected with 70% ethanol and using sterile forceps the egg shell was broken and allantoic and amniotic fluid aspirated into a vial. To clarify and reduce possible bacterial contamination the fluid was centrifuged at

1000*g* for 10 min and the supernatant was kept for either further inoculation and/or checked for HA activity. If no HA activity was detected the sample was passed once more for 4 days as described previously.

3.2.4 Haemagglutination test

Most APMVs and AIVs haemagglutinate chicken RBCs and this characteristic of these viruses was used to determine their presence in the allantoic/amniotic fluid. The HA test, following the procedure as described by Alexander (1996b), was performed as follows. Doubling dilutions of clarified allantoic/amniotic fluid collected from the inoculated embryonated eggs were made in 0.025 ml volume in PBS across a 96-well microtitre "V" bottom plate. After the addition of 1% chicken RBCs to the wells the plate was left at room temperature for the RBCs to settle (c. 40 min). The presence of haemagglutinating virus was detected when no button was formed on the bottom of the well and when the settled cells ran when the plate was tilted at c. 45 degrees. The titre of the sample was read as the highest dilution giving complete agglutination and this represented 1 HA unit. If haemagglutination was confirmed in the sample it was most likely due to the presence of APMV or AIV.

All allantoic fluid samples with HA were tested in parallel by the antigen capture ELISA for the detection of ND viral antigens (JCU Tropical Biotechnology Pty Ltd, Australia) and the antigen capture ELISA for the detection of AIV (described below). Samples that were negative in the NDV or AIV ELISA, were further tested by the HI test using a panel of reference antisera to all nine known types of APMV (Alexander 1996b). All AIVs were tested to determine the HA and NA glycoprotein subtypes by established procedures (Alexander 1996a).

3.2.5 Antigen capture ELISA for the detection of AIV

The ELISA was based on that described elsewhere (Siebinga & de Boer 1988) with some modifications. Briefly, the Nunc Maxisorp plates were precoated with 50 µl of purified AIV egg yolk antibody (A/Chicken/Vic/85-H7N7 obtained from Paul Selleck, AAHL, Geelong, Australia) diluted 1:2000 in 0.5*M* carbonate buffer (pH 9.6). Plates were incubated for 2 h at 37°C on a plate shaker at a speed of 400 rpm. After washing

(0.1% Tween 20 in PBS), 50 µl of allantoic fluid to be tested (diluted 1:10 in washing buffer with 1% bovine serum albumin) was added to each well and incubated for a further 30 min as above. Plates were washed again and 50 µl of a 1:1000 dilution of purified monoclonal antibody (against influenza A nucleoprotein obtained from hybridomas mAb anti-NP, ATCC No. HB65, H16-L10-R5 using the procedures of CELLMAX™ QUAD Artificial Capillary Cell Culture System, Cellco Inc, MD, United States and Z2-SEP™ Pharmacia Biotech) was added and incubated for 30 min, washed again, and a further 50 µl of anti-mouse IgG HRP conjugated antibody (DACO A/S, Denmark) diluted 1:3000 was added and incubated for a further 30 min as above. After the last washing 100 µl of TMB substrate (Shannon et al. 1991) was added and after 10 min the reaction was stopped by adding 50 µl of 1*M* H_2SO_4. The optical density (OD) was read at 450 nm using Multiscan, Flow, ELISA reader (Labsystems Multiskan® Multisoft) and the results were interpreted as described (Siebinga & de Boer 1988).

3.2.6 Haemagglutination inhibition test (HI)

All allantoic/amniotic fluid samples that were negative for AIV and NDV were further tested by the HI test (Alexander 1996b) using antigens and antisera specific for APMV-2 to -9 (except APMV-5) provided by D. J. Alexander from the ND and AI Reference Laboratory, United Kingdom. Briefly, 4 HA units of all tested samples, as determined in the HA test, were used in the HI test against reference antisera. The specific reaction with one of the reference sera was used to determine the serotype of APMV in the sample. APMV HA antigens of known specificity were tested in parallel to the sample to assure validity of the typing.

3.2.7 Subtyping of influenza viruses

The next step in the identification procedure was to determine the antigenic subtype of the HA and NA surface antigens. The HA antigen was identified using the HI test as described (Alexander 1996a) using a panel of 15 distinct HA antisera obtained from the AI Reference Laboratory, United Kingdom and the Australian Animal Health Laboratory (the last 15 HA subtype antiserum).

The NA subtype was determined following the procedure of a micro-NI test as described by Van Deusen et al. (1983) using nine known NA subtype antisera. Additionally, AIV isolates representing all determined subtypes were sent to the AI Reference Laboratory for confirmation.

3.3 RESULTS

A total of 116 cloacal swabs from 19 caged bird species and 175 cloacal swabs from 24 wild bird species (other than waterfowl) were tested with negative results for AIVs and APMVs and the only isolates obtained in this study were from mallard ducks.

A total of 321 tracheal and 321 cloacal samples was investigated for the presence of AIV and/or APMV from mallard ducks and 33 haemagglutinating viruses were isolated. Six of them were characterised as AIV (two H5N2 and four H4N6 subtypes), 10 APMV-1 (NDV), and 17 APMV-4. Isolates were obtained from both adult and juvenile and male and female ducks but only from three of the five sampling sites (Kaituna, Carterton, and Temuka). Of the isolates, 23 were from cloacal swabs and 10 were from tracheal swabs (Table 3.1 and Appendix A).

3.4 DISCUSSION

This current study is the most comprehensive study ever carried out on AIV and APMV in New Zealand for caged birds, wild birds, and waterfowl. Although the number of samples differs significantly from those collected in other countries in similar studies (particularly in waterfowl), it provides valuable information about these viruses from this part of the world.

In mallard ducks a total of 33 viruses were isolated: 6 AIV (two H5N2 and four H4N6), 10 APMV-1 (NDV), and 17 APMV-4. Most of the isolates (31/33) were made from ducks sampled at sites where juvenile ducks made up the majority of the duck population (Kaituna and Carterton). Juvenile ducks are most susceptible to AIV and

APMV infection and this higher rate of isolation from sites where they were present is consistent with the findings of researchers overseas (Hinshaw et al. 1980b).

Table 3.1 Number of avian influenza viruses (AIV) and avian paramyxoviruses (APMV) isolated from tracheal and/or cloacal swabs collected from mallard ducks in January and March 1997 in New Zealand.

Site	No. of samples	No. of isolates (% of total samples)	Isolates from tracheal/cloacal swabs	Isolates		
				AIV	APMV-1	APMV-4
North Island						
Kaituna (Bay of Plenty)	70	20(28.6)	6/14	4	1	1
Feilding (Manawatu)	68	0	0	0	0	0
Carterton (Wairarapa)	60	11(18.3)	4/7	0	9	2
South Island						
Temuka (Milford Lagoon)	43	2(4.7)	0/2	2	0	0
Invercargill (Lake Murihiku)	80	0	0	0	0	0
Total	321	33(10.3)	10/23	6	10	17

A greater proportion of isolates was obtained from cloacal (23/33) than from tracheal swabs (10/33) and 20/33 of the isolates were made from ducks of the Kaituna wetland in the Bay of Plenty where juvenile ducks were predominant. Additionally, in this wetland complex at this time of the year there was a lot of duck movement where ducks from various locations around the complex were flocking for better habitats.

By contrast, at the Invercargill site, where no viruses were isolated, the majority of ducks were moulting adult ducks with no possibility of flying for a number of weeks. Therefore, at Invercargill there was very little bird movement at this time of the year, compared to the Kaituna site.

The limited AIV subtypes isolated during this study may not reflect the true situation with AIV in the New Zealand duck population as a year-to-year fluctuation of the subtype circulation in waterfowl has been confirmed in other similar studies in other

countries (Hinshaw et al. 1980b). In addition, the samples were collected during one day at a particular site and sampling did not continue for 2 or 3 months, for example, which could also make a difference and result in more AIV isolates including those of other HA/NA combinations.

The APMV isolates obtained in this waterfowl study confirms the findings reported in similar studies in New Zealand (Austin & Hinshaw 1984) and in other parts of the world (Hinshaw et al. 1980b), that the most commonly APMV isolates obtained from waterfowl, particularly from ducks, are APMV-1, APMV-4, and APMV-6. Although APMV-6 was not isolated in this study, there was convincing evidence of the presence of the virus in New Zealand ducks from the serological investigation (see Chapter 4).

The lack of isolation of AIVs and of APMVs from caged and wild birds could be due to a number of reasons. The majority of AIVs and APMVs that have been isolated from caged birds were made from birds in quarantine (Alexander et al. 1982b; Senne et al. 1983) and New Zealand has strict quarantine procedures to prevent the entry of avian viruses in imported birds or bird products. Although the MAF bird importation protocols are very effective at preventing the entry of exotic viruses including AIV and APMV, illegal importation of birds and birds products (e.g., fertile eggs) does occur sporadically keeping open the possible entry of these viruses by this means.

A greater number of samples collected over longer period than those used in this study are required to better assess the prevalence, because there is little information on the susceptibility, duration of infection, and virus shedding.

3.5 SUMMARY

No AIV or APMV viruses were isolated from caged birds (from 19 species) and wild birds (24 species) in this study, which may be due to low prevalence of these viruses in New Zealand in the birds specie sampled. However, different results were obtained in this study which fully reinforce findings by researches in other countries that waterfowl, ducks in particular, are the reservoir of all known AIVs and selective APMVs. Most of

the isolates were APMV-1 and APMV-4 obtained form both juvenile and male and female ducks at the sites where juvenile ducks made up the majority of the duck population. Juvenile ducks are more susceptible to infection with AIV and APMV and this is consistent with the results of surveillance studies in other part of the world. Although, only 6 of 33 of the total isolates were AIV, two of them were subtype H5N2, one of two subtypes (H5 and H7) that cause outbreak of disease with devastating outcome in poultry.

Chapter 4 Surveillance for avian paramyxovirus antibodies in mallard ducks, caged birds, wild birds, and poultry

4.1 INTRODUCTION

The first report of serological evidence of APMVs in New Zealand was in poultry flocks in 1966 (Anon. 1978), which coincided with the isolation of the V4 strain of APMV-1 from chickens in Queensland, Australia (Simmons 1967) in the same year. This finding probably precipitated investigations to determine the status of APMV-1 in New Zealand poultry and later on to determine the NDV status.

In passive surveillance for avian APMVs between 1972 and 1977, about 800 samples from chickens with clinical signs of respiratory disease and/or egg production drop were submitted to Ruakura and the Central Animal Health Laboratory (present NCDI) for virus isolation. No virus was isolated from the samples under investigation (Carter 1977).

In serological surveys carried out between 1972 and 1977, APMV-1 infection in poultry was detected by the HI test at very low levels in 2 out of 37 commercial chicken flocks in the Christchurch area (Carter 1977). Pre-export testing of pheasants (*Phasianus colchicus*) in 1973 and of peafowl (*Pavo cristatus*) in 1976, revealed antibody reactive with APMV-1 in 75 of 220 and 4 of 6 respectively (Carter 1977).

Following the isolation of APMV-1 in 1978 (Anon. 1978), the Poultry Industry uses serology on a routine basis particularly to monitor their breeding stocks (B. Jones & D. Marks pers. comm.) or to exclude APMV-1 infection in birds with clinical signs (Howell 1990). For example in 1997, 8376 sera from commercial poultry flocks were

tested and 202 had positive titres (Anon. 1998) whereas in 1998, 8113 sera were tested and 12 were positive (Anon. 1999). Samples from one of the serologically positive surveillance broiler-breeder farms were cultured for virus in 1995. APMV-1 was isolated from cloacal swabs collected from birds (with no clinical signs) in the early stages of infection. No routine serology was performed for the other eight known APMVs in New Zealand except occasional testing associated with imported birds (Stanislawek unpubl. data).

Despite rapid developments with new laboratory diagnostic tests, the HI test still remains as the test of choice in serological research/investigations, which aim to distinguish antibodies to any of the nine known APMVs, because of the ease of performance and high reproducibility when quality control procedures are in place in a laboratory.

This chapter describes an investigation of mallard ducks, caged birds, wild birds (other than waterfowl), and poultry for the presence of HI antibodies using up to eight different APMV antigens per type of bird listed above as appropriate.

4.2 MATERIALS AND METHODS

4.2.1 Haemagglutination inhibition test

Serum samples were examined in the HI test using standard procedures (Alexander 1996a) for antibodies to the following viruses: the New Zealand APMV-1 isolate (7579/78) obtained from commercial poultry; APMV-2 (Ck/Cal/Yucaipa/56); APMV-3, (Turkey/Wisconsin/68); APMV-4 P/Duck/HK/D3//75; APMV-6 P/Duck/HK/199/77; AMPV-7 P/Dove/Tenn/4/75; AMPV-8 P/Goose/Del/1053/76; and AMPV-9 P/DomDuck/NY/22/78.

Sera from species other than poultry were first absorbed with chicken RBCs by gently mixing the serum with 20% v/v of packed RBCs that had been washed 3 times in PBS. The mixture was left for 30 min at room temperature and then pelleted by

centrifugation at 800*g* for 5 min. Such absorbed sera were heat-inactivated at 56°C for 30 min. All poultry sera were heat-inactivated only.

Table 4.1 Haemagglutination inhibition (HI) test results for avian paramyxovirus (APMV) types 1, 2, 3, 4, 6, 7, 8, and 9 antibodies of 315 mallard duck serum samples collected in January and March 1997 from five sites of New Zealand.

Titre	No. (%) of sera reacted with HI antigen							
	APMV-1	APMV-2	APMV-3	APMV-4	APMV-6	APMV-7	APMV-8	APMV-9
1:4	46(14.6)	35(11.1)	14(4.4)	87(27.6)	94(29.8)	106(33.7)	120(38.1)	41(13)
1:8	101(32.1)	7(2.2)	5(1.6)	57(18.1)	125(39.7)	33(10.5)	45(14.3)	6(1.9)
1:16	90(28.6)	0	0	14(4.4)	38(12.1)	7(2.2)	8(2.5)	3(1.0)
1:32	40(12.7)	0	0	5(1.6)	6(1.9)	2(0.6)	6(1.9)	0
≥1:64	16(5.1)	0	0	0	5(1.6)	0	0	0
Total	293(93.0)	42(13.3)	19(6.0)	63(51.7)	268(85.1)	148(47)	179(56.8)	50(15.9)

The HI test was performed in V-bottom, 96-well microtitre plastic plates. Doubling dilutions of sera in PBS, starting from 1:2 (25 µl of tested serum and 25 µl of PBS), were made across the plate. Four units of relevant APMV HI antigen in a 25 µl volume was then added to each well (except the first well as a serum control) and the plates were incubated at room temperature for 30 min. Following this incubation 25 µl of 1% chicken RBCs in PBS was added to every well and the plates were incubated at room temperature for a further 40 min.

The agglutination was assessed by tilting the plate c. 45 degrees. All results were expressed as the reciprocal of the highest dilution of serum inhibiting 4 HA units of the antigen.

4.3 RESULTS

A total of 315 duck sera was tested by the HI test and the antibodies most commonly found, and with the highest titres, were to APMV-1 (93.1%), followed by APMV-6 (85.1%), APMV-8 (56%), APMV-4 (51.7%), APMV-7 (47%), APMV-9 (15.9%), APMV-2 (13.3%), and APMV-3 (6.0%) (Table 4.1).

4.3.1 Caged birds

A total of 231 caged birds representing 25 species from many areas of New Zealand were sampled during the period December 1997–April 1999 (Chapter 2, Table 2.3).

Antibodies to APMV-1 were detected in 11 sera from six species, with titres ranging from 1:8 to 1:1024. Antibodies to APMV-2 were found in four sera from two species, with titres ranging from 1:4 to 1:32, and antibodies to APMV-3 were found in six sera from two species, with titres ranging from 1:4 to 1:8 (Table 4.2).

4.3.2 Wild birds

A total of 522 wild birds representing 24 species was sampled between December 1997 and February 1999 (Chapter 2, Table 2.2).

Antibodies to APMV-1 were detected in sera from nine birds of four different species, to APMV-2 in sera from 22 birds of nine different species, and to APMV-3 in sera from only four birds of two species (Table 4.3). HI titres were low (from 1:4 to 1:8) with the exception of one sample from a rainbow lorikeet with a titre of 1:16 for APMV-2.

4.3.3 Poultry

Only five chicken samples (one from a broiler chicken, four from broiler-breeders; from five different farms) had low titres to APMV-3 (1:8 to 1:16) and titres ranged from 1:16

to 1:1024 when tested with APMV-1 antigen. No HI reactions with APMV-2 were detected (for types of samples collected see: Chapter 2, Table 2.4).

Table 4.2 Serum samples collected from caged birds between December 1997 and April 1999 from throughout New Zealand. Haemagglutination inhibition (HI) test results for avian paramyxovirus (APMV) types 1, 2, and 3 antibodies. (a = sera which reacted to APMV-1 antigen with titres 1:8 to 1:1024; b = sera which reacted to APMV-2 antigen with titres 1:4 to 1:32; c = sera which reacted to APMV-3 antigen with titres 1:4 to 1:8. N/T = not tested. - = no antibodies detected at a serum dilution of 1:4.)

Bird species	No. of sera tested	No. (%) of sera reacted with HI antigen		
		APMV-1[a]	APMV-2[b]	APMV-3[c]
Budgerigar (*Melopsittacus undulatus*)	48	-	-	-
Cockatiel (*Nymphicus hollandicus*)	34	-	-	-
Bourke's parrot (*Neophema bourkii*)	15	-	-	-
Turquoise parrot (*Neophema pulchella*)	5	-	-	-
Rosella (*Platycercus* spp.)	2	-	-	-
Lovebird (*Agapornis* spp.)	7	1 (14.3)	-	-
Elegant parrot (*Neophema elegans*)	4	-	-	-
Red-crowned parakeet (*Cyanoramphus novaezelandiae*)	1	-	-	-
Ring neck parakeet (*Psittacula krameri*)	3	-	-	-
Red rump parrot (*Psephotus haematonotus*)	3	-	-	-
Lorikeet (*Glossopsitta* sp.)	1	-	-	-
Cockatoo (*Cacatua galerita*)	3	-	-	-
Domestic canary (*Serinus canarius domesticus*)	26	2 (7.7)	1 (3.9)	2 (7.7)
Zebra finch (*Poephila guttata*)	15	-	-	-
Java finch (*Lonchura oryzivora*)	6	-	-	-
Bengalese finch (*Lonchura striata domestica*)	4	-	-	-
Peafowl (*Pavo cristatus*)	3	-	-	-
Turkey (*Meleagris gallopavo*)	3	1 (33.3)	-	-
Helmuted guinea fowl (*Numida meleagris*)	10	5 (50)	3 (30)	4 (40)
Bantam (*Gallus* spp.)	3	-	-	-
Chinese silkey (*Gallus* spp.)	2	-	-	-
Rhode Island Red X (*Gallus* spp.)	2	1 (50)	-	-
Chinese quail (*Coturnix chinensis*)	12	1 (8.3)	-	-
Dove (*Streptopelia* spp.)	12	-	-	-
Pigeon (*Columba* spp.)	7	-	-	-
Total	231	11 (4.8)	4 (1.7)	6 (2.6)

Table 4.3 Serum samples collected from wild birds between December 1997 and February 1999 from throughout New Zealand. Haemagglutination inhibition (HI) test results for avian paramyxovirus (APMV) types 1, 2, and 3 antibodies. (a = sera which reacted to APMV-1 antigen with titres 1:4 to 1:8; b = sera which reacted to APMV-2 antigen with titres 1:4 to 1:16; c = sera which reacted to APMV-3 antigen with titres 1:4 to 1:8. - = no antibodies detected at a serum dilution of 1:4.)

Bird species	No. of sera tested	No. (%) of sera reacted with HI antigen		
		APMV-1[a]	APMV-2[b]	APMV-3[c]
House sparrow (*Passer domesticus*)	171	-	7 (4)	-
Greenfinch (*Carduelis chloris*)	127	-	1 (0.8)	-
Chaffinch (*Fringilla coelebs*)	60	-	1 (1.7)	-
Goldfinch (*Carduelis carduelis*)	24	-	-	-
Yellowhammer (*Emberiza citrinella*)	30	-	1 (3.3)	-
Redpoll (*Carduelis flammea*)	15	-	-	-
Blackbird (*Turdus merula*)	12	-	3 (25)	-
Silvereye (*Zosterops lateralis*)	13	-	-	-
Lesser knot (*Calidris canutus*)	26	6 (23)	4 (15.4)	3 (11.5)
Rainbow lorikeet (*Trichoglossus haematodus*)	17	1 (5.9)	3 (17.6)	1 (5.9)
Rock pigeon (*Columba livia*)	4	-	-	-
New Zealand pigeon (*Hemiphaga novaeseelandiae*)	2	-	-	-
Australian magpie (*Gymnorhina tibicen*)	4	-	-	-
Australasian harrier (*Circus approximans gouldi*)	3	-	-	-
Pukeko (*Porphyrio porphyrio*)	3	-	-	-
Red-billed gull (*Larus novaehollandiae scopulinus*)	3	-	-	-
Black-billed gull (*Larus bulleri*)	1	-	-	-
Australasian gannet (*Morus serrator*)	1	-	-	-
Bar-tailed godwit (*Limosa lapponica*)	1	-	-	-
Kingfisher (*Halcyon sancta vagans*)	1	-	-	-
Shag (*Phalacrocorax* sp.)	1	-	-	-
Swan (*Cygnus* sp.)	1	-	-	-
Pheasant (*Phasianus colchicus*)	1	-	-	-
Australian coot (*Fulica atra australis*)	1	1 (100)	1 (100)	-
Total	522	9 (1.7)	22 (4.2)	4 (0.8)

4.4 DISCUSSION

The APMV serological study clearly shows the variations of paramyxovirus antibodies in different bird species. The APMV antibodies were found most commonly in mallard ducks and this was reflected in the number of APMV isolates obtained in this study. In contrary, antibodies were detected very sporadically and very often with unclear interpretation in wild birds and caged birds (with a few exceptions such as "fancy" poultry) as well as in poultry.

