CHAPTER ONE

LITERATURE REVIEW

en·zo·ot·ic ("en-z&-'wä-tik)
Function: *adjective*
Etymology: *en-* + epi*zootic*
of animal diseases **:** peculiar to or constantly present in a locality
- **enzootic** *noun*
Merriam-Webster Online Dictionary http://www.m-w.com

1.1 AVIAN INFLUENZA

1.1.1 Introduction

The severe form of avian influenza (AI), historically known as "fowl plague", was first described by the Italian scientist Perroncito in the late 19th century (Perroncito, 1878). AI can cause devastating losses in poultry, with flock mortalities of up to 100%. Coupled with the added economic impact of trade restrictions and embargoes placed on infected areas, all H5 and H7 AI strains and the severe forms of Newcastle disease are notifiable to the World Animal Health Organization (Office International des Epizooties). Highly pathogenic avian influenza (HPAI) is one of the most feared zoonotic diseases today, because of the purported potential of the Asian HPAI H5N1 strain to be involved in the next, supposedly imminent, human influenza pandemic (Webster *et al*., 2006).

1.1.2 Aetiology

Influenza viruses, the aetiological agents of AI, are segmented, negative strand RNA viruses that belong to the family *Orthomyxoviridae*. There are three genera of influenza viruses: type A, type B and type C. Phylogenetically, influenza A and B viruses are more closely related to each other than to influenza C virus (Gammelin *et al*., 1990; Krossoy *et al*., 1999). Type A influenza viruses (but not types B and C) are further divided into subtypes based on the antigenic relationships in the surface glycoproteins haemagglutinin (HA) and neuraminidase (NA). At present 16 HA subtypes (H1-H16) and nine neuraminidase subtypes (N1-N9) have been identified. Each virus has one HA and one NA antigen, apparently in any combination (Röhm *et al*., 1996).

1.1.3 Morphology and genome organization of influenza A viruses

Influenza A virions are enveloped and highly pleiomorphic, varying from roughly spherical shapes 80-120 nm in diameter (300x10^6 Da) to filamentous particles. The two surface glycoproteins, rod-shaped HA and mushroom-shaped NA, are dispersed as spikes in the host-derived lipid bilayer of the envelope (Fig. 1.1) (Levy *et al*.,

1994). The viral envelope encloses the nucleocapsid containing the genome. The influenza A virus genome is distributed over eight different segments (Fig. 1.2) and encodes at least ten proteins: polymerase A (PA), polymerase B2 (PB2), polymerase B1 (PB1), the two viral surface glycoproteins haemagglutinin (HA) and neuraminidase (NA), matrix proteins M1 and M2, non-structural (NS) proteins NS1 and NS2 and nucleocapsidprotein (NP). The minimal replication unit, the ribonucleoprotein complex, is composed of the viral RNA, NP and three polymerase proteins PB2, PB1 and PA (Neumann *et al*., 2003). Influenza A virus nucleoproteins are antigenically related (and are used to serologically distinguish between influenza types A, B and C), as are their matrix proteins (Zhou *et al*., 1998).

The organization of the eight single-stranded RNA segments within the virion is still poorly understood. Biochemical and biological studies support the idea that each RNA segment exists as a distinct ribonucleoprotein (RNP) complex (Compans *et al*, 1972; Palese & Ritchey, 1977), whereas electron microscopic studies have shown that the internal component released from a disrupted influenza virion is a single large helix (Almeida & Brand, 1975). Genomic RNAs of influenza virus are held in a circular conformation in virions and in infected cells in a terminal panhandle structure form that might play an important role in replication (Hsu *et al*., 1987). Large helical structures have also been observed (Murti *et al*., 1980), and it is possible that multiple RNPs arise from the degradation of helices (Wright & Webster, 2001).

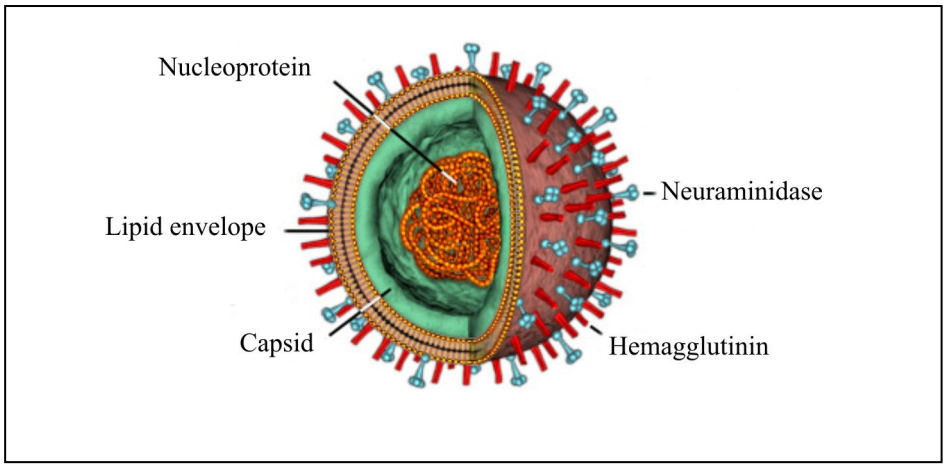

Figure 1.1 Structure of the influenza A virus

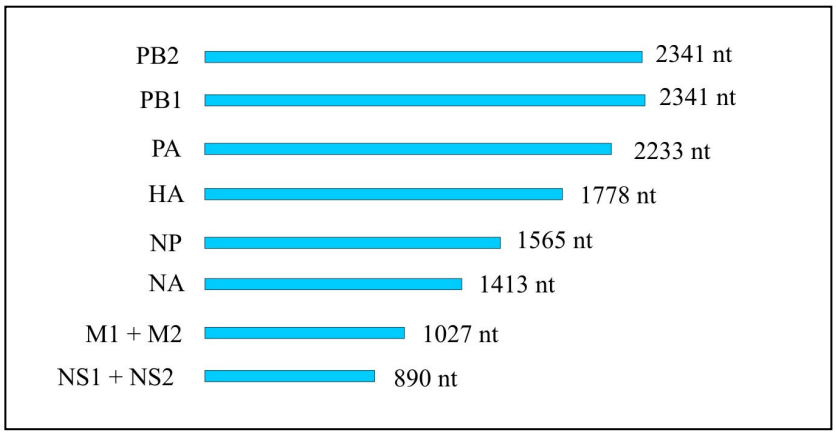

Figure 1.2 Relative sizes of AIV RNA segments

1.1.4 Host range

Type A influenza viruses include the avian, swine and equine strains, as well as human influenza A viruses, which cause pandemics. Natural infections of type A influenza in seals, whales, mink and cats have also been reported. Type B influenza viruses cause influenza epidemics (but not pandemics) in humans, but natural infections of seals are also known to occur. Type C influenza viruses cause mild disease in humans, but have never been associated with large scale epidemics, and have been isolated from dogs and swine (Aiello & Mays, 1998).

Type A are the only influenza viruses known to infect birds, and have been isolated from a large number of avian species covering 12 of the 50 orders (Stallknecht, 1998). The largest numbers of influenza A viruses have been isolated from wild waterbirds including ducks, geese, terns, shearwaters, gulls, as well as a wide range of domestic avian species such as turkeys, chickens, quail, pheasants, geese, ducks, and less frequently, from passerine birds such as starlings and budgerigars (Wright & Webster, 2001). The disease signs associated with influenza A infections vary considerably with the strain of virus and the species of the bird. In ducks, the majority of avian strains of AIV replicate in the lungs and in the cells lining the intestinal tract and infected birds usually show no signs of disease (Webster *et al*., 1978). The viruses gain access by passage through the digestive tract, despite the low pH of the gizzard, and are shed in high concentration in the faeces. The asymptomatic infections in waterfowl, together with the fact that all HA and NA subtypes of influenza A viruses have been isolated from wild waterfowl in most combinations of subtypes, and that mammalian influenza viruses are directly or indirectly derived from this reservoir,