Eleven caged birds from a total of 231 reacted with APMV-1 antigen and five of these were from guinea fowl kept in one bird sanctuary. Most of the six titres to APMV-3 were lower than titres of the same samples to APMV-1, and these probably represent cross-reactions resulting from infection with APMV-1, as has been recorded previously (Smit & Rondhuis 1976). The situation is slightly different for APMV-2 because cross-reactions between AMPV-2 and APMV-1 do not occur to the same degree as between APMV-3 and APMV-1. Therefore these reactions may indicate past infection with APMV-2, or with another APMV such as APMV-6, which cross-reacts most significantly with APMV-2 (Shortridge et al. 1980).

With the canary sera, one sample reacted with all three HI antigens, with titres of 1:32 and another reacted with APMV-1 and APMV-3 HI antigens with titres of 1:8. It is probable that these are non-specific reactions, or possibly, a cross-reaction to other APMVs. The assumption of this results were made on the basis that cross-reactions to such high titres (1:32) occur very rarey (see Appendix A for mallard duck results as a example in addition to my 16 years of experience) and testing with other APMVs could put more light onto this case. Additionally we should also interpret these results through the epidemiology of APMVs in New Zealand, low prevalence of APMV-1 in poultry, wild birds, and caged birds and the unconvincing presence of APMV-2 and -3.

In wild birds, the highest number of reactors was found amongst passerine birds, which made up 87% of the birds tested in this study. All were reactions to APMV-2 antigen. However, these results should be treated cautiously because of the low titres obtained

(1:4 to 1:8), so cross-reactions to other APMVs (or non-specific reactions) cannot be excluded. The finding that the titres in sera from the lesser knot were the same for all three APMV antigens used suggests a non-specific reaction. There is one exception—in a rainbow lorikeet sample with a titre of 1:16 to APMV-2 antigen but no reaction with APMV-1 or -3. These data do not provide convincing evidence for the presence of APMV-3 in wild birds and are consistent with reports that APMV-3 has not been found in wild passerine birds (Alexander 1993b).

In the survey of commercial poultry in this study, four of the five sera that reacted with APMV-3 antigen had higher titres when tested with APMV-1 (ranging from 1:128 to 1:1024), suggesting that these were cross-reactions to APMV-3 antigen. The one remaining chicken serum had a titre of 1:16 to APMV-3 antigen, which may have been a non-specific reaction. Only APMV-2 and APMV-3 antigens were used for testing poultry samples because sufficient information on APMV-1 antibodies is provided by routine surveillance. In 1997, for example, 202 of 8376 poultry sera tested were found positive to APMV-1 (Anon. 1998). In 1998, only 12 sera of 8113 tested were positive (Anon. 1999). APMV-1 vaccine is not used in New Zealand and the seropositive flocks were probably the result of infection with non-pathogenic APMV-1 strains circulating in waterfowl and occasionally infecting poultry flocks. Such a hypothesis is entirely consistent with the finding of many positive reactions to APMV-1 in the duck sera as shown in Table 4.1. It also highlights the need to ensure biosecurity on poultry farms to exclude direct or indirect contact between chickens and wild waterfowl, particularly so since it now appears that highly virulent strains of APMV-1 might arise from strains originally derived from wild birds, once those strains are circulating in domestic chickens (Garcia et al. 1996; Westbury 2001).

In mallard ducks most serological reactions, and those with the highest titres, were to APMV-1 (93%), followed by APMV-6 (85.1%) and APMV-4 (51.7%). Such results are not surprising because APMV-1, -4, and -6 are the most commonly isolated viruses from ducks in other parts of the world (Deibel et al. 1985; Hinshaw et al. 1985). There is insufficient information to make similar deductions for other APMVs by analysing the serological results.

The results for APMV-2, -3, -7, -8 (despite the high percentage of reactors, 56.8%), and -9 should also be interpreted cautiously because the titres obtained with these antigens were the same or lower than with APMV-2, -3, -7, and -9—except in a few samples with higher titres to APMV-8. Cross-reactions to other APMVs or non-specific reactions cannot be excluded and there are reports that APMV-2 cross-reacts with other APMVs, particularly with APMV-6 (Shortridge et al. 1980). Also, APMV-3 has been shown to cross-react with APMV-1 (Smit & Rondhuis 1976).

At sites where there were mainly adult ducks, a higher number of APMV reactors and higher APMV titres were found. This pattern would be expected because adult ducks have many more opportunities to be exposed to APMV over time.

The serological results indicate that the presence of APMV-1 infection is very low in the caged and wild birds but very high in mallard ducks. This is also confirmed by the high number of APMV-1 isolates obtained from ducks (see Chapter 3). There is no convincing evidence for the occurrence of APMV-3, and we cannot confidently assess the true prevalence of APMV-2 in New Zealand in species tested from these results. A greater number of samples collected over longer time periods than those used in this study are required to better assess the prevalence of APMV-2 and -3 in these populations because there is little information on the susceptibility for some of the caged and wild birds in particular, as well as the duration of infection, viral shedding, and immune response. Therefore, it is difficult to interpret the negative results or very low titres obtained in this survey. A similar study in Australia (Garnett & Flanagan 1989) failed to detect APMV-1 antibody or virus in the wild bird population of northern Queensland, and that study, together with the survey reported here, indicates that there is low prevalence of APMVs in some bird species.

4.5 SUMMARY

Variations of APMV antibodies in different bird species were detected. APMV antibodies were found most commonly in mallard ducks in particularly to APMV-1 and APMV-6 viruses. In contrary in wild birds and caged birds (with a few exceptions such

as fancy poultry where antibodies to APMV-1 virus were detected) as well as poultry, antibodies were detected very sporadically and very often with unclear interpretation.

There is no conclusive evidence of the presence of APMV-2 and APMV-3 in poultry or APMV-3 in wild birds. The results do not provide conclusive evidence for the presence of APMV-2 in wild birds in New Zealand.

Chapter 5 Prevalence of AIV antibodies in mallard ducks

5.1 INTRODUCTION

Clinical signs attributed to influenza A infection have not been recorded in poultry or other types of birds in New Zealand. This situation is perhaps the most important reason why there are little data on AIV serology in contrast to APMV. In the past virus isolation was always the preferred means to determine whether influenza virus was present in birds in New Zealand (Austin & Hinshaw 1984; Stanislawek 1992) because of the more precise biological, antigenic, and genetic information that could be gained from any isolates that were obtained.

The number of AIV subtypes (15 H and 9 N subtypes currently recognised) and the variation within the subtypes creates a number of technical problems for the serological diagnosis of influenza with techniques involving surface antigens such as HA or NA unless the known HA subtype is targeted and the specific HI antigen used. Therefore, the most practical tests are group-specific tests such as AGID against the group antigen of influenza A viruses carried by nucleocapsid or matrix protein (Beard 1970). Specific antibodies are produced and are detectable by the AGID test in turkeys, chickens, Canada geese, pheasants, quails, and flamingos but they may not be detectable in other species including ducks (Slemons & Easterday 1972; Hinshaw et al. 1980a; Alexander & Spackman 1981; Alexander & Allan 1982). However, the ease with which antibodies are detected may vary not only within the host species but also between AIVs, and be a reflection of their virulence and antigenicity (Alexander 1993a). Therefore in addition to the AGID test, other type-specific tests, perhaps more sensitive than AGID such as ELISA have been developed and chosen to screen duck sera for antibody to AIV.

5.2 MATERIALS AND METHODS

5.2.1 Agar gel immunodifusion test (AGID)

The AGID test was performed following the procedure of Alexander (Alexander 1996a) using the New Zealand isolate A/Mallard/1/90 (H4N6) (Stanislawek 1992) as an antigen.

The AGID antigen was produced from infectious allantoic fluid harvested from embryonating eggs that had been inoculated with AIV H4N6 subtype and incubated for 4 days at 37°C. Allantoic fluid was inactivated for c. 12 h at 37°C by adding formalin in a final volume 0.1%. After clarification at 1000*g* for 10 min, the supernatant was centrifuged at 40,000*g* for 1.5 h and the pellet was resuspended (1:100 to the original volume) in glycine-sarcosyl buffer consisting of 1% (w/v) sodium lauryl sarcosinate buffered to pH 9 with 0.5*M* glycine. Such prepared antigen contained both nucleocapsid and matrix polypeptide antigens (Alexander 2000a).

The test was carried out using a gel of 1% w/v agarose and 8% NaCl in 0.1*M* PBS pH 7.2–4. Twenty ml of gel was poured into a Petri dish and, using a template, sets of seven wells, 5 mm in diameter and c. 3 mm apart, were cut. AIV positive control sera, and antigen were placed in such a manner that the test serum was adjacent to them. Approximately 50 µl of each test serum was added to each well. The Petri dishes were incubated at room temperature in a humidified box and the results were read after 24 and 48 h using bright, angled light against a dark background (Alexander 2000a). The appearance of a precipitin line of identity between the known positive serum, tested serum and antigen was recorded as a positive result.

5.2.2 Nucleoprotein-blocking ELISA (NP-B-ELISA) procedure

The sera were also tested by AIV nucleoprotein-blocking ELISA (NP-B-ELISA) using procedures similar to those previously described (de Boer et al. 1990). The NP-B-ELISA was performed as follows: Nunc Maxisorp plates were precoated with 50 µl of purified AIV egg yolk antibody (A/Chicken/Vic/85-H7N7 obtained from Paul Selleck,

AAHL, Geelong, Australia) diluted 1:1000 in 0.5*M* carbonate buffer (pH 9.6). Plates were incubated for 2 h at 37°C on a plate shaker at a speed of 400 rpm. After washing at least 4 times in washing buffer (0.1% Tween 20 in PBS), 25 µl of NP-40 treated antigens (equal volumes of 1 µg/ml of H1N1 95/2918 human isolate (obtained from David Featherston, CDC, Porirua, New Zealand) and H4N6 duck isolate (Stanislawek 1992)) purified as described elsewhere (Meulemans et al. 1987)) were added. At the same time, 25 µl of tested serum (diluted 1:10 in washing buffer with 2% bovine serum albumin) was added to the same well and incubated for 60 min. Plates were washed again and 50 µl of purified monoclonal antibody (against influenza A nucleoprotein obtained from hybridomas mAb anti-NP, ATCC No. HB65, H16-L10-R5 using the procedures of CELLMAX™ QUAD Artificial Capillary Cell Culture System, Cellco Inc, MD, United States and Z2-SEP™ Pharmacia Biotech) was diluted 1:1000 and incubated for 30 min, washed again, and a further 50 µl of anti-mouse IgG HRP conjugated antibody (DACO A/S, Denmark) diluted 1:3000 was added and incubated for a further 30 min as above. After the last washing 100 µl of TMB substrate (Meulemans et al. 1987) was added and after 10 min the reaction was stopped by adding 50 µl of 1*M* H_2SO_4. The optical density (OD) was read at 450 nm using Multiscan, Flow, ELISA reader (Labsystems Multiskan® Multisoft) and the results were interpreted following the formula:

$$\% \text{ inhibition} = \frac{\text{OD control negative serum} - \text{OD test serum}}{\text{OD control negative serum} - \text{OD control high titre serum}} \times 100$$

where OD = optical density at 450 nm. The mean of at least four positive and negative control well sera were used in this formula. Sera with inhibition of 50% or greater were considered positive.

5.2.3 Nucleoprotein-blocking ELISA (NP-B-ELISA) evaluation trial

Ten 8-week-old SPF chickens were inoculated intraocularly and intranasally with 100 µl of AIV subtype H6N4 virus (50 µl in each route) and blood samples were collected (from wing vein) for testing at Day: 0 (before inoculation), 1, 2, 3, 4, 5, 7, 10, 14, 21,

28, and 30 (days after inoculation). Serum samples were tested using the HI test (with AIV subtype H6N4 HA antigen), AGID test, and NP-B-ELISA, to study analytical specificity and sensitivity of the NP-B-ELISA using 2 × 2 contingency table as part of Win Episcope 2.0 computer program. True/false positive and negative was determined by evaluating HI and AGID results following OIE recommendations (Alexander 2000a).

5.3 RESULTS

5.3.1 Mallard duck study

A total of 335 duck sera were tested by AGID and NP-B-ELISA. Only five sera tested by AGID were positive but 109 (32.5%) including the five positive tested by AGID, were positive by NP-B-ELISA. The number of positive samples detected by NP-B-ELISA at each sampling site varied, and this includes:

Kaituna 12/67 (17.9%);
Feilding 24/68 (35.3%);
Carterton 13/66 (19.7%);
Temuka 19/41 (46.3%); and
Invercargill 41/93 (44.1%) (Appendix A).

5.3.2 Nucleoprotein-blocking ELISA (NP-B-ELISA) evaluation trial

The NP-B-ELISA detected AIV antibodies in chickens as early as 4 days after inoculation, where two other tests, HI and AGID antibodies were detected at 5 and 7 days after inoculation respectively (Table 5.1).

The analytical sensitivity and specificity of the NP-B-ELISA at the 95% confidence level were as follows: sensitivity 100%, specificity 84.6%, positive predictive value 85.5%, and negative predictive value 100%.

Table 5.1 Detection of antibody by HI, AGID, and NP-B-ELISA in sera of chickens inoculated with AIV A/Mallard/NZ/2/91 (subtype H6N4).

Chicken ID	Time of sampling (days post inoculation)	HI results using H6N4 antigen (titres)	AGID results	ELISA results (% inhibition)
1010	0	<2	Negative	0.0
	1	<2	Negative	0.0
	2	<2	Negative	11.9
	3	<2	Negative	0.0
	4	<2	Negative	0.0
	5	<2	Negative	31.7
	7	128	Positive	94.1
	10	256	Positive	93.1
	14	512	Positive	94.6
	21	>2048	Positive	98.1
	28	256	Positive	95.3
	30	64	Positive	93.7
1016	0	<2	Negative	6.6
	1	<2	Negative	0.0
	2	<2	Negative	0.0
	3	<2	Negative	33.8
	4	<2	Negative	64.7
	5	2	Negative	84.2
	7	>2048	Positive	98.7
	10	>2048	Positive	96.9
	14	>2048	Positive	96.9
	21	>2048	Positive	99.9
	28	>2048	Positive	98.6
	30	>2048	Positive	98.7
1052	0	<2	Negative	0.0
	1	<2	Negative	1.2
	2	<2	Negative	7.1
	3	<2	Negative	0.0
	4	<2	Negative	11.1
	5	<2	Negative	69.9
	7	8	Positive	77.9
	10	256	Positive	82.6
	14	256	Positive	89.7
	21	256	Positive	96.3
	28	512	Positive	98.9
	30	512	Positive	96.8
1057	0	<2	Negative	4.9
	1	<2	Negative	5.6
	2	<2	Negative	5.1
	3	<2	Negative	1.0
	4	<2	Negative	5.1
	5	<2	Negative	40.1
	7	64	Positive	97.0
	10	256	Positive	97.0
	14	1024	Positive	97.2
	21	256	Positive	99.7
	28	128	Positive	97.4
	30	256	Positive	97.9
1079	0	<2	Negative	0.0
	1	<2	Negative	0.0
	2	<2	Negative	0.0
	4	<2	Negative	0.0
	5	<2	Negative	59.3
	7	64	Positive	96.6
	10	>2048	Positive	96.2
	14	>2048	Positive	96.1
	21	1024	Positive	98.6
	28	512	Positive	98.6
	30	>2048	Positive	97.3

Chicken ID	Time of sampling (days)	HI results using H6N4 antigen (titres)	AGID results	ELISA results (% inhibition)
1080	0	<2	Negative	0.0
	1	<2	Negative	0.3
	2	<2	Negative	5.8
	3	<2	Negative	0.0
	4	<2	Negative	30.5
	5	<2	Negative	61.4
	7	16	Positive	96.9
	10	>2048	Positive	98.3
	14	>2048	Positive	98.3
	21	>2048	Positive	98.1
	28	1024	Positive	98.9
	30	>2048	Positive	98.1
1082	0	<2	Negative	0.0
	1	<2	Negative	3.9
	2	<2	Negative	0.3
	3	<2	Negative	0.0
	4	<2	Negative	4.6
	5	<2	Negative	26.9
	7	64	Positive	92.6
	10	>2048	Positive	96.3
	14	>2048	Positive	97.6
	21	>2048	Positive	98.9
	28	256	Positive	99.3
	30	64	Positive	98.6
1122	0	<2	Negative	0.0
	1	<2	Negative	0.0
	2	<2	Negative	2.7
	3	<2	Negative	0.0
	4	<2	Negative	56.2
	5	<2	Negative	60.1
	7	16	Positive	95.6
	10	256	Positive	88.6
	14	256	Positive	90.3
	21	256	Positive	98.3
	28	128	Positive	96.4
	30	256	Positive	96.9
1127	0	<2	Negative	0.9
	1	<2	Negative	0.0
	2	<2	Negative	0.0
	3	<2	Negative	0.0
	4	<2	Negative	8.0
	5	2	Negative	67.8
	7	32	Positive	94.9
	10	256	Positive	93.7
	14	256	Positive	95.4
	21	256	Positive	97.7
	28	256	Positive	98.5
	30	256	Positive	97.1
1128	0	<2	Negative	0.0
	1	<2	Negative	5.9
	2	<2	Negative	0.0
	3	<2	Negative	0.0
	4	<2	Negative	37.4
	5	2	Negative	61.4
	7	64	Positive	95.1
	10	>2048	Positive	95.3
	14	>2048	Positive	96.1
	21	>2048	Positive	100
	28	512	Positive	100
	30	512	Positive	98.5

5.4 DISCUSSION

The results obtained in this study varied in reactor rates between the NP-B-ELISA and AGID tests, which is not surprising as each of the tests used differs in analytical sensitivity which could be the most important factor to explain the results obtained in this study.

Frequency of AIVs circulating in waterfowl, as determined by the NP-B-ELISA, may have a direct implication for the poultry industry with regard to biosecurity in farms. Monitoring of the AIV prevalence will be warranted to determine the frequency of AIV infection in the poultry and the subtype involved. This information will be directly important for the poultry industry to predict influenza outbreaks as well as for human epidemiologists to monitor the possible transmission of chicken AIV influenza to humans.

From 32.5% of the positive serum samples there were big differences between sampling sites. In Invercargill where the majority of ducks were adult, 41/93 (44.1%) were found positive but in Kaituna where juvenile ducks predominated only 12/67 (17.9%) had antibody to influenza A and this was consistent with other sampling sites. Coincidentally, similar serological results were obtained from ducks when tested for APMV antibodies indicating different virus behaviour in populations of different age cross-sections and possibly immune status in a flock.

Unfortunately, the immune response of ducks to AIVs is still not fully understood but, from many reports from a number of experiments studying the virological and serological behaviour of AIVs, we know that ducks do not always produce concentrations of antibody that are detectable with tests such as HI and AGID (Slemons & Easterday 1972; Kida et al. 1980; Alexander et al. 1986; Suss et al. 1994; Easterday et al. 1997). The titres of antibody in ducks are generally very low in comparison to those in chickens and decline to an undetectable concentration within 4–8 weeks after AIV infection (Kida et al. 1980; Suss et al. 1994).

Keeping in mind that the titres of AIV antibodies in ducks are very low and do not last long, sensitivity is a very important issue in any survey designed to estimate the prevalence of exposure to AIV. In the chicken experiment as a part of the NP-B-ELISA, evaluation of the results shows that the NP-B-ELISA detected antibody to AIV earlier than either of the other tests. The antibodies were detected 4 days after AIV inoculation when tested by NP-B-ELISA but 5 days using the HI test and 7 days using the AGID (Table 1). This indicates that the NP-B-ELISA gives a positive reaction in the presence of smaller concentrations of antibody in serum than do either of the other tests. This suggests that more sera in any survey for AIV in ducks would be detected as positive using this test and the presence of AIV infection would be more likely to be detected.

5.4.1 NP-B-ELISA evaluation

The evaluation of the NP-B-ELISAs were carried out using chickens as the only suitable species available at the time and clearly show a very good correlation between these three tests (Table 5.1). There was 100% agreement on positive sera tested by all three tests and negative sera up to 3 days post-infection providing evidence of the suitability of this test to be used for detection of NP antibodies. Proper evaluation of the ELISA will require much more experimentation (preferably in ducks) with other AIV subtypes or testing a higher number of samples with known AIV subtype status. The most critical step in the ELISA is the blocking step using mAb HB 65 derived from hybridomas originally established by Yewdell et al. (1981) who used influenza A/WSN/33 (H1N1) and A/PR8/34 (H1N1) to immunise BALB/c mice. This mAb was found superior when testing other Influenza A mAb against influenza NP (Boer et al. 1990) to be used in NP-B-ELISA. Additionally, high sensitivity was obtained using disrupted antigen and this was confirmed by Boer et al. (1990) and when testing equine or swine sera in other studies (Stanislawek 2001, unpubl. data).

5.5 SUMMARY

High prevalence of AIV antibodies (32.5%) was detected in mallard ducks using NP-B-ELISA by only 1.5% when AGID test was used and this is consistent with published finding that ducks do not always produce concentrations of antibody that are detectable with tests such as HI and AGID.

This information will be directly important for the poultry industry with regard to biosecurity in the farms and predicting influenza outbreaks, as well as for human epidemiologists to monitor the possible transmission of chicken AIV influenza to humans.

The NP-B-ELISA was found to be a useful screening test for AIV antibodies in mallard ducks and may have an imported role in the surveillance of AIV in wild birds and poultry with conjunction with other tests such as HI.

Chapter 6 Pathogenicity assessment of New Zealand APMV-1 and influenza H5N2 isolates

6.1 INTRODUCTION

APMV-1 and AI virus infections occur in domestic poultry in many countries and there is evidence that wild birds, in particular waterfowl, play a role in the introduction of these viruses into poultry. Introduction of these viruses does not always cause outbreaks of diseases, although mild to severe clinical signs have been associated with infection by pathogenic strains of APMV-1 and AI viruses in poultry and sporadically in other birds (Alexander 1989, 1996a,b). Sometimes infection with other bacterial and/or viral agents may produce clinical signs similar to infection with APMV-1/AIV or exacerbate such infections leading to much more serious disease (Alexander 1997; Easterday et al. 1997).

Therefore, isolation of APMV-1 or AIV (all HPAI strains isolated to date have possessed either H5 or H7 haemagglutinin) is usually inadequate for unequivocal diagnosis and as a result it has been necessary to develop laboratory techniques to assess pathogenicity of these virus isolates for susceptible poultry flocks (Easterday et al. 1997; Alexander 1989, 2000b). The definition of pathogenicity of the APMV-1/AIV isolates is critical for local authorities to combat outbreaks if pathogenic strains are confirmed and for the purpose of trade to prevent spread of these viruses to other countries (Alexander 2000a; OIE 2000).

A number of tests have been developed and recognised by the OIE to be used for the assessment of pathogenicity. *In vivo* tests include MDT in eggs, ICPI, and IVPI. *In vitro* tests include RT-PCR using primers targeting the gene sequence of the cleavage region of the F0 protein of APMV-1 and the HA genome for H5 and H7 of AIV (Alexander 2000a; OIE 2000).

For APMV-1, another characteristic influencing pathogenicity has been identified from an analysis of the HN0 protein. It has been confirmed that there is variability in amino acid extension at the C-terminus. This may vary from a total length of 571 amino acids, in the case of Australian pathogenic APMV-1 strain Albiston-Gorrie, to 616 amino acids, in the case of V4 isolates (Sakaguchi et al. 1989). The function of this C-terminus variability is not clear but an involvement with virus-cell activity in conjunction with the F0 protein is suggested (Lamb & Kolakofsky 1996) and thought to be associated with the virulence of the virus. For example, the Australian pathogenic strain AusVic/32 has an HN C-terminal extension with 0 amino acids (Collins et al. 1993) whereas non-pathogenic strains such as QueV4/66 have an extension of 45 amino acids (Sato et al. 1987; Toyoda et al. 1989).

In this present study, ICPI and RT-PCR were used to target the F and HN genome and determine the pathogenicity of APMV-1 virus isolates obtained in this and previous studies in New Zealand. In addition, IVPI and RT-PCR were used to assess the pathogenicity of the AIV isolates.

6.2 MATERIALS AND METHODS

6.2.1 Intracerebral pathogenicity index

Fresh infective allantoic fluid (confirmed free of bacterial contamination) containing the APMV-1 virus with an HA titre no less that 1:16 was diluted 1/10 in PBS. 50 µl was injected intracerebrally into each of ten 24- to 40-h-old SPF chickens. Two chickens were inoculated with PBS only and were used as a control. The birds were observed for 8 days and scored every 24 h of the original injection. The individual birds were scored as follows:

"0" if normal (alert, moving without incoordination);
"1" if sick (including chickens exhibiting signs of paralysis or those that were prostrate, but excluding chickens that were only dull); and

"2" if dead.

The ICPI was calculated as a mean score per bird per observation over the 8 days (Allan et al.1978; Alexander 1996b).

6.2.2 Intravenous pathogenicity index

Fresh, bacteria-free, infective allantoic fluid (containing AIV H5N2 virus) with a titre not less than 1:16 was diluted 1/10 in PBS and 100 µl of the diluted virus was injected intravenously into each of ten 6-week-old SPF chickens. The inoculated chickens were observed daily and scored:

"0" if healthy;
"1" if sick;
"2" if paralysed or showing other nervous signs; and
"3" if dead.

The IVPI was expressed as the weighted value over the number of observations made (Allan et al. 1978).

Both the ICPI and IVPI were performed independently at NCDI, Upper Hutt and CVL Weybridge, United Kingdom (the ND and AI Reference Laboratory).

6.2.3 Molecular basis for pathogenicity using RT-PCR

RNA from all isolates of APMV-1 and AIV H5N2 was extracted directly from infective allantoic fluid after clarification at 1000*g* for 10 min and using TRISOL® LS Reagent (Life Technologies GIBCO BRL) (Chomczynski & Sacchi 1987) following the manufacturer's procedure. Briefly: after clarification, 250 µl of supernatant was mixed with 750 µl of TRISOL-LS and left to stand for 5 min at room temperature. 200 µl of chloroform was added, the tube shaken vigorously, centrifuged at 10,500 rpm for 15 min at 4°C, and incubated for 15 min at room temperature. The top layer formed after

centrifugation was carefully transferred to a clean tube and 500 µl of isopropanol was added and centrifuged as before to pellet the RNA. The RNA pellet was rinsed with 1 ml of 75% cold ethanol, vortexed briefly, and centrifuged again at 8000 rpm for 5 min. The pellet was air-dried for 5–10 min and redissolved finally with 50 µl of RNAse-free distilled H_2O. This extracted RNA was stored at –70ºC until tested.

6.2.4 RT-PCR to determine sequences of the F genome

Primers for the F gene for all APMV-1 isolates (except the NZ/1/97 virus) were identical to those published by other researchers, i.e., F315- 5′-CTT TGC TCA CCC CCC TTG G-3′ and reverse primer F589*r*- 5′-CTG CAT CTT ACC TAC GGC AAC-3′ (Jestin & Jestin 1991), and corresponded to sequences around the fusion protein cleavage site at the position 315–589. For APMV-1, NZ/1/97, new primers based on the comparison of the published H group NDV fusion protein gene nucleotide sequences had to be designed to omit problems occurring with the previous primers which did not bind to the RNA of G group viruses because of nucleotide differences in the targeted region (Collins et al. 1998). The primers were as follows:

Primer Forward: F302 5´-AGGACGCTTACAACCCTCC-3´
Primer Reverse: F596*r* 5´-CTGCATCTTACCTACGGCAAC-3′

For AIV isolates, the following RT-PCR primers were used (Senne et al. 1996):

Primer Forward: H5-968 5′-CCAT(TC)GG(AG)GA(AG)TG(CT)CCCAAATA-3′
Primer Reverse: H5-1262*r* 5′- CTTTCCCAACGGCTTCGAATTTG-3′

For the F gene of APMV-1 and the AIV reactions, a single tube RT-PCR was performed using Superscript 2 (Life Technologies GIBCO BRL) for 30 min at 50°C for AIV and 54°C for APMV-1 followed by denaturation at 94°C for 2 min. This was followed by 25 cycles of denaturation at 94°C for 45 s, primer annealing at 60°C for 1 min, primer extension at 72°C for 2 min, and final extension at 72°C for 10 min. A Mastercycler® gradient thermocycler (Eppendorf-Nethele-Hinz GmbH22331,

Hamburg) was used for the amplification of all products. The RT-PCR products were separated by 1.5% agarose gel electrophoresis, stained with ethidium bromide, and photographed during UV transillumination (Tham & Stanislawek 1992).

It is a policy of the New Zealand Ministry of Agriculture and Forestry (my employer), that any results that may have a direct implication on the exotic disease freedom of New Zealand must be confirmed by another laboratory. RNA extracted from New Zealand APMV-1 isolates was sent to the Australian Animal Health Laboratory (AAHL), Geelong, Australia for confirmation of the F genome sequences, and in addition, HN genome sequences were obtained from that laboratory.

6.2.5 RT-PCR to determine sequences of the HN genome

This RT-PCR was performed by AAHL, Geelong, Australia and the sequences obtained were sent to me for further analysis. The RT-PCR to determine sequences of HN genome was prepared as described by Gould et al. (2001). The primers used in the RT-PCR and nested PCR were as follows:

Primer Forward: NDV HN-304 5′-TTTTTCTTAAGTGACT-3′
Primer Forward: NDV HN-314 5′-ATATCCCGCAGTCGCATAAC-3′
Primer Reverse: NDV HN-330*r* 5′-GCAGCATACACAACATCAACATG-3′

Primary PCR was performed with primers HN-304 and HN-330 using the reaction conditions of 94°C for 1 min, annealing at 37°C for 2 min, and an extension time of 2 min at 72°C for 35 cycles. Nested PCR reactions were done using the primers HN-HN-304 and HN-330 with the conditions as described above with the exception of the number of cycles which was reduced to 25. The sequencing was performed as described in section 6.2.6.

6.2.6 Direct nucleotide sequencing of RT-PCR products

RT-PCR products were purified with QIAquick PCR Purification Kit (QIAGEN Pty Ltd, Clifton Hill, Victoria 3068, Australia) and quantitated spectrophotometrically to obtain 10–20 ng/μl of the template. Direct sequencing of the RT-PCR product was completed by ABI PRISM 377 DNA Sequencer using dye terminators (Applied Biosystemes, Foster City, CA, United States) by Massey University DNA Analysis Services. The sequences obtained were translated to amino acids using various programs available from the Australian National Genomic Information Services (WebANGIS) (Littlejohn et al. 1996).

6.3 RESULTS

The ICPIs of the New Zealand isolates of APMV-1 in chickens ranged from 0.00 to 0.16 (Table 6.1) and the clinical signs observed in some of the inoculated birds included the huddling together with no inclination to move, feed, or drink, These values are typical for non-pathogenic isolates.

Table 6.1 Characterisation of New Zealand avian paramyxovirus type 1 (APMV-1) isolates obtained in this and previous studies by Intracerebral Pathogenicity Index (ICPI) and amino acid sequences of the fusion protein (F2/F1) cleavage site from position 112–117. [a]Austin & Hinshaw (1984); [b]Durham et al. (1980); [c]virus isolated from birds of a broiler breeder flock without any disease symptoms from a farm where routine serological surveillance for APMV-1 detected seropositive flocks; [d]isolate obtained from a healthy brown teal (*Anas chlorotis*) during routine surveillance. N/T = not tested.)

APMV-1 identification	ICPI	Deduced amino acid sequences
APMV-1/mallard/NZ/51/76[a]	0.11	^{112}GKQGR–L^{117}
APMV-1/mallard/NZ/131/76[a]	0.02	GKQGR–L
APMV-1/mallard/NZ/132/76[a]	0.14	GKQGR–L
APMV-1/chicken/NZ/7579/78[b]	0.00	GKQGR–L
APMV-1/chicken/NZ/8038/78[b]	0.00	GKQGR–L
APMV-1/parrot/NZ/3528/78[b]	0.04	GKQGR–L
APMV-1/mallard/NZ/1/97	0.00	ERQGR–L
APMV-1/chicken/NZ/8043/95[c]	0.00	GKQGR-L
APMV-1/mallard/NZ/2/97	0.00	GKQGR–L
APMV-1/mallard/NZ/3/97	0.00	GKQGR–L
APMV-1/mallard/NZ/4/97	0.00	GKQGR–L
APMV-1/mallard/NZ/5/97	0.00	GKQGR–L
APMV-1/mallard/NZ/6/97	0.00	GKQGR–L
APMV-1/mallard/NZ/7/97	0.00	GKQGR–L
APMV-1/mallard/NZ/8/97	0.10	GKQGR–L
APMV-1/mallard/NZ/9/97	0.10	GKQGR–L
APMV-1/mallard/NZ/10/97	0.16	GKQGR–L
APMV-1/brown teal/NZ/983/01[d]	N/T	EKQGR–L

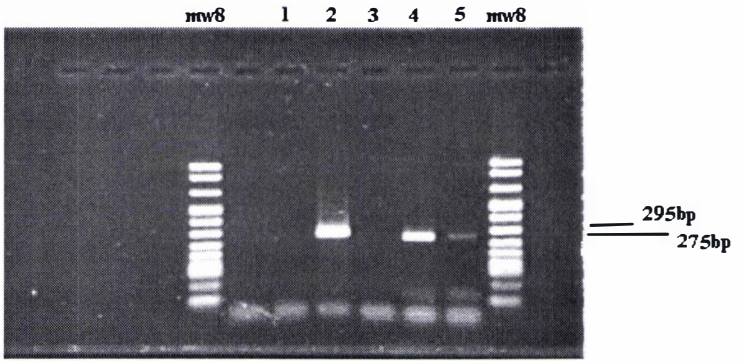


Fig. 6.1 Agarose gel electrophoresis of the 295 bp and 275 bp RT-PCR products from the F gene of avian paramyxovirus type 1 (APMV-1) using F302–F589*r* and F315–F589*r* primers respectively.

```
            1                                                    50
NZ76-78       VSSSSTKAAY TTSTCFKVVK TNKTYCLSIA EISNTLFGEF RIVPLLVEIL
NZ2,3,4,8,10/97.......... .......... .......... .......... ..........
NZ5/97        .......... .......... .......... .......... ..........
NZ6,7/97      .......... .......... .......... .......... ..........
NZ9/97        .....A.... .......... .......... .......... ..........
NZ8043/95     .......... .......... .......... .......... ..........
Que/66        .......... .......... .......... .......... ..........
Aus0655/99    .......... .......... .......... .......... ..........
AusVic/32     ....R..... .......... .......... .......... ..........

              51                                                   101
NZ76-78       KDDGVREARS SRSSQLREGW KDDIVSPIFC DAKNQTEYRR ELESYAASWP*
NZ2,3,4,8,10/97 .......... .......... .......... .......... ..........*
NZ5/97        .......... .........* .......... .......... ..........*
NZ6,7/97      .......... .......... .......... .......... ..........*
NZ9/97        .......... .......... .......... .......... ..........*
NZ8043/95     .......... .......... .......... .......... ..........*
Que/66        .......... ..L....... .......... .......... ..........*
Aus0655/99    .......... ..L.*..... .......... .......... ..........*
AusVic/32     .....*K... ..L...*... .......... S........P RARILRCQSVI
```

Fig. 6.2 Deduced amino acid sequence alignment of the C-termini of the HN glycoprotein of the New Zealand avain paramyxovirus type 1 (APMV-1) strains and comparison to recent pathogenic APMV-1 isolated from an outbreak in Australia and two reference Australian APMV-1 strains. (Dots, amino acids identical to NZ76-78 isolates; asterisks, termination codons; highlights, HN amino acid extension.)

The predicted 275 bp and 295 bp fragments were visualised (Fig. 6.1) and the deduced amino acid sequences in the cleavage region of the F0 protein of APMV-1 isolates were GKQGR-L, ERQGR-L, or EKQGR-L (Table 6.1). This is typical of non-pathogenic viruses, without a pair of basic amino acids at residues 112–116 and with leucine at residue 117.

All New Zealand AMPV-1 isolates had 45 amino acid extensions at the C-terminus of the HN0 protein when compared to the virulent AusVic/32 virus, with the exception of isolate NZ5/97. This latter isolate had an extension of 14 amino acids (Fig. 6.2).

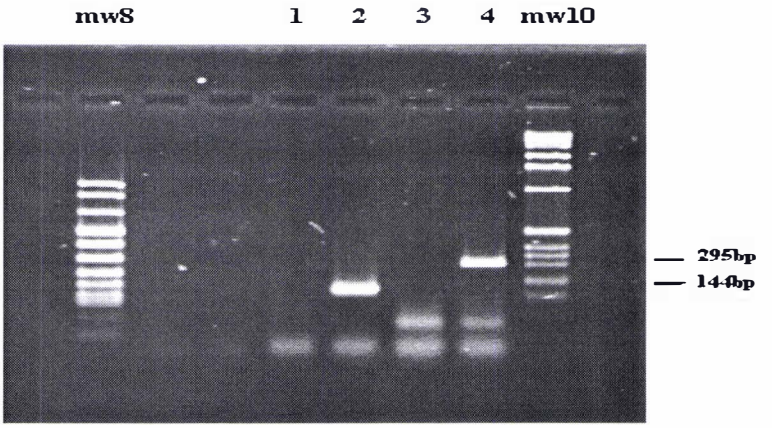


Fig. 6.3 Agarose gel electrophoresis of the 295 bp RT-PCR products from the HA gene of avian influenza virus (AIV) using H5-985 and H5-1262*r* primers.

The two H5N2 subtypes of AIV isolates each had an IVPI score of 0.00 in tests carried out in both laboratories. The predicted 295 bp fragment was visualised (Fig. 6.3) and the deduced amino acid sequence at the haemagglutinin cleavage site of both isolates was PQRETR*G, a typical pattern for non-pathogenic H5 subtype strains.

6.4 DISCUSSION

ICPI and IVPI are regarded as the most accurate tests to determine pathogenicity of APMV-1 and AIV isolates for poultry (Alexander 2000a; OIE 2000), although attempts have been made to replace them with *in vitro* tests including molecular tests. The methods of ICPI and IVPI are described in detail in the OIE manual (the most renowned and recognised worldwide selection of tests) and various other publications, however it is known that results obtained by two different laboratories may differ slightly. Providing that the chickens used for this assessment are brooded correctly the classification of inoculated chickens as normal, sick, or paralysed is very subjective and

can influence the final score because of the interpretation made by the observer (Allan et al. 1978). This situation was observed in the ICPI experiments for APMV-1, carried out by CVL Weybridge, United Kingdom and by myself, but the score was below 0.2 for all isolates even though it varied between laboratories for some individual isolates. For the two AIV H5N2 isolates there was no variation between laboratories in the pathogenicity assessments and both IVPI indexes were 0.00.

Using molecular techniques to assess the pathogenicity of the New Zealand APMV-1 isolates, the F0 cleavage site sequences were consistent with low virulent isolates in the same region as that recognised by the OIE, having deduced amino acid sequences ^{112}G/E-K/R-Q-G/E-R^{116} at the C-terminus of the F2 protein and L (leucine) at residue 117, the N-terminus of the F1 protein (OIE 2000). This method proved to be very accurate in the assessment of pathogenicity, although problems to initially detect the NZ1/97 isolate is one of a variety of reasons why this method is not yet in general use. An additional set of primers specific for the mAb H group isolate (Alexander et al. 1997) had to be designed to provide the right product. Although molecular methods have some advantages in pathogenicity assessment, such as speed (18 h was required for Gould et al. (2001) to obtain and analyse partial sequencing for F and HN gene), full replacement of biological methods is not recommended at this stage because of the sensitivity and nucleotide variations of the cleavage site for different APMV-1 isolates (OIE 2000).

Analysis of the APMV-1 HN protein showed that all New Zealand isolates had amino acid extensions of 45 amino acids, except for isolate NZ5/97, which had an extension of 14 amino acids resulting from a single nucleotide mutation at the position 1838 (Fig. 6.4) and this is in line with the theory of generation of different size HN0 extensions (Sakaguchi et al. 1989). The sequences encoding the extra C-terminal peptides with the HN0 616 type isolates (45 amino acids) are highly conserved. Analysis of pathogenic isolates obtained over the last 50 years, with very short or no HN0 extension, reveal that the corresponding region up to nucleotide 1939 of the HN gene accumulated a number of changes indicating a low selection pressure on these non-coding regions (Sakaguchi et al. 1989) (Fig. 6.4).

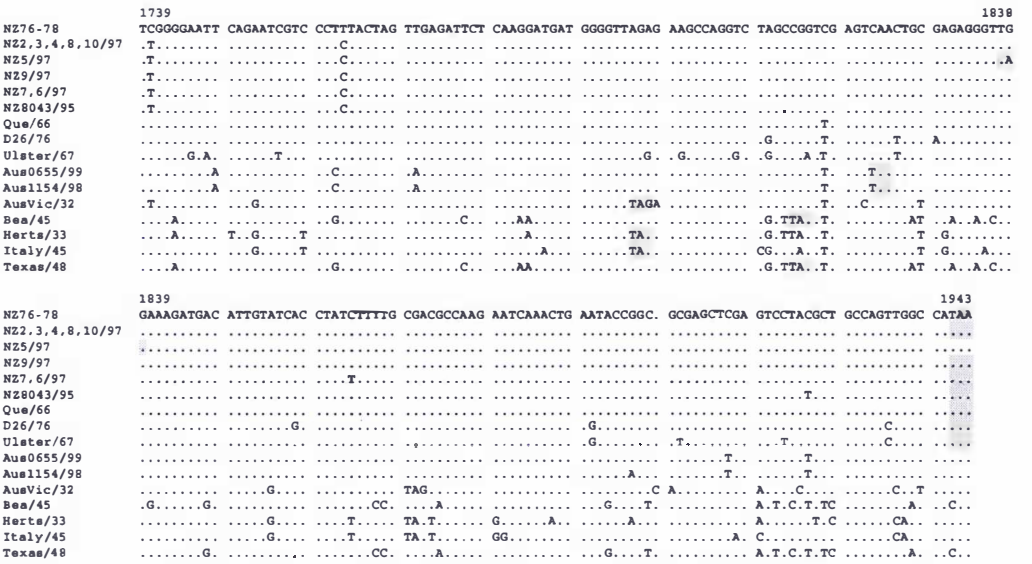


Fig. 6.4 Nucleotide sequence alignment at the translation termination site of the HN protein gene for avian paramyxovirus type 1 (APMV-1) isolates. Termination sites for HN translation are highlighted.

Isolates with amino acid extensions of 7, 9, and 14 amino acids have been associated in Australia with viruses that can cause a severe upper respiratory disease (Hooper et al. 1999a,b). However, the New Zealand isolate (NZ5/97) does not appear to have such characteristics as determined from the ICPI studies and argues against the length of the HN0 protein being a consistent determinant for pathogenicity.

Both of the tests used for AIV H5N2 virus pathogenicity assessment were in full agreement and neither of the isolates, when inoculated into chickens, produced any clinical signs. The deduced amino acid sequences of the HA cleavage site had no more than two basic amino acids at the corresponding -1 to -4 position of the HA_1. This is characteristic for non-pathogenic AIVs, and both positions were represented by arginine (the most common residue in these positions) (Collins et al. 1993; Steinhauer 1999). Similarly for the determination of the cleavage site of the F0 protein of APMV-1, sequencing or other methods will eventually become most preferred method to assess the pathogenicity of AIVs in the future. However, negative results obtained by this method could still require confirmation because of the possibility of, for example, mixed infection of non-pathogenic and pathogenic strains. Consequently, *in vivo* tests will continue to play an important role in the meantime (Alexander 2000a).

6.5 SUMMARY

A pathogenicity study was carried out on APMV-1 obtained in this and from previous studies in New Zealand and on two H5N2 AIV isolates also obtained in this study.

In vivo tests, such as ICPI and IVPI in SPF chickens, were used to determine the virulence of these viruses for chickens. The ICPIs for all APMV-1 isolates ranged from 0.00 to 0.16 and the two AIV isolates of H5N2 subtype each had an IVPI score of 0.00, which are typical for non-pathogenic viruses.

In vitro assessment by RT-PCR was used to target cleavage sites of the F and HN genes of APMV-1 and the HA gene of the H5N2 AIV isolates. The deduced amino acid sequences in the cleavage region of the F0 protein of APMV-1 isolates were GKQGR-L, ERQGR-L, or EKQGR-L, which are typical sequences of non-pathogenic viruses. All AMPV-1 isolates had 45 amino acid extensions at the C-terminus of the HN0 protein when compared to the virulent AusVic/32 virus, except for isolate NZ5/97. This latter isolate had an extension of 14 amino acids and this argues against the length of the HN0 protein being a consistent determinant for pathogenicity.