strongly suggest that waterfowl, shorebirds and gulls are the natural hosts and biological reservoirs of AIV (Webster *et al*., 1992). In contrast, influenza viruses that have become established in mammals show a restricted combination of HA and NA types, limited to H1, H2, H3, N1 and N2 types. Certain avian influenza viruses have been transmitted directly to and have caused epidemics in other mammals including H3N8 in horses, H7N7 in seals, and H1N1 in pigs (Baigent & McCauley, 2003). Phylogenetic analyses of amino acid changes led to the surprising discovery that avian influenza viruses, unlike mammalian strains, display low evolutionary rates (Gorman *et al.,* 1990). In fact, influenza viruses in wild aquatic birds appear to be in evolutionary stasis. Nucleotide changes have continued to occur at a similar rate in avian and mammalian influenza viruses, but these changes no longer result in amino acid changes in the avian viruses, whereas all eight mammalian influenza gene segments continue to accumulate changes in amino acids. The high level of genetic conservation suggests that avian influenza viruses in their natural reservoirs are approaching an adaptive optimum, wherein nucleotide changes no longer provide any selective advantage (Wright & Webster, 2001).

1.1.5 Disease

Influenza A viruses infecting poultry can be divided into two distinct groups on the basis of their ability to cause disease. The very virulent viruses cause HPAI (fowl plague) in which flock mortality may be as high as 100%. Common signs of HPAI are slight to severe drop in egg production, increased mortality, respiratory difficulties, excessive lacrimation, sinusitis, oedema of the head, cyanosis of the unfeathered skin and diarrhoea. However, none of these signs can be considered pathognomonic, as similar symptoms are often seen with acute fowl cholera, velogenic Newcastle disease and other respiratory diseases. HPAI is associated only with H5 and H7 AIV strains (Alexander, 2000a).

Low pathogenic avian influenza (LPAI) viruses (all subtypes, including H5 and H7) cause a much milder disease consisting primarily of mild respiratory symptoms, depression and egg production problems in laying birds. The host species, age of the bird, immune status (particularly if the host is concomittently infected with other pathogens), and environmental conditions also contribute to the severity of the disease (Capua *et al*., 2000a). The OIE terrestrial code (2004) differentiates between

notifiable avian influenza (NAI) and strains that are non-notifiable to the OIE. Notifiable avian influenza is defined as an infection of poultry caused by influenza A virus of the H5 or H7 subtype or by any AI virus with an intravenous pathogenicity index (IVPI) greater than 1.2 (or as an alternative at least 75% mortality). The notifiable strains can be further divided into highly pathogenic notifiable avian influenza (HPNAI) and low pathogenicity notifiable avian influenza (LPNAI). Therefore, all H5 and H7 strains, whether highly pathogenic or of low pathogenicity, are notifiable to the OIE. The OIE definition also allows a molecular basis for confirming HPNAI, this is discussed in 1.1.8 (p12).

1.1.6 Diagnosis

1.1.6.1 Virus isolation and identification

The classical method of AIV diagnosis is virus isolation in embryonated fowl eggs. Tracheal or cloacal swabs, faeces from live birds or homogenized organs of dead birds are used. The sample or pooled samples are treated with antibiotics and the clarified supernatants are then inoculated into the allantoic sac of nine to eleven-day-old embryonated specific pathogen free (SPF) eggs, or specific antibody negative (SAN) eggs. At least five eggs are inoculated per sample, and incubated for four to seven days at 35-37ºC. Allantoic fluid is harvested from eggs containing dead or dying embryos, and then tested for hemagglutinating (HA) activity. Detection of HA activity (HA test) indicates a high probability of the presence of influenza A virus or of an avian paramyxovirus. The presence of influenza A virus can be confirmed in various other serological tests, including the agar gel immunodiffusion (AGID) that demonstrates the presence of the NP or M antigens, HI tests, and various commercially-available ELISAs. Alternatively, the presence of influenza virus, and subtyping, can be confirmed with the use of reverse-transcription polymerase chain reaction (RT-PCR) or real time reverse-transcription PCR (rRT-PCR). rRT-PCR is able to detect the presence of AIV nucleic acids even if the viruses are no longer viable, and is therefore considered to be a more sensitive method than virus isolation (Swayne 2003; OIE Terrestrial Manual 2004; Cattoli *et al*., 2006).