The two H5N2 subtypes of AIV isolates each had an IVPI score of 0.00. The deduced amino acid sequence at the haemagglutinin cleavage site of both isolates was PQRETR-G, which is a typical pattern for non-pathogenic H5 subtype strains.

Chapter 7 Characterisation of New Zealand APMV-1 isolates using monoclonal antibody banding patterns and phylogenetic relationships

7.1 INTRODUCTION

The effectiveness of control measures relies on quick identification of the virus involved and the determination of the origin of the disease outbreak. A number of techniques were used to characterise strains on the basis of biological and physicochemical (Alexander 1997), but only a few provide epizootiologically meaningful results. The first APMV-1 strain classification which included group-shared antigenic, biological, and epizootiological properties was determined by Russell & Alexander (1983) using nine mAbs and resulted in the division of isolates into eight distinct "groups". However, 14 distinct binding "patterns" were obtained for the viruses using the same nine mAbs, where more isolates were tested (Alexander et al. 1997). Alexander et al. (1997) further extended the number of mAbs used to 26 and 39 different binding patterns were seen. In many instances all the viruses tested from the same group obtained with nine mAbs had identical patterns with the extended panel, e.g., group A (pattern 1) grouping viscerotropic velogenic (VV) viruses isolated during the 1970–72 outbreaks in England and the United States, and group G (pattern 22) where all the isolates were lentogenic derived from aquatic birds worldwide. In other groups the extended mAb panel could further divide the isolates within the group obtained by nine mAbs. For example, in group H, three distinctly different binding patterns were obtained: pattern 24 (grouping isolates obtained in a survey of waterfowl in Germany over a period 1980–89); pattern 25 (grouping viruses isolated in France, Northern Ireland, Canada, and the United States between 1976 and 1993); and pattern 26 (a single highly virulent virus) (Alexander et al. 1997).

Comparing the F and HN gene sequences among 11 and/or 13 strains of NDV isolated during the last 50 years, the sequence variability demonstrated the existence of at least three distinct lineages, which must have co-circulated for considerable periods (Sato et al. 1987; Sakaguchi et al. 1989).

Using restriction enzyme (RE) analysis of PCR product Ballagi-Pordany et al. (1996) obtained, after the amplification of the F gene of viruses isolated during the last c. 50 years, six different groups. Group 1 includes lentogenic strains closely related to reference strains such as D26/76 and Que/66 isolated from waterfowl and chickens all over the world. Groups 2, 3, and 4 comprise isolates responsible for the first epizootic, and for example, group 2 is made up of mostly North American isolates with various virulence (e.g., LaSota, B1, Beaudette C, and Texas GB). In group 3, viruses include AusVic/32 and Miyadera/51 and group 4 includes European strains e.g., Herts/33 and Italian/45. Group 5 is quite distinct and comprises isolates derived from imported psittacines and from chickens during the 1970–72 epizootic in Great Britain and the United States and as well as isolates obtained in Hungary, Croatia, and Germany. Middle East and Greece isolates which caused epizootic in the late 1960s were classified in group 6 together with pigeon isolates although as a separated cluster within the 6th group (Ballagi-Pordany et al. 1996).

Lomniczi et al. (1998), in addition to RE analysis, used nucleotide sequences of the F gene of NDV strains isolated from outbreaks during an epizootic between 1992 and 1999 in Western European countries to compare them with each other and APMV-1 sequences of the F gene available from GenBank. They concluded that NDV strains responsible for these epizootics belong to two distinct genotypes and classified them into genotype 6 (isolate obtained from Denmark, Sweden, Switzerland, and Austria), the same group which caused outbreaks in the Middle East and Greece in the late 1960s and Hungary in the 1980s. However, isolates obtained from Germany, Belgium, The Netherlands, Spain, and Italy could be classified into novel genotypes 7 (Lomniczi et al. 1998). Both methods, RE and sequence analysis of the F gene, were generally in good agreement with APMV-1 classification, however, RE failed to detect minor differences in quantitative bases which can be very crucial in microepidemiological investigations

and could only be evaluated by comparing the genome sequences, e.g., doing phylogenetic analysis (Lomniczi et al. 1998).

In this study, partial genomic and antigenic analyses were carried out to determine the relationship of the New Zealand APMV-1 viruses with each other and their relationship to reference isolates obtained worldwide using mAbs and phylogenetic analysis.

7.2 MATERIALS AND METHODS

7.2.1 APMV-1 isolates

In total 17 APMV-1 New Zealand isolates were used: six obtained in studies carried out in 1976–78 (Durham et al. 1980; Austin & Hinshaw 1984) and 11 APMV-1 isolates obtained in this study (see Chapter 3).

7.2.2 Grouping of APMV-1 isolates using monoclonal antibodies

The mAbs used in this study were produced from hybridomas raised in balb/C mice as described by Russell & Alexander (1983). A panel of 26 monoclonal antibodies was employed to determine the binding pattern of all New Zealand APMV-1 isolates by the indirect IPX test as described in detail elsewhere (Alexander et al. 1997). Briefly, culture microtitre plates containing c. 300 infected Vero cells amongst a background of uninfected cells were prepared by overnight infection with tested APMV-1 isolate and fixed with 10% (v/v) formol-saline. mAbs were added to the fixed cell and binding visualised as a fine brown staining of cell membranes and/or cytoplasm by treatment with peroxides conjugated anti-mouse antiserum and 3-amino-9-ethyl carbazole substrate (Russell & Alexander 1983; Alexander et al. 1997).

7.2.3 RNA extraction from APMV-1 isolates

APMV-1 viral RNA was extracted from infectious allantoic fluid as described in Chapter 6.2.3.

7.2.4 Reverse Transcriptase Polymerise Chain Reaction (RT-PCR)

The RT-PCR reaction was carried out as described in Chapter 6.2.4.

7.2.5 Direct nucleotide sequencing of RT-PCR products and phylogenetic analysis

RT-PCR products were purified and sequenced as described in Chapter 6.2.6.

The DNA sequences were compiled using various programs available from the Australian National Genomic Information Service (ANGIS) (Littlejohn et al. 1996) of which the majority of the programs are the Phylogeny inference package (PHYLIP) (Felsenstein 1989) and ported to the GCG program as a EPHYLIP by the EGCG team for user-friendly interface. A multiple sequence alignment on the nucleotide sequences generated in this study and reference strains available from GenBank (D26 76(M24692), Que 66(M24693), Ulster 67(M24694), Bea 45(M24697), Texas 48(M24698), AusVic 32(M24700), Herts 33(M24702), Italy 45(M24703), MC110 77(AF003726)) was created using the PILEUP part of the GCG program suite (Anon. 1994). The nucleotide distances were computed using Kimura's 2-parameter method (Kimura 1980) in DNAdist (PHYLIPS). Phylogenetic trees were constructed using the neighbour-joining (NJ) method in NEIGHBOR (PHYLIPS). The tree was drawn using Tree View software version 1.6.1 (Page 1996). The confidence values of the internal lineages within the phylogenetic trees were determined by bootstrap analysis (Felsenstein 1985) with 1000 replicates using SEQBOOT (PHYLIPS) program and a consensus of the created trees was made using CONSENSUS (PHYLIPS).

7.2.6 Comparisons of the New Zealand APMV-1 nucleotide sequences of the F gene with those available in GenBank

All New Zealand APMV-1 nucleotide sequences of the F gene were compared with those available in GenBank using the BLAST_FASTA programme (Altschul et al. 1997).

7.3 RESULTS

7.3.1 Grouping of APMV-1 isolates using monoclonal antibodies

Of the total of 17 New Zealand APMV-1 isolates tested using a panel of 26 mAbs, all (except isolate NZ1/97) have binding pattern 22 or group G (Tables 7.1 and 7.2). NZ1/97 had mAbs binding pattern 25 which is one of three patterns belonging to group H (Table 7.1).

7.3.2 RT-PCR, sequencing, and phylogenetic analysis

Nucleotide sequences of the F gene region covering positions 315–589 for 17 APMV-1 isolates and 302–596 for NZ1/97 were obtained and analysed together with reference strains from GenBank (Fig. 7.1A and 7.2) (sequences at the position 334–571 were used for all analyses). The New Zealand isolates formed three groups. Group 1 comprised 11 isolates that consisted of nine obtained from the 1997 duck survey, the chicken isolate obtained in 1995, and an isolate obtained from brown teal (*Anas chlorotis*) in 2001. Group 2 comprised six isolates obtained during 1976–78 from wild ducks, chickens, and a parrot. There was only one isolate (NZ1/97) in Group 3. This was very distinct from the other New Zealand isolates, but was very closely related to the reference strain, MC 110/77, which was obtained from a shelduck in France in 1977. Comparing the New Zealand isolates from Group 1 and 2 with the other

reference strains, the most similar were the lentogenic reference strains, D26/76 originally isolated in Japan and also the Que/66 isolated in Australia.

Table 7.1 Monoclonal antibody binding pattern results for New Zealand avian paramyxovirus type 1 (APMV-1) isolates using panel of 26 mAbs (Alexander et al. 1997).

APMV1 isolate	NZ51/76	NZ131/ 76	NZ132/ 76	NZ3528/ 78	NZ7579/ 78	NZ8038/ 78	NZ8043/ 95	NZ1/97	NZ2/97
MAb									
14(A)	+	+	+	+	+	+	+	-	+
479(G)	+	+	+	+	+	+	+	+	+
424(E)	+	+	+	+	+	+	+	+	+
445(F)	+	+	+	+	+	+	+	-	+
481(H)	+	+	+	+	+	+	+	+	+
688(I)	-	-	-	-	-	-	-	-	-
11(J)	-	-	-	-	-	-	-	-	-
23(K)	+	+	+	+	+	+	+	-	+
32(L)	+	+	+	+	+	+	+	-	+
45(M)	+	+	+	+	+	+	+	+	+
48	-	-	-	-	-	-	-	-	-
55(O)	+	+	+	+	+	+	+	-	+
57(P)	-	-	-	-	-	-	-	-	-
67(Q)	+	+	+	+	+	+	+	-	+
68(R)	+	+	+	+	+	+	+	-	+
69(S)	+	+	+	+	+	+	+	+	+
70(S)	+	+	+	+	+	+	+	-	+
79(U)	+	+	+	+	+	+	+	-	+
85(V)	+	+	+	+	+	+	+	-	+
3(W)	-	-	-	-	-	-	-	-	-
38(X)	-	-	-	-	-	-	-	-	-
43(Y)	-	-	-	-	-	-	-	ND	-
54(Z)	+	+	+	+	+	+	+	+	+
83	+	+	+	+	+	+	+	ND	+
161£	-	-	-	-	-	-	-	-	-
165($)	+	+	+	+	+	+	+	-	+
Group	G	G	G	G	G	G	G	H	G

Similarly, phylogenetic analyses were done on the nucleotide sequences from the HN gene of APMV-1 isolates and reference strains from GenBank (Fig. 7.1B) (sequences at the position 1639–1943 were used for all analyses (Fig. 7.3)). The groupings of APMV-1 isolates from the phylogenetic analysis of the HN gene sequences essentially mirrored that seen with the F gene analyses.

7.3.3 Comparisons of the New Zealand APMV-1 nucleotide sequences of the F gene with those available in GenBank

Comparisons of the New Zealand APMV-1 sequences of the F gene with those available in GenBank using the BLAST_FASTA programme (Altschul et al. 1997), showed that the D26/76 strain was most similar to the New Zealand isolates (except NZ1/97) with 98% nucleotide identity for the 1976–78 isolates and 97% for the 1995–97 isolates. Que/66 strain had 97% nucleotide identity for both groups. In the same analysis the MC110/77 strain had 95% nucleotide identity to the NZ1/97strain (data not shown). In the same comparison using HN gene sequences, the Que/66 strain had 99% nucleotide identity for the New Zealand 1976–78 isolates and 98% for the 1995–97 strains (Appendix B).

Table 7.2 Monoclonal antibody binding pattern results for New Zealand avian paramyxovirus type 1 (APMV-1) isolates using panel of 26 mAbs (Alexander et al. 1997).

APMV1 isolate	NZ3/97	NZ4/97	NZ5/97	NZ6/97	NZ7/97	NZ8/97	NZ9/97	NZ10/97
MAb								
14(A)	+	+	+	+	+	+	+	+
479(G)	+	+	+	+	+	+	+	+
424(E)	+	+	+	+	+	+	+	+
445(F)	+	+	+	+	+	+	+	+
481(H)	+	+	+	+	+	+	+	+
688(I)	-	-	-	-	-	-	-	-
11(J)	-	-	-	-	-	-	-	-
23(K)	+	+	+	+	+	+	+	+
32(L)	+	+	+	+	+	+	+	+
45(M)	+	+	+	+	+	+	+	+
48	-	-	-	-	-	-	-	-
55(O)	+	+	+	+	+	+	+	+
57(P)	-	-	-	-	-	-	-	-
67(Q)	+	+	+	+	+	+	+	+
68(R)	+	+	+	+	+	+	+	+
69(S)	+	+	+	+	+	+	+	+
70(S)	+	+	+	+	+	+	+	+
79(U)	+	+	+	+	+	+	+	+
85(V)	+	+	+	+	+	+	+	+
3(W)	-	-	-	-	-	-	-	-
38(X)	-	-	-	-	-	-	-	-
43(Y)	-	-	-	-	-	-	-	-
54(Z)	+	+	+	+	+	+	+	+
83	+	+	+	+	+	+	+	+
161£	-	-	-	-	-	-	-	-
165($)	+	+	+	+	+	+	+	+
Group	G	G	G	G	G	G	G	G

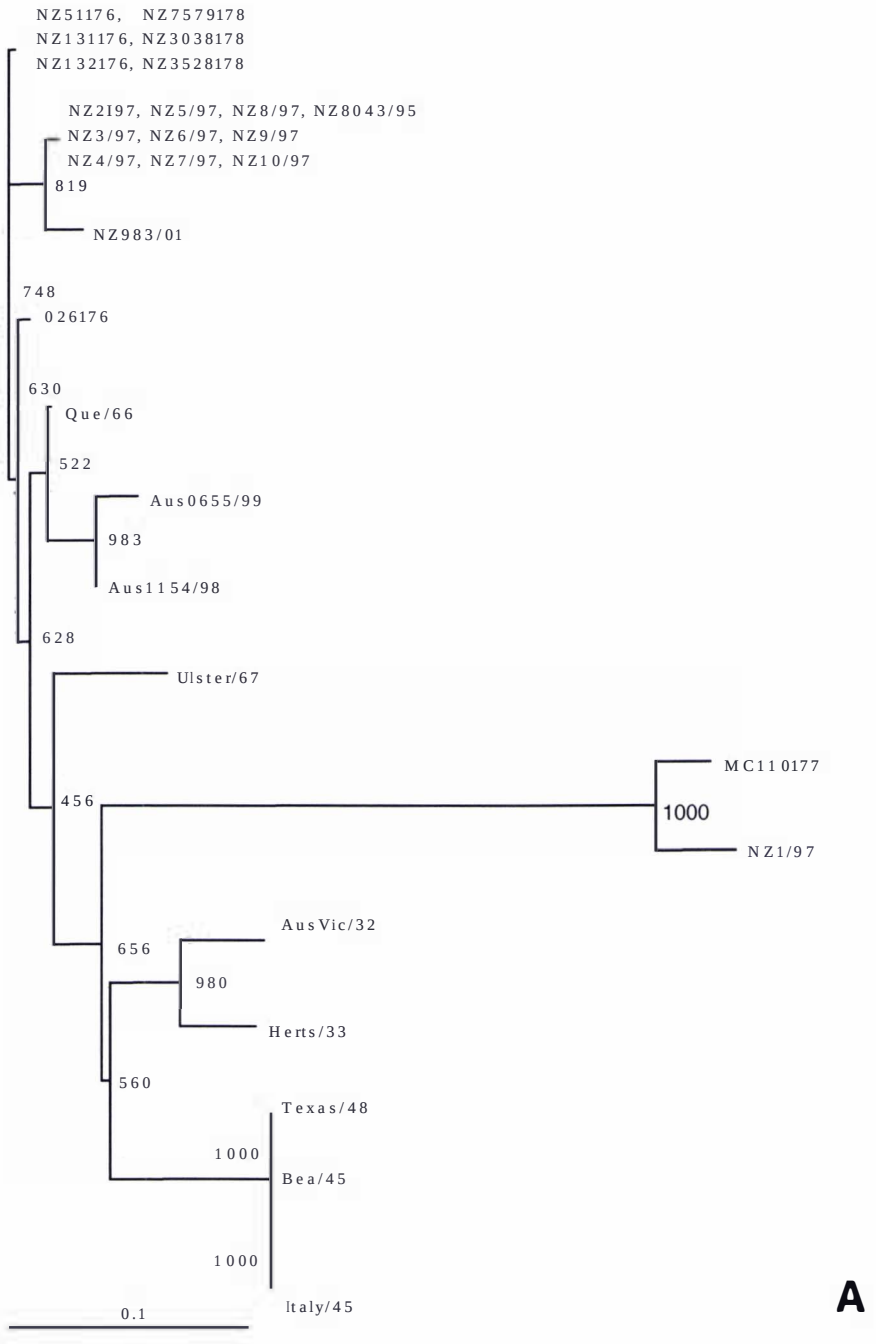
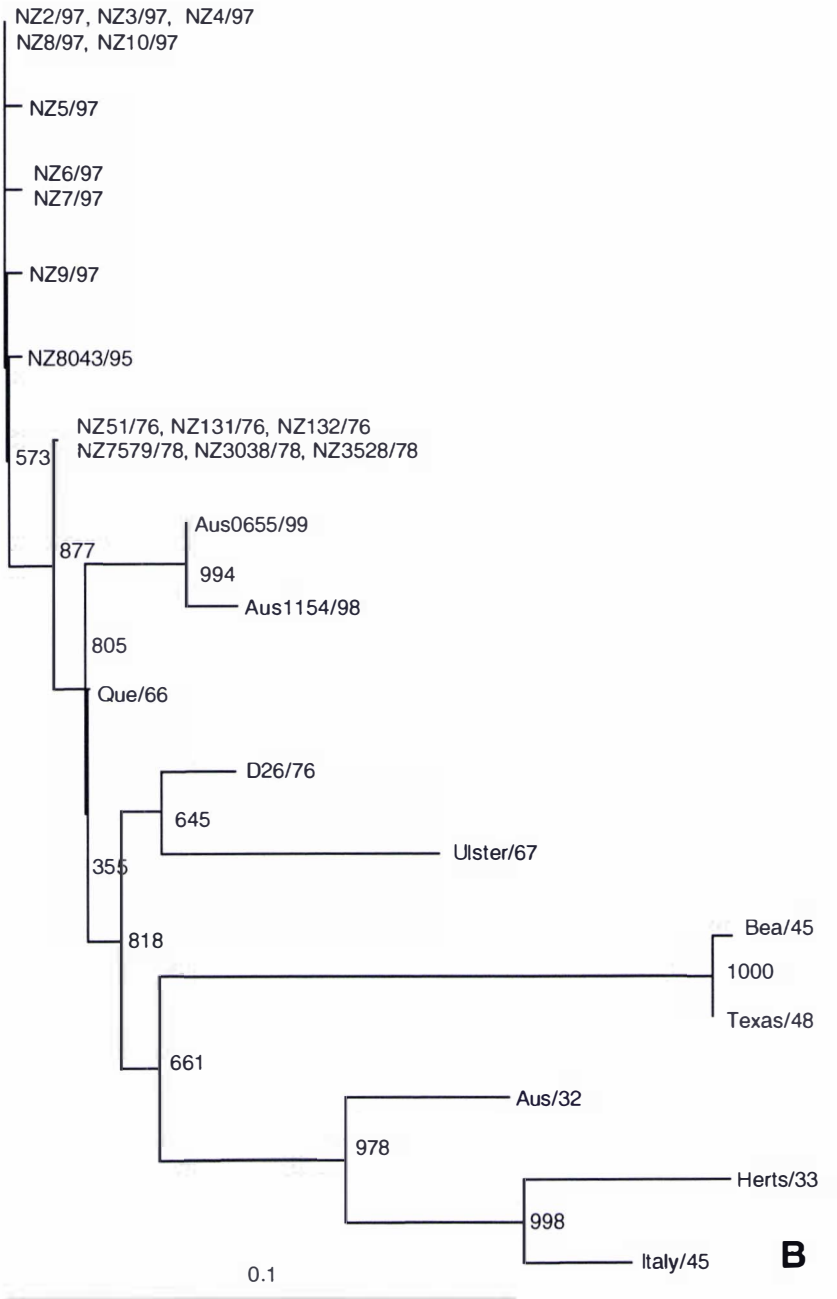


Fig. 7.1 Phylogenetic relationship by the Kimura-2/neighbour-joining method of New Zealand avian paramyxovirus type 1 (APMV-1) strains with those isolated from other parts of the world based on: **A**, a 238 bp region (position 334–571) of the F gene; and **B** (next page), a 304 bp region (position 1639–1943) of the HN gene. Confidence values as shown at the nodes of the internal lineage within the phylogenetic trees were determined by bootstrap analysis with 1000 replicates. Accession numbers of reference strain nucleotide sequences available from GenBank for F and HN genes are respectively: D26/76 (M24692 and M24705), Que/66 (M24693 and M24706), Ulster/67 (M24694 and M24707), Bea/45 (M24697 and M24710), Texas/48 (M24698 and M24711), AusVic/32 (M24700 and M24712), Herts/33 (M24702 and M24714), Italy/45 (M24703 and M14715), MC110/77 (AF003726 (F gene)). Accession numbers of New Zealand APMV nucleotide sequences submitted to GenBank are: AMPV-1 F gene AF438364 to AF438381 and HN gene AF438384 to AF438399. Accession numbers of the Australian APMV-1 isolates submitted to GenBank, F and HN gene respectively, are: Aus1154/98 (AF438382 and AF438400) and Aus0655/99 (AF438383 and AF438401).