1.1.6.2 Assessment of pathogenicity

The IVPI test is used as a method of clinically assessing virulence of AIVs. Cultivated virus is injected intravenously into each of ten six-week-old SPF chickens, and the birds are examined at 24-hour intervals for ten days. At each observation, each bird is scored (0) if normal, (1) if sick, (2) if severely sick, and (3) if dead (dead individuals are scored as (3) at each of the remaining daily observations after death). The IVPI is the mean score per bird per observation over the ten-day period. An index of 3.0 means that all birds died within 24 hours, and an index of 0.00 means that no birds showed any clinical sign during the ten-day observation period (OIE Terrestrial Manual, 2004). The OIE and European Union (EU) have adopted the following definition to confirm disease for the purposes of disease control: 'HPAI is defined as 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 H5 or H7 subtype for which nucleotide sequencing has demonstrated the presence of multiple basic amino acids at the cleavage site of the haemagglutinin' (OIE Terrestrial Manual, 2004). The amino acid sequence at the hemagglutinin cleavage site as a determinant of virulence is discussed in 1.1.8.

1.1.6.3 Nomenclature

Strains of influenza viruses are described by their type, host, place of first isolation, strain number (if any), year of isolation, and antigenic subtype (Fenner *et al*., 1987; Acha & Szyfres 2003) e.g. A/Ostrich/South Africa/2004 (H5N2). For human strains, the host is omitted.

1.1.7 The infection cycle

1.1.7.1 The surface glycoproteins

The main functions of the glycoproteins are to facilitate the entry into and release of viruses from the host cell. HA is the major viral antigen against which neutralizing antibodies are formed. Each HA spike is composed a trimer of rod-shaped protein molecules which, depending on the environment, may be split by a specific peptide bond cleavage into two chains (HA-1 and HA-2) of roughly 20 and 25kDa, held

together by disulphide bonds. The proteolytic cleavage exposes HA-1 at the tip of the spike that carries most of the oligosaccharides of the virion. The oligosaccharides, which compose about 20% of the protein, consist of fructose, galactose, branch-structured mannoses and most importantly, several glucosamines, one of which is attached by N-glucosidic linkages to an asparagine on the protein surface. The sialic (N-acetylneuraminic) acid (SA), usually present at the end of such oligosaccharides is absent in influenza A, owing to the presence of neuraminidase activity on the virion. Erythrocytes of many species also bear polysaccharides on their cell surfaces, to which influenza A viruses bind, thereby causing hemagglutination. The hemagglutination reaction is characteristic of influenza A and is shared only by the paramyxoviruses and a few strains of reovirus, EDS 76 adenovirus, infectious bronchitis virus (if treated with neuraminidase) and bacteria. This characteristic forms the principle on which the hemagglutination (HA) and hemagglutination inhibition (HI) diagnostic tests are based. The HA test will indicate the presence of the virus in a sample, usually alantoic fluid, whereas the HI test is used to detect the presence of specific neutralizing antibodies in serum. In practice, the test serum is incubated with a known influenza A subtype antigen. If any neutralizing antibodies are present, they will bind the HA and NA surface glycoproteins, and prevent the natural hemagglutination reaction. The test involves a serial dilution of the serum against a known concentration of antigen, expressed in HA units (HAU). Agglutination is visually judged against positive and negative controls. The HI titer is defined as the highest dilution of serum causing complete inhibition of 4 HAU of antigen, and may be regarded as positive if there is inhibition at a serum dilution of 1:16 (OIE Terrestrial Manual, 2004).