NZ2/97, NZ3/97, NZ4/97
NZ8/97, NZ10/97
NZ5/97
NZ6/97
NZ7/97
NZ9/97
NZ8043/95
NZ51/76, NZ131/76, NZ132/76
NZ7579/78, NZ3038/78, NZ3528/78
573
Aus0655/99
877
994
Aus1154/98
805
Que/66
D26/76
645
Ulster/67
355
818
Bea/45
1000
Texas/48
661
Aus/32
978
Herts/33
998
Italy/45
B
0.1

```
            334                                                                                                 433
NZ76-78     TGATTCTATC CGTAGGATAC AAGAGTCTGT GACCACGTCC GGAGGAGGGA AACAGGGACG TCTTATAGGA GCCATTATCG GTGGTGTAGC TCTCGGGGTT
NZ95-97     .........T .......... .......... .......... .......... .......... .......... .......... .......... ..........
NZ983/01    .......... .......... .......... .......... .......A.. .G........ .......... .......... .......... ..........
D26/76      .......... .......... .......... ......A... .......... .......... .........C .......... .......... ..........
Que/66      .......... .......... .......... .......... .......... .......... .........C .......... .......... ..........
Ulster/66   .........T .......... .......... ...T..A..T .......... .......... C........C .......... .C....C... ..........
Aus0655/99  .......... .......... ......T... .......... ......A... G....A.G.. .T.......C .......... .......... .........G
Aus1154/98  .......... .......... .......... .......... ......A... G......G.. .........C .......... .......... .........G
AusVic32    .......... ..C....... .......... ...T..A... ......A... G....AA... CT.......T ..T....... .CA....... ...T......
Bea/45      ...C...... .......... .......... A..T..A..T .....GA... G....AA... CT.......C ........T. .C.....G.. ...T......
Herts/33    .......... ..C....A.. .......... ...T..T... ......A... G....A.... CT.......T .......... .CA....... ...T......
Italy_45    ...C...... .......... .......... A..T..A..T .....GA... G....AA... CT.......C ........T. .C.....G.. ...T......
Texas/48    ...C...... .......... .......... A..T..A..T .....GA... G....AA... CT.......C ........T. .C.....G.. ...T......
MC110/77    ......A... A.A..A..C. .......A.. A.....A..A ..G....AAC GG....AG.. ...GG.G..G ..A..A..A. .A..C..C.. AT.A..T..A
NZ1/97      C.....A..T A.G..A..C. .......A.. A..T..A..A .......AAC GG.....G.. .T.GG.G..G ..A..A..A. .A..C..C.. AT.A..T..A

            434                                                                                                 533
NZ76-78     GCAACCGCTG CACAAATAAC AGCAGCCTCG GCTCTGATAC AAGCCAATCA AAATGCTGCC AACATCCTCC GGCTCAAAGA GAGCATTGCT GCAACCAATG
NZ95-97     .......... ....G..... .......... .......... .G........ .......... .......... .......... .......... ..........
NZ983/01    .......... ....G..... .......... .....T.... .G........ .......... .......... .......... .......... ..........
D26/76      .......... ....G..... .......... .......... .......... .......... .......... .......... .......... ..........
Que/66      .......... ....G..... .......... .......... .......... .......... .......... T......... .......... ..........
Ulster/66   .......... ....G..... ......T... .......... TT.....C.. .........T .......... ....T..... .......... ..........
Aus0655/99  .......... ....G..... .......... .T.T...... .......... .......... .....A.... ....A..G.. .......... ..........
Aus1154/98  .......... ....G..... .......... .......... .......... .......... .....A.... ....A..G.. .......... ..........
AusVic/32   .....A.... ....G..... .......... ..C....... .......... G......... .......... ....T..G.. .......A.. .......T..
Bea/45      .....T.... .......... ...G...G.A .......... .......A.. .......... .......... .A..T..... .........C ..........
Herts/33    .....A.... ....G..... G......... ..C....... .......C.. G......... .......... ....T..G.. .......... ..........
Italy/45    .....T.... .......... ...G...G.A .......... .......A.. .......... .......... .A..T..... .........C ..........
Texas/48    .....T.... .......... ...G...G.A .......... .......A.. .......... .......... .A..T..... .........C ..........
MC110/77    ..C.....A. .G..G..C.. ......T..T ..C..C.... .......C.. G........G .....A..GA AAT.A..G.. ...T.....C ..T.......
NZ1/97      ..C.....A. ....G..C.. ...G..T..T ..C..C.... .......C.. G.....C..G .....AT.GA AAT.A..G.. ...T.....C ..T.......

            534                                 571
NZ76-78     AGGCTGTGCA CGAGGTCACT GACGGATTAT CACAACTA
NZ95-97     .......... T......... .......... ........
NZ983/01    .......... T......... C......... ........
D26/76      .......... .......... .......... ........
Que/66      .......... .......... A.T....... ........
Ulster/66   .......... .......... ..T....... ........
Aus0655/99  .......... .......... A.T....... ........
Aus1154/98  .......... .......... A.T....... ........
AusVic/32   .A.....A.. T......... .......... ........
Bea/45      ....C..... T......... .......... .G......
Herts/33    .A........ .......... .......... .....A..
Italy/45    ....C..... T......... .......... .G......
Texas/48    ....C..... T......... .......... .G......
MC110/77    .A..A..... T........A A.T..G..G. .C..G..G
NZ1/97      .A..A..... T........A A.T..G..G. .C..G..G
```

Fig. 7.2 Nucleotide sequence alignment at the cleavage site of the fusion protein gene for avian paramyxovirus type 1 (APMV-1) isolates. See Fig. 7.1 for GenBank accession numbers.

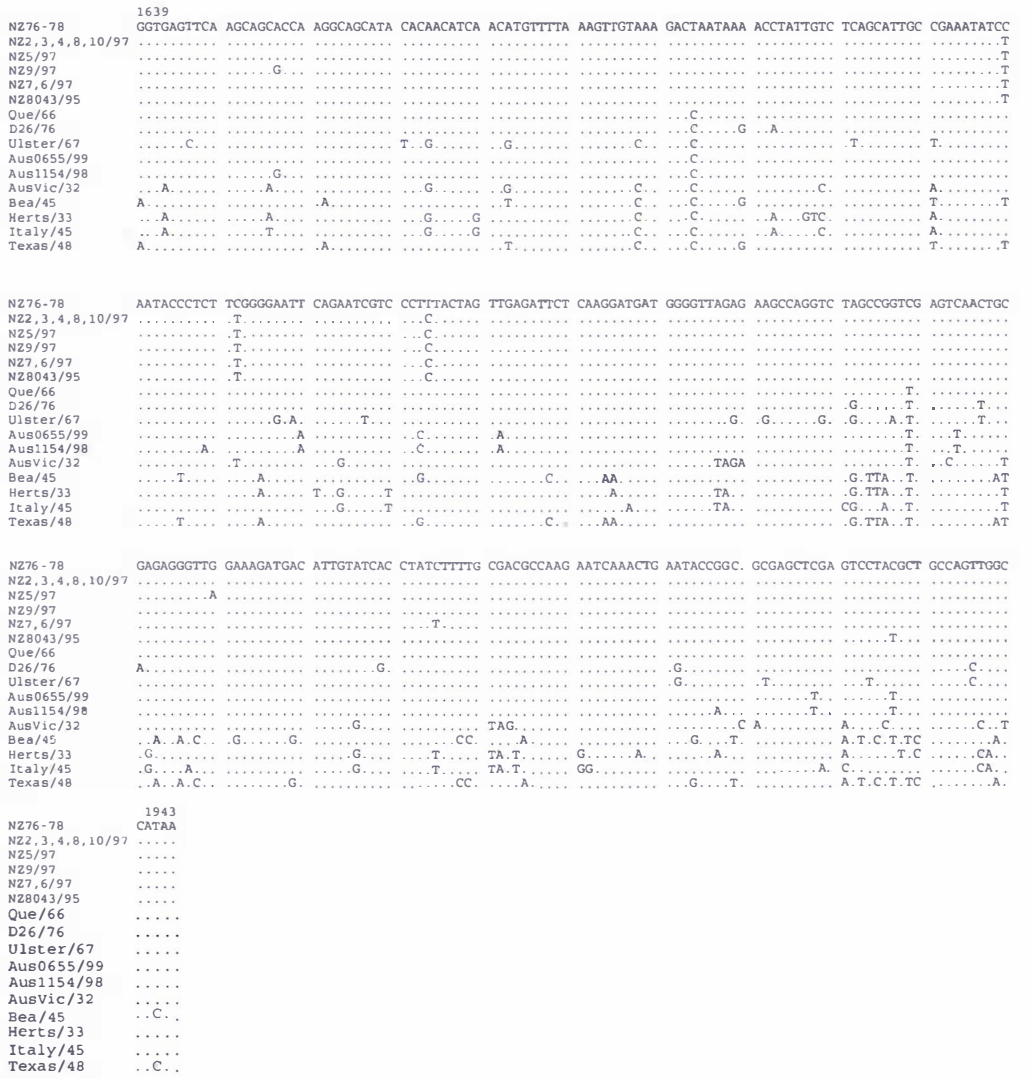


Fig. 7.3 Nucleotide sequence alignment at the translation termination site of the HN protein gene for avian paramyxovirus type 1 (APMV-1) isolates. Termination sites for HN translation are highlighted. See Fig. 7.1 for GenBank accession numbers.

7.4 DISCUSSION

The results obtained by the mAbs binding patterns and phylogenetic analysis of New Zealand APMV-1 strains were very much in agreement with respect to their antigenic and epizootiological properties.

The mAbs binding pattern grouped all New Zealand APMV-1 isolates (except NZ1/97) in group G (pattern 22) (Alexander et al. 1997) which comprises only lentogenic strains isolated from waterfowl and chickens and includes strains such as D26/76, Que/66, and Ulster/67. The NZ1/97 had mAbs binding pattern 25 which is one of three patterns belonging to group H (Alexander et al. 1997) and comprises lentogenic strains isolated from feral birds in France (including strain MC110/77), Northern Ireland, Canada, and the United States.

All New Zealand AMPV-1 isolates obtained during the 1976–78 study had the same nucleotide sequences in the regions examined. Similar homogeneity was seen for the sequences of all APMV-1 isolates obtained during the 1995–97 study, with the exception of NZ1/97. For the mAb group G viruses, there was only a difference of four nucleotides in the F gene between the isolates from 1976–78 and 1995–97 (Fig. 7.2). It therefore seems likely that the viruses isolated from chickens, and possibly the parrot, in 1978 (Durham et al. 1980) had been introduced from wild birds as ostensibly the same virus had been present in mallards in 1976 (Austin & Hinshaw 1984). A similar conclusion could be made from the analysis of the sequences of the genomes of the isolates obtained from mallards in 1997 and the chicken isolate obtained in 1995 as these isolates also had the same nucleotide sequences in the F gene.

Although the sequences of relatively few viruses similar to the New Zealand isolates are available, when considered in conjunction with the evidence available from mAb studies (Alexander et al. 1997), it would seem that both types of viruses isolated in New Zealand are typical of ubiquitous NDV strains of low virulence for chickens that have been isolated from ducks and other wild birds across the world. Both types have been reported in Australia (Alexander et al. 1986).

The phylogenetic analysis of both the F and HN genome sequences analysis of APMV-1 (Fig. 7.1A,B) shows a close relationship of the New Zealand isolates with Australian APMV-1 including Que/66 strain, the "intermediate" strain Aus1154/98, and the pathogenic strain Aus0655/99 and this is particularly obvious when HN genome sequences are compared. Comparisons of the New Zealand APMV-1 sequences of the HN gene with those available in GenBank using the BLAST_FASTA programme (Altschul et al. 1997), showed that the Que/66 strain had 99% nucleotide identity for the New Zealand 1976–78 isolates and 98% for the 1995–97 isolates, and where F genome sequences were included in the same comparison, Que/66 strain had 97% nucleotide identity for both groups (Appendix B). This may indicate that the introduction of Australian APMVs to New Zealand occurs occasionally through limited migration or by vagrant birds from Australia (Heather & Robertson 1996).

A number of factors could effect the overall tree topology including, e.g., length of sequences and tree-making methods used. In this study I have limited phylogenetic analysis to 238 nucleotides of the F gene and 304 nucleotides of the HN region of the APMV-1 genome and it is unlikely that substantially different results would have been obtained using a larger segment. It was found that relatively short sequences of any subgenic region were suitable for identification of hepatitis C virus (HCV) genotypes and subtypes and that the size of the fragment between 220 and 1093 bp of HCV NS-5 did not significantly influence the values of genetic analysis (Mellor et al. 1995).

There are many different tree-making approaches that are the subject to great discussion amongst scientists in this field. In broad categories, there are three ways of creating phylogenetic trees: distance methods, maximum parsimony (MP), and maximum likelihood (ML) (Gaeta & Balding 1997). In distance methods, the distance matrix is calculated by comparing the similarities of two sequences and taking into account the possibility of mutation, following constriction of the tree using the assumption that the distance value between sequences directly reflected to the similarities of the sequences examined (Gaeta & Balding 1997). A number methods are available for this step including neighbour joining (NJ).

The MP method requires the fewest character changes to explain the differences amongst sequences under analysis and tree topology is chosen to show the smallest number of evolutionary changes from all three obtained (Masatoshi 1991). The MP tree does not contain branch length.

The ML method is based on conditional probabilities and the nucleotides of all DNA sequences at each nucleotide site are examined separately to calculate the log-likelihood of having these nucleotides. This log-likelihood is added for all nucleotide sites, and the sum of the log-likelihood is maximised to estimate branch length. This procedure is repeated for all trees and the tree that shows the highest likelihood is chosen (Masatoshi 1991). This method requires enormous computer time and a small number of sequences is recommended (under 11) (Gaeta & Balding 1997).

Comparing all the phylogenetic methods, Masatoshi concluded that the MP method is as efficient as the other methods, only when the number of nucleotides examined is very large and the number of substitutions is low (Masatoshi 1991). The ML method is probably the most efficient method from all three, providing that underling assumptions are satisfied, but if not, the NJ method is more efficient to create the most correct tree (Masatoshi 1991). Taking into account all of the considerations presented above, the NJ method was used in this study.

The phylogenetic tree can be further tested using statistical methods to put confidence intervals on the phylogeny. One of the most commonly statistical method used for phylogeny estimation is the bootstrap which involves new alignment containing small random deviations from one's own data, to create phylogenetic trees from this modified alignments (Felsenstein 1985). Only a "robust" tree will be supported from most of the modified alignments. For DNA sequences at least 1000 replicates is recommended (Gaeta & Balding 1997). The bootstrap confidence limit obtained in these two trees varies for F gene sequences from 456 to 1000 and 355 to 1000 in HN gene (Fig. 7.1A,B). This means that in 1000 trees examined, in some cases only in 456 trees e.g., Ulster/67 and MC110/77 and NZ1/97 were grouped together but MC110/77 and NZ1/97 sequences were grouped together 1000 times.

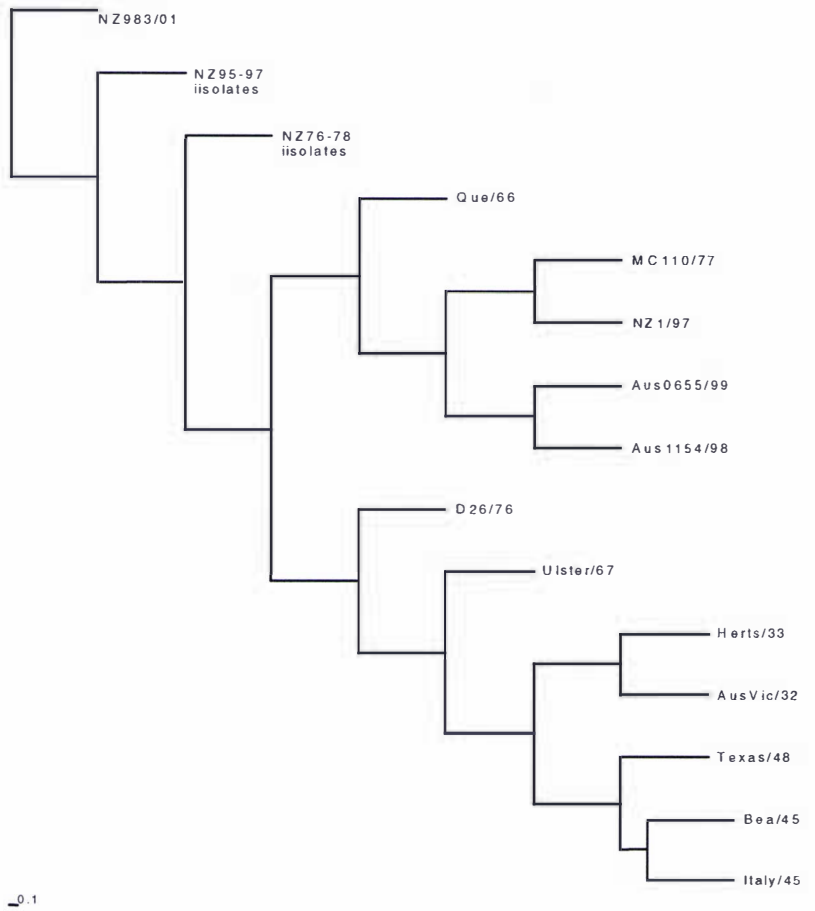


Fig. 7.4 Phylogenetic analysis of portion of F gene sequences from New Zealand and reference strains of avian paramyxovirus type 1 (APMV-1) using the maximum parsimony (MP) method.

To further illustrate the confidence of creating the most correct tree, the maximum parsimony method was used to analyse this data (Fig. 7.4). All New Zealand isolate sequences were grouped together branching to Que/66 and D26/76 isolates. However, the grouping of NZ1/97 together with MC110/77 isolates is in a different position and is grouped together with Australian isolates Aus0655/99 and Aus1154/98 which directly reflect the bootstrap confidence limit given for these groups in Fig. 7.1A. Of course we must remember that in this method no length of branches is given.

No phylogenetic analysis were carried out for two H5N2 AIV isolates as only a limited number of H5 influenza A strains, primarily from North America, have been submitted to GenBank. However, comparisons of the influenza A/Mallard/NZ/1/97 and

A/Mallard/NZ2/97 (H5N2) isolates (GenBank Accession numbers of HA gene: AF439407 and AF439408) with sequences of H5 viruses available in GenBank showed that the most similar viruses were A/Mallard/Ohio/556/1987 (H5N9) and A/Turkey/Minnesota/10734/95 (H5N2) with 92% and 90% nucleotide identity respectively (Appendix B). This could suggest that New Zealand H5N2 strains have quite a different lineage, although a greater comparison with H5 strains from different sites around the world is required to reach any conclusions about the similarity or uniqueness of the New Zealand isolates.

7.5 SUMMARY

Partial genomic and antigenic analyses were carried out to determine the relationship of the New Zealand APMV-1 viruses among themselves and their relationship to reference isolates obtained worldwide using mAbs and phylogenetic analysis.

All New Zealand APMV-1 isolates tested using a panel of 26 mAbs all (except isolate NZ1/97) have binding pattern 22 or group G which is comprised of only lentogenic strains isolated from waterfowl and chickens and includes strains such as D26/76, Que/66, and Ulster/67. The NZ1/97 had mAbs binding pattern 25 is one of three patterns belonging to group H which comprises lentogenic strains isolated from feral birds in France (including strain MC110/77), Northern Ireland, Canada, and the United States.

Partial nucleotide sequences of the F gene region covering positions 315–589 for 17 APMV-1 isolates and 302–596 for NZ1/97 were analysed together with reference strains from GenBank. The New Zealand isolates formed three groups. Group 1 comprised 11 isolates that consisted of nine obtained from the 1997 duck survey, the chicken isolate obtained in 1995, and an isolate obtained from brown teal (*Anas chlorotis*) in 2001. Group 2 comprised six isolates obtained during 1976–78 from wild ducks, chickens, and a parrot. There was only one isolate (NZ1/97) in Group 3. This was very distinct from the other New Zealand isolates, but was very closely related to the reference strain, MC 110/77, which was obtained from a shelduck in France in

1977. Comparing the New Zealand isolates from Group 1 and 2 with the other reference strains, the most similar were the lentogenic reference strains, D26/76 originally isolated in Japan and also the Que/66 isolated in Australia.

Phylogenetic analyses carried out on the nucleotide sequences from the HN gene of APMV-1 isolates essentially mirrored that seen with the F gene analyses.

Comparisons of the New Zealand APMV-1 sequences of the F gene with those available in GenBank showed that the D26/76 strain was most similar to the New Zealand isolates (except NZ1/97) with 98% nucleotide identity for the 1976-78 isolates and 97% for the 1995-97 isolates. Que/66 strain had 97% nucleotide identity for both groups. In the same analysis MC110/77 strain had 95% nucleotide identity to the NZ1/97 strain. In the same comparison using HN gene sequences, the Que/66 strain had 99% nucleotide identity for the New Zealand 1976–78 isolates and 98% for the 1995–97 strains.

The close relationship of the New Zealand to the Australian APMV-1 isolates may indicate that the introduction of Australian APMVs to New Zealand occurs occasionally through limited migration or by vagrant birds from Australia despite the geographical isolation of New Zealand.

Chapter 8 General discussion: Is New Zealand likely to have an outbreak of Newcastle disease and/or highly pathogenic avian influenza?

New Zealand has never experienced an outbreak of ND or HPAI and there is great pressure from various organisations such as the Ministry of Agriculture and Forestry, Poultry Industry, Department of Conservation, and a number of ornithological organisations, to characterise the myxoviruses and paramyxoviruses that are present in the New Zealand bird population. Sound scientific knowledge of the APMVs and AIVs present in New Zealand birds will assist in the development of protocols to reduce the risk of introducing virulent strains of these viruses to New Zealand in imported birds or avian products. Also it will provide information on whether or not the presently circulating viruses will increase their virulence through e.g., mutation and/or reassortment. In addition to the potential threat posed by NDV or HPAI to poultry and aviaries, there is also concern about the impact of these viruses on already endangered native avian species of New Zealand.

There have been a number of attempts, through limited surveys in the past, to provide data on these viruses in New Zealand birds (Durham et al. 1980; Austin & Hinshaw 1984; Stanislawek 1990, 1992) and although a number of APMVs and AIVs were isolated, none of these studies provided satisfactory information for a full assessment of these viruses with regard to their pathogenicity and/or epidemiology.

The present study is, on one hand, a continuation of research done in the past to build up knowledge with regard to AIVs and APMVs in New Zealand, but at the same time extends some aspects, particularly virulence and epidemiology of these viruses, which is the most critical information of interest for all parties.

In the very early stages of designing this project it was realised that it would be difficult to achieve all goals without compromise, including the number of samples collected from different species and the duration of sampling and location (Chapter 2). Although samples were collected from all over New Zealand, the design was based on "convenience" (e.g., associated with bird banding operations or access to big aviaries) rather that random sampling to guarantee that the samples would be representative. However, sampling of mallard ducks was probably an exception, because the estimated prevalence of APMV and AIV in the duck population could be predicted and this reflects very well the results that we obtained (Chapter 3). Other complicating factors which could influence the results, such as time of the year and/or ratio of susceptible birds (e.g., juvenile), have been well documented for waterfowl (Hinshaw et al. 1980a, 1985; Stallknecht et al. 1990a) but not for other bird species. In general, isolation of a particular virus in this study correlated well with the serological results and reinforced the fact that waterfowl, ducks in particular, are a reservoir for AIV and most types of APMVs (Chapters 3, 4, and 5).

In contrast, the failure to isolate APMV and AIV from wild and caged birds and the very sporadic detection of APMV antibody in these birds (with a few exceptions, such as fancy poultry) shows that, other than for APMV-1, the data do not provide us with satisfactory evidence to make any meaningful statements about the presence of these viruses in New Zealand (Chapters 3 and 4). A more statistically sound sampling regimen would be required to make conclusive statements about the prevalence of these viruses.

The situation is much clearer for commercial poultry, where negative serological results for APMV-2 and APMV-3 obtained in this study, together with data for APMV-1 provided from routine testing carried out by the poultry industry, clearly show that there is a very low prevalence of APMV-1 but the results do not support the hypothesis that APMV-2 and APMV-3 are present in commercial poultry (Chapter 4).

There is a real possibility that New Zealand's "free" status from ND and HPAI and the lack of evidence of pathogenic APMV-1 and AIVs in the bird population, as defined in this and previous studies, may change. Despite the geographical isolation of New

Zealand, we could identify at least three possibilities by which virulent strains of APMV-1 and HPAI viruses could emerge in New Zealand: (1) introduction by migratory birds; (2) importation of live birds and avian products; and (3) mutation in endemic viruses of low virulence.

The majority of birds migrating to New Zealand are shorebirds of the family Scolopacidae (sandpipers and allies) in the order Charadriiformes, which breed in the Arctic regions of Europe, Asia, and North America and migrate south for the boreal winter (Heather & Robertson 1996). The migratory routes are not completely understood, but breeding occurs at low latitudes in the Arctic, and birds fly non-stop over the west Pacific Ocean between only a few staging areas such as in the Gulf of Carpentaria. For example, the subpopulation of lesser knots that migrates to New Zealand breeds on the Chukotsky Peninsula of eastern Siberia. These birds begin their journey south in late August arriving in New Zealand from September to December (Higgins & Davies 1996). The possibility for these birds to introduce viruses is limited because of the long migration time. They would have to be infected before migration or during migration, such as at the staging grounds in the Gulf of Carpentaria where a large number of birds from different flight groups congregate. The virus would then be shed on arrival in New Zealand. In addition to large-scale migration, a number of Australian vagrants have been recorded in New Zealand including sporadic guests such as ducks and geese (see Chapter 2), which could also potentially introduce APMV-1 and AIVs to New Zealand.

Legally imported birds to New Zealand go through quarantine and a number of tests are carried out to detect and exclude exotic organisms including APMV-1 and AIV (particularly H5 and H7). However, there are still illegal attempts to bring live birds or embryonated eggs to New Zealand despite the very tight biosecurity and it would be difficult to state how may such attempts are successful. In legally imported avian products, a MAF risk assessment concluded that there was a remote chance that these viruses will be introduced to New Zealand (Christensen et al. 1999).

The third possibility for the appearance of ND or HPAI in New Zealand is for non-pathogenic strains of APMV-1 and AIV, presently circulating in the New Zealand bird

population, to become more pathogenic through mutation. From an evolutionary point of view, neither APMV-1 nor AIV show that mutation of these viruses occurs to such a degree in wild birds that the pathogenicity is altered, especially that there are no advantages that would result in selection (Kida et al. 1987; Sakaguchi et al. 1989; Bean et al. 1992; Murphy & Webster 1996). However, viruses isolated from ND outbreaks in Ireland in 1990 and in Australia during the 1990s have suggested provided strong evidence that non-pathogenic viruses may became pathogenic through mutation (Collins et al. 1998, 1993; Gould et al. 2001; Westbury 2001).

The pathogenic viruses (34/90) isolated during the outbreak in Ireland were very closely related, antigenically and genetically, to non-pathogenic viruses such as MC110/77 isolated from waterfowl in many countries and from chickens in Northern Ireland in 1986 (Collins et al. 1998; McNulty et al. 1988). In the phylogenetic analysis, the New Zealand APMV-1 isolate NZ1/97 was very closely related to MC110/77 virus (Chapter 7) with 95% nucleotide identity. Comparison of the sequences of these three viruses at the F0 cleavage site shows that only four nucleotide changes are required for the MC110/77 isolate to become pathogenic. For NZ1/97, four nucleotide changes are also required for the virus, from an analysis of the F0 cleavage site, to change pathogenicity (Table 8.1).

Table 8.1 Comparison of nucleotide/amino acid sequences at the F0 cleavage site of closely related low and high virulent avian paramyxovirus type 1 (APMV-1) viruses.

	F0 cleavage site		
Virus	Nucleotides	Amino acids	Virulence
NZ1/97	GAA CGG CAG GGG CGT TTG	^{112}ERQGR*L^{117}	Low
MC110/77	GAA CGG CAG GAG CGT CTG	ERQGR*L	Low
34/90	AAA CGG CAG AAG CGT TTT	KRQKR*F	High

A similar conclusion can be drawn from a comparison of other New Zealand APMV-1 isolates obtained between 1976 and 1997 and two Australian viruses responsible for the outbreak during 1998–2000. The mutation of only two nucleotides was required for the

"intermediate" strain Aus1154/98 to become pathogenic and the mutation of four nucleotides would be required for the New Zealand isolates to follow the same path (Table 8.2).

The phylogenetic analysis of both the F and HN genome sequences of APMV-1 (see Chapter 7) shows a very close relationship of the New Zealand isolates to strains of Australian APMV-1 including the "intermediate" strain Aus1154/98 and the pathogenic strain Aus0655/99, reinforcing further the possibility of evolutionary changes in the New Zealand APMV-1 isolates.

Table 8.2 Comparison of nucleotide/amino acid sequences at F0 cleavage site of closely related low and high virulent avian paramyxoviris type 1 (APMV-1) viruses isolated in New Zealand and Australia.

	F0 cleavage site		
Virus	Nucleotides	Amino acids	Virulence
NZ76-97	GGG AAA CAG GGA CGT CTT	^{112}GKQGR*L^{117}	Low
Aus1154/98	AGG AGA CAG GGG CGT CTT	RRQGR*L	Low
Aus0655/99	AGG AGA CAG AGG CGT TTT	RRQRR*F	High

However, the mutation theory of non-pathogenic viruses becoming pathogenic is still unclear despite the fact that the last Australian ND outbreak provides some good evidence of such a possibility (Gould et al. 2001; Westbury 2001). It is more likely that mutation of viruses occurs within poultry rather than the introduction of pathogenic APMV-1 viruses from wild birds because generally there is lack of such viruses in wild birds (Alexander 2000b).

Westbury (2001) presented a view of APMV-1 evolution and the creation of pathogenic viruses, using Australian experiences with these viruses over several decades (Westbury 2001). A number of facts would contribute to such mutation including: the introduction of new chicken breeds; poor management of very intensive meat and egg production; emergence of virulent viruses such as Marek's disease; and natural selection of APMV-

1 viruses, to avoid neutralisation, due to immunological pressure in flocks infected with low pathogenic strains (Westbury 2001).

In an attempt to analyse the New Zealand situation, a number of features are different from those in Australia. First, there is much higher biosecurity in New Zealand to prevent the introduction of viruses circulating in wild birds. This is reflected in the very low prevalence of APMV-1 in New Zealand commercial poultry (Chapter 4) and this trend continues since no positive samples were detected in 2001 after testing 5557 sera (Anon. 2002). In addition, no vaccination for NDV is practised in New Zealand suggesting there have been limited opportunities for viruses to enter poultry and mutate. There are further differences in the health status of poultry such as lower virulence of Marek's disease virus controllable by vaccination programmes (B. Jones & D. Marks pers. comm.) and freedom from infectious bursal disease (although there was a lapse in this freedom most likely due to virus accidentally introduced through contaminated vaccine in 1993) as confirmed by serological surveillance in the commercial poultry testing of 19,225 samples in 2001 (Anon. 2002).

No dedicated research has been conducted to study the evolution/mutation of APMV-1 in New Zealand but an analysis of the data obtained in this study suggest that mutation does occur, only perhaps at a slow rate. All APMV-1 isolates except NZ1/97 and NZ983/2001 had the some deduced amino acids of the F0 protein, however there was a difference of four nucleotides between isolates obtained in 1976–78 and 1995–97. Analysis of the HN genome, found that all had 45 amino acid extensions except the NZ5/97 isolate, which had only 14 amino acids. This is comparable to the results of a study conducted in Australia analysing the F and HN genomes of 87 isolates, from 1976 to 1999, where different HN extensions were observed in virus groups with a homologous F genome to the V4 strain but with 14 different amino acid deduced HN extensions (Peroulis-Kourtis et al. 2002).

Similar hypotheses can be applied to the New Zealand AIV H5N2 isolate to investigate the possibility for the virus to become pathogenic. AI viruses of H5 and H7 subtypes isolated from wild birds, apart from the South African isolate that caused death in terns, are invariably of low pathogenicity and most of the HPAI isolates have been associated

with contact of infected poultry (Swayne & Suarez 2000). Also, a phylogenetic study of H5 and H7 subtypes concluded that viruses of low and high pathogenicity shared the same phylogenetic branches and support the theory that HPAI viruses arise from non-pathogenic viruses (Rohm et al. 1995; Banks et al. 2000).

The molecular characterisation of AIV H5N2 isolates obtained before and during the 1995 outbreak of HPAI in Mexico clearly show that mutation was the cause of the creation of HPAI virus from non-pathogenic viruses (Garcia et al. 1996). The mutation resulted in the insertion of six nucleotides coding for two basic amino acids (R and K) in addition to a single nucleotide mutation (G to A) (Table 8.3). The insertion most likely happened as the result of a transcription fault of the host polymerase complex and the single mutation could have occurred at the same time (Garcia et al. 1996). The New Zealand isolate has the same deduced amino acid sequences at the HA cleavage site as the Mexican non-pathogenic virus (Table 8.3) (differs only by one silent nucleotide) and can potentially mutate in a similar manner as the Mexican isolate, providing that the environment is right. The assumption is that this transcription fault occurs more readily with chicken and turkey enzymes than with those of wild birds.

Table 8.3 Comparison of nucleotide/amino acid sequences at the HA cleavage site of influenza H5N2 viruses of low and high virulence isolated in New Zealand and Mexico 1993–95.

Isolate	Nucleotide	Amino acids	Virulence
NZ1/97	CCTC - - - - - - AAAGAGAAACAAGG*GGT	PQ - - RETR* G	Low
ME93-4	CCCC - - - - - - AAAGAGAAACAAGA*GGC	PQ - - RETR*G	Low
ME94-5	CCCCAAAGAAAAAGAAAAACAAGA*GGC	PQRKRKTR*G	High

No information is available on the prevalence of AIVs in commercial poultry in New Zealand because no surveillance has been carried out to determine this. We can only assume that its prevalence is likely to be very similar to that for APMV-1 because these two viruses have very similar epidemiology. The Ministry of Agriculture and Forestry

is currently in the planning stage to determine the prevalence of AIV in commercial poultry as part of a trade requirement and for the development of importation protocols.

The possibility of outbreaks of ND and HPAI does exist in New Zealand. The findings from this study and elsewhere emphasise the importance of good biosecurity measures on poultry farms, to prevent the introduction of viruses of low virulence, as well as monitoring for the presence and type of APMV-1 and AIV in wild and domestic birds. The situation is likely to be dynamic with new strains emerging and the occurrence of clinically important introductions is a real possibility.

Mallard ducks in the pond at Massey University—a potential reservoir of avian influenza and paramyxoviruses.

Flock of lesser knots in northern New Zealand—are they bringing exotic viruses to New Zealand?

Appendices

APPENDIX A Avian paramyxovirus (APMV) serology (by HI test) and avian influenza (AI) serology by NP-B-ELISA as well as virus isolation on mallard duck samples collected in January–March 1997

APPENDIX B BLAST results

APPENDIX C Buffers and solutions

APPENDIX D A survey for paramyxoviruses in caged birds, wild birds, and poultry in New Zealand (Published results, 2001)

APPENDIX E Avian paramyxoviruses and influenza viruses isolated from mallard ducks (*Anas platyrhynchos*) in New Zealand (Published results, 2002)

APPENDIX A Avian paramyxovirus (APMV) serology (by HI test) and avian influenza (AI) serology by NP-B-ELISA as well as virus isolation on mallard duck samples collected in January–March 1997. (Where HI titres obtained were <4, no data are recorded. n/t, not tested. K, Kaituna; F, Feilding; C, Carterton; T, Temuka; I, Invercargill. J, juvenile; A, adult. M, male; F, female.*,Virus isolated from both tracheal and cloacal swabs.)

ID	J/A	M/F	HI test (titres) APMVs								ELISA inhibition (%)	Virus isolation
			-1	-2	-3	-4	-6	-7	-8	-9		
K1	A	F	4				8				100	
K2	J	M	4			4	4				25.3	
K3	J	M	8			4	8	4			8.3	
K4	A	M	8				≥64	8	4		50.5	AIV; H4N6
K5	J	M	4								10.2	APMV-4
K6	J	M	4			4	8	8			4.5	AIV; H4N6
K7	J	M	4			4	8	4	4		14.6	
K8	A	M	≥64				8				n/t	
K9	J	M	16								8.6	
K10	J	F	4			4	4	4		4	8.2	
K11	A	M	4				8				n/t	
K12	J	M	8				≥64				94.8	
K13	A	F	4			4	4				0	
K14	J	M	8								13.5	
K15	A	M	16	4	4	4	8	8	4	4	60.8	
K16	A	F	8					8			36.1	
K17	A	F	4				4				0	
K18	J	F	16				8				80.9	
K19	A	F	8			4	8				19.1	
K20	J	F	8				4		8		n/t	
K21	A	F	4						4		0	
K22	J	M	32			8	4	4	8		34.3	
K23	J	M	8			8	16		8		3.4	APMV-4
K24	J	M	32			4	8		4		43.7	APMV-4
K25	J	F	16			4	8		4		3.6	

ID	J/A	M/F	HI test (titres) APMVs								ELISA inhibition (%)	Virus isolation
			-1	-2	-3	-4	-6	-7	-8	-9		
K26	J	M	16				4	4	4		100	AIV; H4N6
K27	J	M	32			4	16	8	8		100	
K28	J	F	8				8		4		6.1	
K29	J	M	≥64								45.9	
K30	A	F	32				8	4	4		0	APMV-4
K31	J	M	8			4	8				12.6	
K32	J	F	32				4	4	8	4	86.4	APMV-4
K33	J	M	4	4		4	8	8	4	4	1.6	
K34	A	M	8				4				0	
K35	A	M	8			4	4	8	4		56.9	
K36	J	M	8				8				0	APMV-1
K37	J	M	8			4	8	4	4		4.8	
K38	A	F	16				16	4	4		18.2	
K39	A	F	4								18.2	APMV-4*
K40	J	M	<4								4.5	APMV-4
K41	A	F	4			4	8				3.5	
K42	A	F	16	4	4	4	8	8	4		100	
K43	A	M	4				4				0	
K44	J	M	4				4				0	APMV-4
K45	A	F	≥64				4	4	4		71.4	
K46	J	M	8				8				3.8	
K47	J	M	≥64	4	4	8	16	8	4	4	0	
K48	A	F	4						4		0	
K49	A	F					8				0	
K50	J	F	<4								9.3	
K51	J	F	8			4	4	8	4		12.2	APMV-4
K52	J	M	8				8		4	4	8.3	
K53	A	F	8				4				57.1	
K54	A	F	8	4				4			6.5	APMV-4
K55	J	F	8				4	4	4		40.8	
K56	J	M	32			8	8	4	32	8	15.8	
K57	J	M	4				4				11.6	
K58	A	F	4				4				45.6	APMV-4
K59	J	M	16			4	4	4	8		45.4	
K60	J	F	32						16		10.7	APMV-4*

ID	J/A	M/F	HI test (titres) APMVs								ELISA inhibition (%)	Virus isolation
			-1	-2	-3	-4	-6	-7	-8	-9		
K61	A	F	<4								0	
K62	A	F	16		4	4	16	4	4	4	36.6	APMV-4
K63	A	F	8								1.9	
K64	J	M	8				16		4		11.3	
K65	J	F	<4								23.7	
K66	A	M	16		4	8	16		16	4	12.5	
K67	J	M	4				16		4		43.1	
K68	J	M	16				≥64	16	8		27.8	
K69	A	F	16				4	4	4		29.9	
K70	A	F	<4								5.1	
F1	A	F	8	4		8	8	8	4		68.5	
F2	A	F	32			8	16		4		6.1	
F3	A	F	16			16	8	4			2.5	
F4	A	F	32	4		4	8	4	8	4	11.8	
F5	A	F	32				16	4	8	4	45.2	
F6	A	M	32				8				36.8	
F7	A	F	4				4				4.7	
F8	A	F	16			8	8	4	8		100	
F9	A	F	8				4		16		10.4	
F10	A	F	8				8	4	4		11.5	
F11	J	F	8			4					2.2	
F12	A	F	16	4		16	32	8	8	4	100	
F13	A	F	16				16	4	4	4	1.8	
F14	A	F	<4								15.8	
F15	A	F	16			16	8		4		67.9	
F16	A	F	16				8	4	4		30.7	
F17	A	F	8				4				16.7	
F18	A	M	8				8	8	8		18.5	
F19	A	F	<4								85.4	
F20	A	F	16			4	4				23.9	
F21	A	F	8				4	4	4		21.5	
F22	A	F	8								30.9	
F23	A	M	16				8	4	4	4	13.3	
F24	A	F	16				4	4		4	75.9	
F25	A	F	32								100	

ID	J/A	M/F	HI test (titres) APMVs								ELISA inhibition (%)	Virus isolation
			-1	-2	-3	-4	-6	-7	-8	-9		
F26	A	F	8	4		4	8	4	8	4	98.7	
F27	A	F	≥64				8	4	8	4	100	
F28	A	F	8			4	8	4	4		100	
F29	A	F	32				8		8		43.1	
F30	A	F	16			8	8	4	4		100	
F31	A	F	16			8	8	4	4		18	
F32	A	F	8			8	8	4	4	4	32.8	
F33	A	F	≥64			16	8	8	4	8	94.3	
F34	A	F	8			8	8	4	4		93	
F35	A	F	8				8	4	8		100	
F36	A	F	32	4		8	16	4	4		96.4	
F37	J	M	4				4	4	4		24.9	
F38	A	M	16			4	8		4		72.7	
F39	A	M	8			4	4	4	4	4	18.9	
F40	A	M	32			8	4				20.1	
F41	A	M	16			8	8	4	4		29.5	
F42	J	F	16				4		8		100	
F43	A	M	16			8	8	4	4		52.6	
F44	A	M	8			8	8	4	4		71.5	
F45	A	M	32			8	16	4	4		20.8	
F46	A	M	8								14.4	
F47	A	M	16			4	16	4	4		63.8	
F48	A	M	8			4	4				14.3	
F49	A	M	8			4	8	4	8		12.6	
F50	A	F	16				4	4	4		17.2	
F51	A	M	16			4	8	4	4		25.1	
F52	A	F	16				8	4	4		9.8	
F53	A	F	8				16	4	4		14.1	
F54	A	F	8				8	4	8		14.2	
F55	A	F	8			4	8	4	4		27.5	
F56	A	F	16					8			63.6	
F57	A	F	16			4	8	4	4		57.8	
F58	A	F	8			4	4	4			20.8	
F60	J	F	≥64			16	8	4	4	4	100	

ID	J/A	M/F	HI test (titres) APMVs								ELISA inhibition (%)	Virus isolation
			-1	-2	-3	-4	-6	-7	-8	-9		
F61	J	F	8			4	4				20.5	
F62	A	F	16			4	8	8	8		6.6	
F63	A	F	32			8	8	4	32		100	
F64	A	F	8			4	8	4			35.1	
F65	A	F	8			4	8	4	4	4	18.9	
F66	A	F	16			8	8	4	4		48.9	
F67	A	F	16			8	8	4	8	4	23.6	
F68	A	F	16				8				n/t	
F69	J	F	≥64			8	4		4		100	
C1	A	M	32				8		32		84.1	
C2	J	F	8				8		16		1.1	
C3	J	F	8				4		4		39.4	
C4	A	M	16				4	4	4		0	
C5	J	F	4				4		8		12	
C6	J	M	4						8		6.8	
C7	A	M	16			4	4		4		47.7	
C8	J	M	8				8				37.3	
C9	J	M	8				4		8		9.3	APMV-1
C10	J	M	8				4		8		16.5	APMV-1*
C11	A	M	n/t	n/t	n/t	n/t	n/t	n/t	n/t	n/t	n/t	
C12	J	F	32				4	4	4		0	
C13	J	F	8				4		4		1.8	
C14	J	F	16				8	8	8		0	
C15	J	M	4	4				8			83.9	
C16	A	F									82.9	
C17	J	F	4				4	4	4	4	6.6	
C18	J	M	8				4	4	4		88.3	APMV-4
C19	A	M	4			4	4	4	4		1.5	