There are fewer neuraminidase (NA) spikes on the influenza virion surface than hemagglutiniating spikes (about 200 compared with 750). Each NA spike (~200 kDa) consists of a dimer of two identical disulphide-linked 55 kDa glycoproteins. A single oligosaccharide is located on the stalk region, but the terminal knob is the enzymatically active region: the neuraminidase breaks the bond holding sialic acid to the end of many polysaccharide receptors on cell surfaces (although not those for mature influenza virions, because these lack sialic acid). The main function is therefore to remove terminal sialic acid from nascent viral glycoproteins, which facilitates the budding and release of the virions from infected cells, prevention of virus clumping, and the spread of the virus, particularly by helping to penetrate the mucous layer of the host respiratory system. The neuraminidase function therefore

also gradually reverses hemagglutination of erythrocytes. Hemagglutination even occurs at 0°C, but at 37°C the neuraminidase activity is activated and the NA protein cleaves the receptors that hold the virus to the erythrocyte surface. The neuraminidase inhibition (NI) test has also been used to identify the AI neuraminidase type of isolates and to characterize the antibody in infected birds (OIE, 2004).

1.1.7.2 Receptor specificity and attachment

The initial infection involves the attachment of the HA spike to the cellular receptor. The receptor binding site (RBS), which lies near the membrane distal tip of each HA subunit (Weiss *et al*., 1988) binds sialic acid (SA) attached to galactose. Influenza viruses recognise two species of SA (N-acetylneuraminic acid, NeuAc, and N-glycolylneuraminic acid, NeuGlc) which are attached to galactose in SAα-2,3Gal or SAα-2,6Gal linkages (Gambaryan *et al*., 1995). Ability of the virus to replicate in different host species is influenced by both SA species and linkage type in the host, and by amino acids at positions 226 and 228 in the RBS of the virus HA. The host animal might exert selective pressures on receptor specificity of the virus, since the abundance of receptor types on cells at the sites of virus replication varies. Human tracheal epithelium has predominantly NeuAcα-2,6Gal (expressed mainly on noncilliated cells) but recent evidence indicates that ciliated cells, a substantial cellular subset of the respiratory epithelium, express α-2,3 linked sialic acid receptors in sufficient density to allow entry and replication of avian viruses. Ciliated cells therefore most likely serve as primary target cells in those rare cases where avian viruses cause human disease, with sometimes even fatal outcome (Baigent & McCauley, 2003). Equine tissues possess both NeuAc and NeuGc, the major SA-Gal moiety in horse trachea being NeuGcα-2,3Gal; duck intestine contains mainly NeuAcα2,3Gal (also NeuGcα-2,3Gal). This may explain why horses are susceptible to direct transmission of avian viruses (albeit only a single genotype A/Equine/Jilin/1/89, H3N8, has been reported) though apparently not to human viruses. Swine tissues possess both NeuAc and NeuGc, the trachea having both SAα-2,3Gal and SAα-2,6Gal (Ito *et al*., 1998). This confers susceptibility to avian and human viruses, both experimentally and in the field (Baigent & McCauley, 2003), and until recently, it was thought that pigs were required as an intermediate host for transmission of AIV to humans (Gammelin *et al*., 1989; Yasuda *et al*., 1991).

1.1.7.3 Endocytosis and escape into the host cell

In addition to the glycoproteins, a small protein, M2, is also anchored in the viral envelope, and serves as a trans-membrane proton channel (Lamb and Choppin, 1983; Sugrue and Hay, 1991). After the HA spike has docked onto the SA receptor, the cell wall invaginates (mediated by the protein clathrin on the inner wall surface), internalising the virus to a host cell endosome. M2 ion activity is essential for the uncoating process. Once a virion particle has been endocytosed, the low –pH-activated ion channel activity of the M2 protein permits the flow of ions from the endosome to the virion interior, to disrupt protein-protein interactions and free the RNPs from the M1 protein (Lamb & Krug, 2001). Low pH within the endosome also triggers irreversible conformational changes in HA2, exposing the "fusion peptide" and pulling the lipid cell and viral membranes together to fuse them. The virion contents are poured into the host cell, and the vRNPs (viral ribonucleoproteins) are then transported to the nucleus where RNA replication, transcription and assembly of progeny vRNPs occurs (Lamb & Krug, 2001).