ID	J/A	M/F	HI test (titres) APMVs								ELISA inhibition (%)	Virus isolation
			-1	-2	-3	-4	-6	-7	-8	-9		
C20	A	M	16				8	4	4		0	
C21	J	M	<4								0	APMV-1
C22	J	M	16			4	4	4	4		0	
C23	J	F	16				4		4		92.4	
C24	A	F	16				8		4		100	
C25	A	M	≥64				4	4	4		0	
C26	A	M	4				4				0	
C27	A	F	16				8	8	8	4	41	
C28	A	F	8				8		8		0	
C29	A	F	8			4	4				0	
C30	J	M	4				4		4		2.6	
C31	A	M	≥64				8				90.2	
C32	A	M	16				4	4	4		12.4	
C33	A	M	16				8	4	4		77.9	
C34	A	F	≥64		8	16	8	4		16	12.2	
C35	J	F	16				8				7.2	
C36	J	M	16			4	8	8	4		47.6	
C37	J	M	8				8	8	4		0	
C38	J	M	8				4		16		18.8	
C39	J	F	8			4	≥64	8	4		25.5	
C40	J	F	8				4	4	8		0	APMV-1
C41	A	M	8			4	4				4	
C42	J	F	16				8		4		10	
C43	J	F									100	APMV-1*
C44	A	M	16			4	8	4	4		27.3	APMV-1
C45	J	F	8			4	4	4	4		15.6	
C46	A	F									33.6	
C47	J	M	n/t	n/t	n/t	n/t	n/t	n/t	n/t	n/t	n/t	
C48	J	M	4			8	8				4.9	APMV-1
C49	J	F	8			4	8	4			6.6	
C50	A	F	8				4			16	13.4	

ID	J/A	M/F	HI test (titres) APMVs -1	-2	-3	-4	-6	-7	-8	-9	ELISA inhibition (%)	Virus isolation
C51	A	M	32				8				63.2	
C52	A	F	8								0	
C53	A	F	8								2.9	
C54	J	M	8				8		4		23.4	
C55	A	F	32				8				100	
C56	A	M	32			16	4	4	4		23.9	
C57	J	M	8				4	4	4		6.2	
C58	J	M	16			4	4	4			0	
C59	J	M	4			4	4				20.7	
C60	J	F	8			4	4				0	
C61	A	M	16			4	8	4			100	
C62	J	F	8			8	8		8		37.5	
C63	A	F	32			4	4	4			14.7	
C64	J	F	n/t	n/t	n/t	n/t	n/t	n/t	n/t	n/t	n/t	
C65	J	M	n/t	n/t	n/t	n/t	n/t	n/t	n/t	n/t	n/t	APMV-4
C66	J	F	≥64			4	4		4		74.4	
C67	A	F	8				4	4	8		20.6	
C68	J	F	16				4		4		4	
C69	A	F	16				4	4	4		0	
C70	J	F	8			8	4		4		0	
T1	A	F	8	4		8	8	8	4		51.9	
T2	A	M	32	4	4	8	8	4	4	4	n/t	
T3	A	F	8				4	4	4		10.7	
T4	A	F	16	4		4	32	8	8	4	41.8	
T5	A	M				4	4		4		95.6	AIV, H5N2
T6	A	F	8	4		4	16	8	4		100	
T7	A	F	16			16	16	16	4		94.7	
T8	A	F	8			8	16	4	8		54	
T9	A	F	32								9.7	
T10	A	M	16	4	4	8	8	8	8	4	87.4	
T11	A	F	8	4		8	8				47.9	
T12	A	F	16			4	8		4		1.1	
T13	A	M	8			8	8				27.1	
T14	A	M	16	4	4	16	8	8	4	4	12.3	
T15	A	M	16	4	4	16	8	4	4	4	32.1	

ID	J/A	M/F	HI test (titres) APMVs								ELISA inhibition (%)	Virus isolation
			-1	-2	-3	-4	-6	-7	-8	-9		
T16	A	F	16	4	4	8	≥64	4	4		75.7	
T17	A	M	32	8	8	16	16	8	8	8	13.2	
T18	J	M	16	8	8	8	16	16	32	8	20.8	
T19	A	F	≥64	4	4	8	16	32	32	4	62.1	AIV,H5N2
T20	A	F	16	8	8	8	16	4	4		63.7	
T21	A	F									1.5	
T22	A	M	16			4	8	8	4		16.3	
T23	A	F	16			8	8				89.5	
T24	A	F	4								33.2	
T25	A	F	4								55.3	
T26	A	M	4			4	8	4	4		100	
T27	A	F									35	
T28	A	M	8			8	8	8			9.4	
T29	J	M	16			4	8	4	4		100	
T30	A	F	16			8	8	4	4		33.3	
T31	A	M	≥64	4	4	4	32	4	4	4	46.8	
T32	J	M	16			16	16	4	4		38.9	
T33	A	M	16			8	8				47.8	
T34	A	M	16			4	4		4		63.6	
T35	A	M	32	8				8	8		100	
T36	A	M	32	4		4	32	32	8	4	84	
T37	A	F	8			4	4	4	4		23.2	
T38	A	F	8				16				59.7	
T39	A	F	8								98.3	
T40	J	M	16	4		8	16	8	8	4	0	
T41	A	M	8			8	16	4	4		60.5	
T42	A	F	4	4		4	8	8	4		n/t	
T43	A	M	16	8	8	8	16	16			100	
I1	A	F									79	
I2	A	F	8				8				63.5	
I3	A	M	8				16		8	4	56.6	
I4	A	F	4			4	4				34.8	
I5	A	F									100	
I6	A	F	32			16	16			16	87.2	
I7	A	M	4				4				100	

ID	J/A	M/F	HI test (titres) APMVs								ELISA inhibition (%)	Virus isolation
			-1	-2	-3	-4	-6	-7	-8	-9		
I8	A	F	8	4		8	8	4		4	36.7	
I9	A	F	4								56.6	
I10	A	M	4			4	4				6.7	
I11	A	F	32	8	4	4	8	8			100	
I12	A	F	16			4	16		8		97.4	
I13	A	F	8			8	8		8		90.1	
I14	A	F	16			8	8			4	19.8	
I15	A	F	16	4		16	16		16		94.6	
I16	A	F									86.6	
I17	A	F	8			4	4				14.3	
I18	A	F									42.6	
I19	A	M	8			8	4	4	4		47.1	
I20	A	F	32			4	16	16			90.5	
I21	J	M	32				4				100	
I22	A	M	4			4	4				100	
I23	A	M	16	8		8	8	4		4	35.2	
I24	A	F	8	4		8	16	8	4	4	75.6	
I25	A	F	4			32					13.9	
I26	A	F	16			8	8				96.7	
I27	A	M	8				4		16		82.1	
I28	J	M	8			8	16				41.5	
I29	A	F	8				4	4			100	
I30	A	F									21.2	
I31	A	M	16				8	4	4	4	17.3	
I32	A	F									9.8	
I33	A	M	8	4		4	8	8	4	4	12.1	
I34	J	M	8			8	8	4			41.1	
I35	J	M	32			4	8				43.6	
I36	J	M	8	4		4	4	4			58.4	
I37	J	F	16			8	8				27.9	
I38	A	F	16				16		8		24.3	
I39	A	M	32				8	16			16.2	
I40	J	M	8		4						97.9	

ID	J/A	M/F	HI test (titres) APMVs -1	-2	-3	-4	-6	-7	-8	-9	ELISA inhibition (%)	Virus isolation
141	A	M	≥64			4	8	4	16	8	85.5	
142	A	F									n/t	
143	A	M	32					4			31.4	
144	A	M									n/t	
145	A	M									2.9	
146	J	M									45.5	
147	J	M	4						4		8.9	
148	A	F									23.5	
149	J	M	8								91.3	
150	A	F	32			32	16		32		34.8	
151	J	F	8			4	8				23.3	
152	J	M	32				16				100	
153	A	F	8			4	16		4		1.9	
154	J	M									100	
155	A	M	16				16				24.1	
156	J	M	8			4	8		8		3.9	
157	A	M	4				4		4		42.9	
158	A	F	4			4	4				0	
159	A	F	16				32				90.4	
160	J	F									0	
161	J	M	4			8	8				91.2	
162	A	F	16	4	4	8	8	4	4	4	11.2	
163	A	M	16	4		8	8	4			75.3	
164	A	M	16								0	
165	A	M									4.7	
166	A	M									99.4	
167	J	M	8			4	4				0	
168	A	F	16				4	4			37.8	
169	A	F									100	
170	A	F	32			4	32				75.2	
171	A	F	32			8	4	4			35.9	
172	A	F	16			4	8		8		24.4	
173	A	M	16				4		8		97.2	
174	J	F									25	

ID	J/A	M/F	HI test (titres) APMVs -1	-2	-3	-4	-6	-7	-8	-9	ELISA inhibition (%)	Virus isolation
I75	A	M	4				4		4		23.1	
I76	A	M	4			4	8				78.1	
I77	A	F	≥64	4	8	32	4		4	8	17.7	
I78	A	F	16			8					21.2	
I79	A	F	8			32	4		4		87.5	
I80	A	M	8	4		4	8	4	4	4	100	
I81	A	M	n/t	n/t	n/t	n/t	n/t	n/t	n/t	n/t	17.7	
I82	A	M	n/t	n/t	n/t	n/t	n/t	n/t	n/t	n/t	54.3	
I83	A	M	n/t	n/t	n/t	n/t	n/t	n/t	n/t	n/t	100	
I84	A	M	n/t	n/t	n/t	n/t	n/t	n/t	n/t	n/t	42.7	
I85	J	F	n/t	n/t	n/t	n/t	n/t	n/t	n/t	n/t	100	
I86	A	F	32			32	16		32		n/t	
I89	A	F	n/t	n/t	n/t	n/t	n/t	n/t	n/t	n/t	96.1	
I90	A	M	n/t	n/t	n/t	n/t	n/t	n/t	n/t	n/t	100	
I91	A	M	n/t	n/t	n/t	n/t	n/t	n/t	n/t	n/t	20.5	
I92	J	M	n/t	n/t	n/t	n/t	n/t	n/t	n/t	n/t	78.2	
I93	A	M	n/t	n/t	n/t	n/t	n/t	n/t	n/t	n/t	31.3	
I94	J	F	n/t	n/t	n/t	n/t	n/t	n/t	n/t	n/t	13.6	
I95	A	F	n/t	n/t	n/t	n/t	n/t	n/t	n/t	n/t	9.9	
I96	A	M	n/t	n/t	n/t	n/t	n/t	n/t	n/t	n/t	99.6	

APPENDIX C BLAST results

The first three sequences (except for NZ1/97) for the sequences producing significant alignments.

APMV-1 viruses

```
Query= NZ10/97 (F gene)
Beg:1, End:238 (238 letters)
Database: nt 671,573 sequences; -2,051,914,455 total letters
Searchingdone.
                                                                  Score(bits) E Value
gb|M24692.1|M24692 Newcastle disease virus fusion protein gene, ... 432 e-119
gb|M24693.1|NDVFPB Newcastle disease virus fusion protein gene, ... 416 e-114
gb|AF217084.1|AF217084 Newcastle disease virus fusion protein ge... 408 e-112

gb|M24692.1|M24692 Newcastle disease virus fusion protein gene, (strain D26/76) complete
cds, Length = 1823, Score = 432 bits (218), Expect = e-119
Identities = 233/238 (97%)Strand = Plus / Plus

Query: 1   tgattctattcgtaggatacaagagtctgtgaccacgtccggaggagggaaacagggacg 60
           ||||||||| |||||||||||||||||||||||| |||||||||||||||||||||||
Sbjct: 334 tgattctatccgtaggatacaagagtctgtgaccacatccggaggagggaaacagggacg 393

Query: 61  tcttataggagccattatcggtggtgtagctctcggggttgcaaccgctgcacagataac 120
           ||||||||| ||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct: 394 tcttataggcgccattatcggtggtgtagctctcggggttgcaaccgctgcacagataac 453

Query: 121 agcagcctcggctctgatacaggccaatcaaaatgctgccaacatcctccggctcaaaga 180
           |||||||||||||||||||| |||||||||||||||||||||||||||||||||||||
Sbjct: 454 agcagcctcggctctgatacaagccaatcaaaatgctgccaacatcctccggctcaaaga 513

Query: 181 gagcattgctgcaaccaatgaggctgtgcatgaggtcactgacggattatcacaacta 238
           |||||||||||||||||||||||||||||| |||||||||||||||||||||||||
Sbjct: 514 gagcattgctgcaaccaatgaggctgtgcacgaggtcactgacggattatcacaacta 571


gb|M24693.1|NDVFPB Newcastle disease virus fusion protein gene, strain Que/66, complete
cds Length = 1823, Score = 416 bits (210), Expect = e-114
Identities = 231/238 (97%)Strand = Plus / Plus
Query: 1   tgattctattcgtaggatacaagagtctgtgaccacgtccggaggagggaaacagggacg 60
           ||||||||| |||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct: 334 tgattctatccgtaggatacaagagtctgtgaccacgtccggaggagggaaacagggacg 393

Query: 61  tcttataggagccattatcggtggtgtagctctcggggttgcaaccgctgcacagataac 120
           ||||||||| |||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct: 394 tcttataggcgccattatcggtggtgtagctctcggggttgcaaccgctgcacagataac 453

Query: 121 agcagcctcggctctgatacaggccaatcaaaatgctgccaacatcctccggctcaaaga 180
            |||||||||||||||||||| |||||||||||||||||||||||||| |||||||||
Sbjct: 454  agcagcctcggctctgatacaagccaatcaaaatgctgccaacatcctcctgctcaaaga 513

Query: 181  gagcattgctgcaaccaatgaggctgtgcatgaggtcactgacggattatcacaacta 238
            |||||||||||||||||||||||||||||| ||||||||| | ||||||||||||||
Sbjct: 514  gagcattgctgcaaccaatgaggctgtgcacgaggtcactaatggattatcacaacta 571


gb|AF217084.1|AF217084 Newcastle disease virus fusion protein gene, strain V4, complete
cds Length = 1823, Score = 408 bits (206), Expect = e-112
Identities = 230/238 (96%) Strand = Plus / Plus

Query:1    tgattctattcgtaggatacaagagtctgtgaccacgtccggaggagggaaacagggacg 60
           ||||||||| |||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct: 334 tgattctatccgtaggatacaagagtctgtgaccacgtccggaggagggaaacagggacg 393
```

```
Query: 61 tcttataggagccattatcggtggtgtagctctcggggttgcaaccgctgcacagataac 120
          ||||||||| ||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct: 394 tcttataggcgccattatcggtggtgtagctctcggggttgcaaccgctgcacagataac 453


Query: 121 agcagcctcggctctgatacaggccaatcaaaatgctgccaacatcctccggctcaaaga 180
           |||||||||||||||||||| |||||||||||||||||||||| |||||||| |||||
Sbjct: 454 agcagcctcggctctgatacaagccaatcaaaatgctgccaacatactccggctaaaaga 513

Query: 181 gagcattgctgcaaccaatgaggctgtgcatgaggtcactgacggattatcacaacta 238
           |||||||||||||||||||||||||||||| ||||||||| | ||||||||||||||
Sbjct: 514 gagcattgctgcaaccaatgaggctgtgcacgaggtcactaatggattatcacaacta 571
```

Query= NZ132/76 (F gene)
Beg:1, End:238 (238 letters)Database: nt 671,573 sequences; -2,051,914,455 total letters
Searchingdone.

	Score(bits)	E Value
gb|M24692.1|M24692 Newcastle disease virus fusion protein gene, ...	448	e-124
gb|M24693.1|NDVFPB Newcastle disease virus fusion protein gene, ...	432	e-119
gb|AF217084.1|AF217084 Newcastle disease virus fusion protein ge...	424	e-117

gb|M24692.1|M24692 Newcastle disease virus fusion protein gene,strain D26/76,
complete cds, Length = 1823 Score = 448 bits (226), Expect = e-124
Identities = 235/238 (98%) Strand = Plus / Plus

```
Query: 1   tgattctatccgtaggatacaagagtctgtgaccacgtccggaggagggaaacagggacg 60
           |||||||||||||||||||||||||||||||||||| ||||||||||||||||||||||
Sbjct: 334 tgattctatccgtaggatacaagagtctgtgaccacatccggaggagggaaacagggacg 393

Query: 61  tcttataggagccattatcggtggtgtagctctcggggttgcaaccgctgcacaaataac 120
           ||||||||| ||||||||||||||||||||||||||||||||||||||||||| |||||
Sbjct: 394 tcttataggcgccattatcggtggtgtagctctcggggttgcaaccgctgcacagataac 453

Query: 121 agcagcctcggctctgatacaagccaatcaaaatgctgccaacatcctccggctcaaaga 180
           ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct: 454 agcagcctcggctctgatacaagccaatcaaaatgctgccaacatcctccggctcaaaga 513

Query: 181 gagcattgctgcaaccaatgaggctgtgcacgaggtcactgacggattatcacaacta 238
           ||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct: 514 gagcattgctgcaaccaatgaggctgtgcacgaggtcactgacggattatcacaacta571
```

gb|M24693.1|NDVFPB Newcastle disease virus fusion protein gene, strain Que/66, complete
cds, Length = 1823 Score = 432 bits (218),Expect = e-119
Identities = 233/238 (97%) Strand = Plus / Plus

```
Query: 1    tgattctatccgtaggatacaagagtctgtgaccacgtccggaggagggaaacagggacg 60
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct: 334  tgattctatccgtaggatacaagagtctgtgaccacgtccggaggagggaaacagggacg 393

Query: 61   tcttataggagccattatcggtggtgtagctctcggggttgcaaccgctgcacaaataac 120
            ||||||||| ||||||||||||||||||||||||||||||||||||||||||| |||||
Sbjct: 394  tcttataggcgccattatcggtggtgtagctctcggggttgcaaccgctgcacagataac 453

Query: 121  agcagcctcggctctgatacaagccaatcaaaatgctgccaacatcctccggctcaaaga 180
            |||||||||||||||||||||||||||||||||||||||||||||||||| |||||||||
Sbjct: 454  agcagcctcggctctgatacaagccaatcaaaatgctgccaacatcctcctgctcaaaga 513

Query: 181  gagcattgctgcaaccaatgaggctgtgcacgaggtcactgacggattatcacaacta 238
            |||||||||||||||||||||||||||||||||||||| | ||||||||||||||||
Sbjct: 514  gagcattgctgcaaccaatgaggctgtgcacgaggtcactaatggattatcacaacta 571
```

```
gb|AF217084.1|AF217084 Newcastle disease virus fusion protein gene, strain V4, complete
cds Length = 1823 Score = 424 bits (214), Expect = e-117
Identities = 232/238 (97%) Strand = Plus / Plus

Query: 1   tgattctatccgtaggatacaagagtctgtgaccacgtccggaggagggaaacagggacg 60
           ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct: 334 tgattctatccgtaggatacaagagtctgtgaccacgtccggaggagggaaacagggacg 393

Query: 61  tcttataggagccattatcggtggtgtagctctcggggttgcaaccgctgcacaaataac 120
           ||||||||| ||||||||||||||||||||||||||||||||||||||||||| |||||
Sbjct: 394 tcttataggcgccattatcggtggtgtagctctcggggttgcaaccgctgcacagataac 453

Query: 121 agcagcctcggctctgatacaagccaatcaaaatgctgccaacatcctccggctcaaaga 180
           |||||||||||||||||||||||||||||||||||||||||| |||||||| |||||
Sbjct: 454 agcagcctcggctctgatacaagccaatcaaaatgctgccaacatactccggctaaaaga 513

Query: 181 gagcattgctgcaaccaatgaggctgtgcacgaggtcactgacggattatcacaacta 238
           |||||||||||||||||||||||||||||||||||| | ||||||||||||||||
Sbjct: 514 gagcattgctgcaaccaatgaggctgtgcacgaggtcactaatggattatcacaacta 571


Query= NZ1/97 (F gene)
Beg:1, End:238 (238 letters)Database: nt 671,573 sequences; -2,051,914,455 total letters
Searchingdone.
                                                                  Score(bits) E Value
gb|AF003726.1|AF003726 Newcastle disease virus strain PMV-1/shel... 391 e-106
gb|AF003727.1|AF003727 Newcastle disease virus strain PMV-1/chic... 335 5e-90


gb|AF003726.1|AF003726 Newcastle disease virus strain PMV-1/shelduck/France/MC110/77
fusion protein (F) mRNA, partial cds Length = 309 Score = 391 bits (197),
Expect = e-106 Identities = 227/237 (95%) Strand = Plus / Plus

Query: 2   gattcaattaggagaatccaagagtcagtaactacatcaggaggagaacggcaggggcgt 61
           |||||||| || |||||||||||||||||||| |||||||| |||||||||||||| ||||
Sbjct: 34  gattcaatcagaagaatccaagagtcagtaaccacatcaggggagaacggcaggagcgt 93

Query: 62  ttggtgggggcaataataggaggcgtcgcattaggtgtagccaccgcagcacagatcaca 121
           |||||||||||||||||||||||||||||||||||||||||||||||| |||||||||
Sbjct: 94  ctggtgggggcaataataggaggcgtcgcattaggtgtagccaccgcagcgcagatcaca 153

Query: 122 gcggcttctgccctcatacaagccaaccagaatgccgcgaacatattgaaattaaaggag 181
           || |||||||||||||||||||||||||||||| |||||||||| ||||||||||||||
Sbjct: 154 gcagcttctgccctcatacaagccaaccagaatgctgcgaacatactgaaattaaaggag 213

Query: 182 agtattgccgctaccaatgaagcagtgcatgaggtcacaaatgggttgtcccagctg 238
           ||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct: 214 agtattgccgctaccaatgaagcagtgcatgaggtcacaaatgggttgtcccagctg 270


gb|AF003727.1|AF003727 Newcastle disease virus strain PMV-1/chicken/Republic of
Ireland/34/90 fusion protein (F) mRNA, partial cds Length = 309 Score = 335 bits (169),
Expect = 5e-90 Identities = 220/237 (92%) Strand = Plus / Plus

Query: 2   gattcaattaggagaatccaagagtcagtaactacatcaggaggagaacggcaggggcgt 61
           ||||| |||||||||||||||||||||| |||||||||||| |||||||| ||||
Sbjct: 34  gattcgattaggagaatccaagagtcagtaaccacatcaggaggaaaacggcagaagcgt 93

Query: 62  ttggtgggggcaataataggaggcgtcgcattaggtgtagccaccgcagcacagatcaca 121
           || || |||||||||||||||||||||||||||||| |||||||||||| |||||||||
Sbjct: 94  tttgtaggggcaataataggaggcgtcgcattaggtgtcgccaccgcagcgcagatcaca 153

Query: 122 gcggcttctgccctcatacaagccaaccagaatgccgcgaacatattgaaattaaaggag 181
           || |||||||||||||||||||||||||||||| || |||||| ||||||||||||||
Sbjct: 154 gcagcttctgccctcatacaagccaaccagaatgctgcaaacatactgaaattaaaggag 213
```

```
Query: 182 agtattgccgctaccaatgaagcagtgcatgaggtcacaaatgggttgtcccagctg 238
           ||||||||||||||||||||||||||| |||||||||||||| ||| ||||||||||||
Sbjct: 214 agtattgccgctaccaatgaagcagtacatgaggtcacaagcgggctgtcccagctg 270
```

Query NZ131/76 (HN gene)

```
Beg:1, End:304 (304 letters)Database: nt 942,448 sequences; -363,420,810 total letters
Searchingdone.
                                                                Score(bits) E Value
gb|M24706.1|NDVHANAB Newcastle disease virus QUE/66 hemagglutini... 587 e-165
gb|J03911.1|NDVGPP Newcastle disease virus hemagglutinin-neurami... 587 e-165
emb|X85971.1|NCVRNAHN Newcastle disease virus mRNA for haemaglut... 579 e-163

gb|M24706.1|NDVHANAB Newcastle disease virus QUE/66 hemagglutinin-neuraminidase gene,
complete cds Length = 2002 Score = 587 bits (296), Expect = e-165
Identities = 302/304 (99%) Strand = Plus / Plus

Query: 1    ggtgagttcaagcagcaccaaggcagcatacacaacatcaacatgttttaaagttgtaaa 60
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct: 1639 ggtgagttcaagcagcaccaaggcagcatacacaacatcaacatgttttaaagttgtaaa1698


Query: 61   gactaataaaacctattgtctcagcattgccgaaatatccaataccctcttcggggaatt 120
             ||| ||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct: 1699 gaccaataaaacctattgtctcagcattgccgaaatatccaataccctcttcggggaatt1758

Query: 121  cagaatcgtccctttactagttgagattctcaaggatgatggggttagagaagccaggtc 180
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct: 1759 cagaatcgtccctttactagttgagattctcaaggatgatggggttagagaagccaggtc 1818

Query: 181  tagccggtcgagtcaactgcgagagggttggaaagatgacattgtatcacctatcttttg 240
             |||||||| ||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct: 1819 tagccggttgagtcaactgcgagagggttggaaagatgacattgtatcacctatcttttg 1878

Query: 241  cgacgccaagaatcaaactgaataccggcgcgagctcgagtcctacgctgccagttggcc 300
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct: 1879 cgacgccaagaatcaaactgaataccggcgcgagctcgagtcctacgctgccagttggcc 1938

Query: 301  ataa 304
             ||||
Sbjct: 1939 ataa 1942


gb|J03911.1|NDVGPP Newcastle disease virus hemagglutinin-neuraminidase mRNA, V4,
complete cds Length = 2002 Score = 587 bits (296), Expect = e-165
Identities = 302/304 (99%) Strand = Plus / Plus

Query: 1    ggtgagttcaagcagcaccaaggcagcatacacaacatcaacatgttttaaagttgtaaa 60
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct: 1639 ggtgagttcaagcagcaccaaggcagcatacacaacatcaacatgttttaaagttgtaaa 1698

Query: 61   gactaataaaacctattgtctcagcattgccgaaatatccaataccctcttcggggaatt 120
             ||| ||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct: 1699 gaccaataaaacctattgtctcagcattgccgaaatatccaataccctcttcggggaatt 1758

Query: 121  cagaatcgtccctttactagttgagattctcaaggatgatggggttagagaagccaggtc 180
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct: 1759 cagaatcgtccctttactagttgagattctcaaggatgatggggttagagaagccaggtc 1818

Query: 181  tagccggtcgagtcaactgcgagagggttggaaagatgacattgtatcacctatcttttg 240
             |||||||| ||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct: 1819 tagccggttgagtcaactgcgagagggttggaaagatgacattgtatcacctatcttttg 1878

Query: 241  cgacgccaagaatcaaactgaataccggcgcgagctcgagtcctacgctgccagttggcc 300
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct: 1879 cgacgccaagaatcaaactgaataccggcgcgagctcgagtcctacgctgccagttggcc 1938
```

```
Query: 301  ataa 304
            ||||
Sbjct: 1939 ataa 1942
```

emb|X85971.1|NCVRNAHN Newcastle disease virus mRNA for haemaglutinin-neuraminidase protein, V4 vaccine strain. Length = 1996 Score = 579 bits (292), Expect = e-163
Identities = 301/304 (99%) Strand = Plus / Plus

```
Query: 1    ggtgagttcaagcagcaccaaggcagcatacacaacatcaacatgttttaaagttgtaaa 60
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct: 1636 ggtgagttcaagcagcaccaaggcagcatacacaacatcaacatgttttaaagttgtaaa 1695

Query: 61   gactaataaaacctattgtctcagcattgccgaaatatccaataccctcttcggggaatt 120
            ||| ||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct: 1696 gaccaataaaacctattgtctcagcattgccgaaatatccaataccctcttcggggaatt 1755


Query: 121  cagaatcgtccctttactagttgagattctcaaggatgatggggttagagaagccaggtc 180
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct: 1756 cagaatcgtccctttactagttgagattctcaaggatgatggggttagagaagccaggtc 1815

Query: 181  tagccggtcgagtcaactgcgagagggttggaaagatgacattgtatcacctatcttttg 240
            |||||||| ||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct: 1816 tagccggttgagtcaactgcgagagggttggaaagatgacattgtatcacctatcttttg 1875

Query: 241  cgacgccaagaatcaaactgaataccggcgcgagctcgagtcctacgctgccagttggcc 300
            |||||||||||||||||||||||||||||||| ||||||||||||||||||||||||||
Sbjct: 1876 cgacgccaagaatcaaactgaataccggcgcgaggtcgagtcctacgctgccagttggcc 1935

Query: 301  ataa 304
            ||||
Sbjct: 1936 ataa 1939
```

Query NZ10/97 (HN gene)
Beg:1, End:304 (304 letters)Database: nt 942,448 sequences; -363,420,810 total letters
Searchingdone.

	Score(bits)	E Value
gb|M24706.1|NDVHANAB Newcastle disease virus QUE/66 hemagglutini...	563	e-158
gb|J03911.1|NDVGPP Newcastle disease virus hemagglutinin-neurami...	563	e-158
emb|X85971.1|NCVRNAHN Newcastle disease virus mRNA for haemaglut...	555	e-156

gb|M24706.1|NDVHANAB Newcastle disease virus QUE/66 hemagglutinin-neuraminidase gene, complete cds Length = 2002 Score = 563 bits (284), Expect = e-158
Identities = 299/304 (98%) Strand = Plus / Plus

```
Query: 1    ggtgagttcaagcagcaccaaggcagcatacacaacatcaacatgttttaaagttgtaaa 60
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct: 1639 ggtgagttcaagcagcaccaaggcagcatacacaacatcaacatgttttaaagttgtaaa 1698

Query: 61   gactaataaaacctattgtctcagcattgccgaaatatctaataccctctttggggaatt 120
            ||| |||||||||||||||||||||||||||||||| |||||||||| ||||||||
Sbjct: 1699 gaccaataaaacctattgtctcagcattgccgaaatatccaataccctcttcggggaatt 1758

Query: 121  cagaatcgtccctctactagttgagattctcaaggatgatggggttagagaagccaggtc 180
            |||||||||||||| ||||||||||||||||||||||||||||||||||||||||||||
Sbjct: 1759 cagaatcgtcccttttactagttgagattctcaaggatgatggggttagagaagccaggtc 1818

Query: 181  tagccggtcgagtcaactgcgagagggttggaaagatgacattgtatcacctatcttttg 240
            |||||||| ||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct: 1819 tagccggttgagtcaactgcgagagggttggaaagatgacattgtatcacctatcttttg 1878

Query: 241  cgacgccaagaatcaaactgaataccggcgcgagctcgagtcctacgctgccagttggcc 300
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct: 1879 cgacgccaagaatcaaactgaataccggcgcgagctcgagtcctacgctgccagttggcc 1938
```

```
Query: 301  ataa 304
            ||||
Sbjct: 1939 ataa 1942
```

gb|J03911.1|NDVGPP Newcastle disease virus hemagglutinin-neuraminidase mRNA, V4, complete cds Length = 2002 Score = 563 bits (284), Expect = e-158
Identities = 299/304 (98%) Strand = Plus / Plus

```
Query: 1    ggtgagttcaagcagcaccaaggcagcatacacaacatcaacatgttttaaagttgtaaa 60
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct: 1639 ggtgagttcaagcagcaccaaggcagcatacacaacatcaacatgttttaaagttgtaaa 1698

Query: 61   gactaataaaacctattgtctcagcattgccgaaatatctaataccctctttggggaatt 120
            ||| ||||||||||||||||||||||||||||||||| |||||||||| ||||||||
Sbjct: 1699 gaccaataaaacctattgtctcagcattgccgaaatatccaataccctcttcggggaatt 1758

Query: 121  cagaatcgtccctctactagttgagattctcaaggatgatggggttagagaagccaggtc 180
            |||||||||||||| ||||||||||||||||||||||||||||||||||||||||||||
Sbjct: 1759 cagaatcgtccctttactagttgagattctcaaggatgatggggttagagaagccaggtc 1818

Query: 181  tagccggtcgagtcaactgcgagagggttggaaagatgacattgtatcacctatcttttg 240
            |||||||| ||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct: 1819 tagccggttgagtcaactgcgagagggttggaaagatgacattgtatcacctatcttttg 1878

Query: 241  cgacgccaagaatcaaactgaataccggcgcgagctcgagtcctacgctgccagttggcc 300
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct: 1879 cgacgccaagaatcaaactgaataccggcgcgagctcgagtcctacgctgccagttggcc 1938

Query: 301  ataa 304
            ||||
Sbjct: 1939 ataa 1942
```

emb|X85971.1|NCVRNAHN Newcastle disease virus mRNA for haemaglutinin-neuraminidase protein, V4 vaccine strain. Length = 1996 Score = 555 bits (280), Expect = e-156
Identities = 298/304 (98%) Strand = Plus / Plus

```
Query: 1    ggtgagttcaagcagcaccaaggcagcatacacaacatcaacatgttttaaagttgtaaa 60
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct: 1636 ggtgagttcaagcagcaccaaggcagcatacacaacatcaacatgttttaaagttgtaaa 1695

Query: 61   gactaataaaacctattgtctcagcattgccgaaatatctaataccctctttggggaatt 120
            ||| ||||||||||||||||||||||||||||||||| |||||||||| ||||||||
Sbjct: 1696 gaccaataaaacctattgtctcagcattgccgaaatatccaataccctcttcggggaatt 1755

Query: 121  cagaatcgtccctctactagttgagattctcaaggatgatggggttagagaagccaggtc 180
            |||||||||||||| ||||||||||||||||||||||||||||||||||||||||||||
Sbjct: 1756 cagaatcgtcccttttactagttgagattctcaaggatgatggggttagagaagccaggtc 1815

Query: 181  tagccggtcgagtcaactgcgagagggttggaaagatgacattgtatcacctatcttttg 240
            |||||||| ||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct: 1816 tagccggttgagtcaactgcgagagggttggaaagatgacattgtatcacctatcttttg 1875

Query: 241  cgacgccaagaatcaaactgaataccggcgcgagctcgagtcctacgctgccagttggcc 300
            |||||||||||||||||||||||||||||||| ||||||||||||||||||||||||||
Sbjct: 1876 cgacgccaagaatcaaactgaataccggcgcgaggtcgagtcctacgctgccagttggcc 1935

Query: 301  ataa 304
            ||||
Sbjct: 1936 ataa 1939
```

Avian Influenza viruses

```
Query= A/Mallard/NZ/1/97 - H5N2, (HA gene)
Beg:1, End:248 (248 letters)Database: nt 690,083 sequences; -2,006,040,756 total letters
Searchingdone.
                                                                  Score(bits) E Value
gb|U67783.1|AIU67783 Avian influenza virus hemagglutinin (HA) mR... 329 3e-88
gb|U79455.1|IAU79455 Influenza A virus strain A/Turkey/Minnesota... 289 3e-76
gb|U28919.1|AIU28919 Avian influenza virus A/Emu/TX/39442/93 par... 287 1e-75
gb|U67783.1|AIU67783 Avian influenza virus hemagglutinin (HA) mRNA, complete cds Length
= 1740 Score = 329 bits (166), Expect = 3e-88 Mallard/Ohio/556/1987(H5N9)
Identities = 227/246 (92%), Gaps = 1/246 (0%) Strand = Plus / Plus


Query: 1    tgtcaaatcagacagactagttcttgcaacagggctaagaaacgtacctcaaagagaaac 60
             ||||||||| |||| ||| || |||||||||||| |||||||||||||| ||||||||||
Sbjct: 963  tgtcaaatcggacaaactggtccttgcaacaggactaagaaacgtaccccaaagagaaac 1022

Query: 61   aaggggtctatttggagcaatagcaggattcatagaaggaggatggcaaggaatggtgga 120
             ||| || ||||||||||||||||||||||||||||||||||||||||||||||||||| ||
Sbjct: 1023 aagaggcctatttggagcaatagcaggattcatagaaggaggatggcaaggaatggtaga 1082

Query: 121  tgggtggtatggataccatcatagcaatgaacaaggaagtggatatgctgcagacagaga 180
             ||| |||||||||||||||||||||||| || |||||||||||||||||||||| |||
Sbjct: 1083 tggatggtatggataccatcatagcaatgagcagggaagtggatatgctgcagacaaaga 1142

Query: 181  atcgtacccagaaagcaatagatgggatcaccaacaagataaattcaatcattgacaaaa 240
             ||| |||||||||||||| |||||||||||||| || |||| ||||||||||||||||
Sbjct: 1143 atc-tacccagaaagcaatcgatgggatcaccaataaagtaaactcaatcattgacaaaa 1201

Query: 241  tgaaca 246
             ||||||
Sbjct: 1202 tgaaca 1207


gb|U79455.1|IAU79455 Influenza A virus strain A/Turkey/Minnesota/10734/95(H5N2)
hemagglutinin gene, partial cds Length = 1647 Score = 289 bits (146), Expect = 3e-76
Identities = 222/246 (90%), Gaps = 1/246 (0%) Strand = Plus / Plus

Query: 1    tgtcaaatcagacagactagttcttgcaacagggctaagaaacgtacctcaaagagaaac 60
             ||||||||| |||| ||| || |||||||||||| | |||||||||||| ||||||||||
Sbjct: 915  tgtcaaatcggacaaactggtccttgcaacaggaccaagaaacgtaccccaaagagaaac 974

Query: 61   aaggggtctatttggagcaatagcaggattcatagaaggaggatggcaaggaatggtgga 120
             ||| || ||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct: 975  aagaggcctatttggagcaatagcaggattcatagaaggaggatggcaaggaatggtgga 1034

Query: 121  tgggtggtatggataccatcatagcaatgaacaaggaagtggatatgctgcagacagaga 180
             ||| ||||| |||||||||||||||| |||| || || |||||||||||||||||| |||
Sbjct: 1035 tggatggtacggataccatcatagctatgagcagggtagtggatatgctgcagacaaaga 1094

Query: 181  atcgtacccagaaagcaatagatgggatcaccaacaagataaattcaatcattgacaaaa 240
             ||| |||||||||||||| ||||| |||||||| | |||||||||||||||||| ||
Sbjct: 1095 atc-tacccagaaagcaatcgatggaatcaccaatgaagtaaattcaatcattgacagaa 1153

Query: 241  tgaaca 246
             ||||||
Sbjct: 1154 tgaaca 1159


gb|U28919.1|AIU28919 Avian influenza virus A/Emu/TX/39442/93 (H5N2) parent virus
hemaglutinin mRNA, partial cds Length = 1644 Score = 287 bits (145),
Expect = 1e-75 Identities = 221/245 (90%), Gaps = 1/245 (0%) Strand = Plus / Plus

Query: 2    gtcaaatcagacagactagttcttgcaacagggctaagaaacgtacctcaaagagaaaca 61
             |||||||| |||| ||| || |||||||||||| | |||||||||||| |||||| |||||
Sbjct: 916  gtcaaatcggacaaactggtccttgcaacaggaccaagaaacgtaccccaaagaaaaaca 975
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```
Query: 62   aggggtctatttggagcaatagcaggattcatagaaggaggatggcaaggaatggtggat 121
            || || ||||||||||||||||||||||||||||||||||||||||||||||||| |||
Sbjct: 976  agaggcctatttggagcaatagcaggattcatagaaggaggatggcaaggaatggtagat 1035

Query: 122  gggtggtatggataccatcatagcaatgaacaaggaagtggatatgctgcagacagagaa 181
             || ||||||||||||||||||||||| || |||||||||||||||||||| ||||
Sbjct: 1036 ggatggtatggataccatcatagcaatgagcagggaagtggatatgctgcagacaaagaa 1095

Query: 182  tcgtacccagaaagcaatagatgggatcaccaacaagataaattcaatcattgacaaaat 241
             || |||||||| ||| || ||||| |||||||| || |||| ||||||||||||||||
Sbjct: 1096 tc-tacccagagagccatcgatggaatcaccaataaagtaaactcaatcattgacaaaat 1154

Query: 242  gaaca 246
             |||||
Sbjct: 1155 gaaca 1159
```

APPENDIX C Buffers and solutions

Avian virus transport media

	For tracheal swabs	For cloacal swabs
MEM	1 ×1 pkt	1 × 1 pkt
$NaHCO_3$	2.2 g	2.2 g
Bovine Serum Albumin	5 g	5 g
Penicillin	2 million units	10 million units
Gentamycin	0.05 g	0.25 g
Amphotericin B	0.4 ml (2 µg)	2 ml (10 mg)
Distilled H_2O	1 litre	1 litre

Mix until thoroughly dissolved, may take up to 4 h.
pH to 7.2–7.4
Aseptically sterilise medium by filtration through a 0.2-micron filter and dispense as required.
Amphotericin B should be added after filtration and the media should be mixed for half an hour and the pH should be checked again.

Tincture of iodine

Dissolve 2.5 g of Potassium iodide in 5 ml of distilled water.
Top up to 100 ml with 95% Ethanol and mix.

Phosphate Buffered Saline (PBS) (Ca^{2+} and Mg^{2+} free)

NaCl	8 g
KCl	0.2 g
Na_2HPO_4 (anhydrous)	1.15
KH_2PO_4	0.2 g
Distilled water	1000 ml

Adjust pH to 7.2–7.4
If necessary, sterilise by autoclaving (15 lbs/15 mins)

ELISA Coating Buffer (pH 9.6)

Na_2CO_3	0.159 g
$NaHCO_3$	0.293 g
dH_2O	100 ml

Adjust to pH 9.6 with NaOH/HCl

ELISA Washing Buffer

PBS	1 litre
Tween 20 (0.1%)	1 ml

ELISA Diluent

PBS	100 ml
Tween 20 (0.1%)	0.1 ml
BSA (2%)	2 g

Acetate/citric acid buffer (10 × concentrated)

1*M* sodium acetate 100 ml
1*M* citric acid. Add to sodium acetate to bring pH to 5.9 (approx. 1.5 ml)

Nonidet P-40 (1%) in PBS

NP-40	50 ul
PBS	4.950 ml

Store in a sterile bottle at 4°C not longer than 1 month

TMB substrate

Dissolve 350 mg of 3'-3'-5'-5'- tetramethylbenzidine (Sigma Cat. No. T2885) in 100 ml of methanol by stirring for several hours at room temperature (25°C). Store in dark bottle in a cupboard at 25°C to avoid crystallisation. TMB is light sensitive.

Electrophoresis Buffers

50 × TAE

2*M* Tris-base	242.0 g
5.7% Glacial acetic acid	57.1 ml
50 m*M* disodium EDTA (pH 8.0)	18.61 g
Distilled H_2O	1000 ml

Working solution 1 × TAE

10 × TBE

0.89*M* Tris-base	108 g
0.89*M* Boric acid	55 g
0.02*M* disodium EDTA (pH 8.0)	7.44 g
Distilled H_2O	1000 ml

Working solution 0.5 × TBE

6 × Gel Loading Buffer

40% sucrose (w/v)	4.0 g
0.25% Bromophenol blue	0.025 g

APPENDIX D A survey for paramyxoviruses in caged birds, wild birds, and poultry in New Zealand (Published results, 2001)

APPENDIX E Avian paramyxoviruses and influenza viruses isolated from mallard ducks (*Anas platyrhynchos*) in New Zealand (Published results, 2002)

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ERRATUM

p. iv, para 4, line 1: Hugh Black should be Hugh Blair (my sincere apologies)
p. 5, para 4, line 3: Hinshaw 1980 should be Hinshaw 1980b
p. 8, para 4, line 1: N5N2 should be H5N2
p. 32, para 2, line 4: NH should be HN
p. 39, para 1, line 1, APMV-2, -4, -5, -7, and -8 should be APMV-2, -4, -6, -7, and -8
p. 40, para 3, line 3: simila should be similar
p. 58, Table 2.1: 20.75 should be 20/75
p. 71, para 3, line 2: haemadsorbtion should be haemadsorption
p. 74, para 1, line 8: DACO should be DAKO
p. 76, Table 3.1, 1st row, last column (under APMV-4), 1 should be 15
p. 100, para 6, lines 2 and 5: TRISOL should be TRIZOL
p. 102, para 5, line 3: HN-HN-304 should be NH-314
p. 105, Fig. 6.3 caption: H5-985 should be H5-968
p. 116, Fig. 7.1, 2nd row, 2nd column from top: NZ3038/78 should be NZ8038/78
p. 119, Fig. 7.3 caption: Delete "Termination sites for HN translation are highlighted"
p. 148: APPENDIX C should be APPENDIX B