1.1.7.4 Transcription, translation and particle assembly

The three types of virus-specific RNAs (mRNAs, template RNAs and vRNAs) are all synthesized in the nucleus. The intact RNPs enter the cell nucleus through a nuclear pore. A unique feature of influenza virus mRNA synthesis is the dependence on host cell nuclear function. This dependence involves the requirement of the virion-associated polymerase on a primer to initiate viral mRNA synthesis. These primers are capped (m^7GpppX^m-containing) RNA fragments, derived by cleavage of host cell RNA polymerase II transcripts. A cap-dependent endonuclease that is intrinsic to the influenza virus polymerase cleaves capped RNAs 10 to 13 nucleotides from their 5' ends (Plotch *et al*., 1981) and the presence of a 5'-methylated cap structure, and not hydrogen bonding between the 12-nt sequence is the actual primer for transcription. The process has been called “cap snatching” (Lamb & Krug, 2001). After priming, mRNAs are transcribed, terminating at the stretch of uridine residues at the 5' end of the gene, which are the templates for the incorporation of polyadenylate (polyA) residues into the new mRNAs. In the nucleus, the viral mRNAs undergo at least some of the same processing steps as cellular RNA precursors. Internal adenosine residues of influenza virus mRNAs are methylated, and two of the viral mRNAs are spliced.

The viral mRNAs travel out through the nuclear pores, and are translated in the cytoplasm (Lamb & Krug, 2001). The virion transcriptase also synthesises viral cRNAs, the template for vRNA synthesis. The second step in replication involves the copying of the template cRNAs into vRNAs, and for this to occur, an alternative type of transcription is utilized that bypasses the need for the capped priming mechanism. Instead, synthesis proceeds due to the presence of 5' triphosphorylated ends of vRNAs (Young & Content, 1971). These new negative-sense viral genomic RNAs become associated with the newly-synthesized nucleoproteins and some M1 proteins that have migrated into the nucleus. The newly formed nucleocapsids and their associated M1 proteins exit the nucleus via nuclear pores (Lamb & Krug, 2001).

The influenza virus integral membrane proteins HA, NA and M2 are synthesized on membrane-bound ribosomes and are translocated across the membrane of the endoplasmic reticulum (ER). Once correctly folded and assembled, proteins are transported out of the Golgi apparatus. These oligosaccharides may be further processed to the complex form, and in HAs containing a furin cleavage site (see 1.1.8), cleavage activation occurs here too. HA (HPAI cleaved, LPAI uncleaved, see 1.1.8), NA and M2 migrate via the Golgi apparatus and become anchored in the regions of the plasma membrane. Although HA appears to be diffusely distributed over the surface, M2 and NA appear to cluster in patches, although the significance of this arrangement is unknown (Lamb & Krug, 2001). Once the integral membrane proteins are in place, matrix protein and new viral nucleocapsids align underneath, and interactions between the cytoplasmic tails of the viral integral membrane proteins likely provide the necessary molecular information for the formation of the budding particle. The progeny virions bud out at the apical surfaces and are initially aggregated at the host cell membrane because they are still sialylated, but the enzymatic activity of NA cleaves these sialic acids, releasing infectious progeny (Lamb & Krug, 2001).

1.1.8 HA, the primary molecular determinant of virulence

Although virulence of avian influenza viruses is multigenic in nature, the HA glycoprotein is recognised as playing a major role (Rott *et al*., 1979; Scholtissek *et al*., 1977). While HPAI viruses appear to exclusively involve H5 and H7 hemagglutinin subtypes, not all H5 and H7 viruses are highly pathogenic. HPAI H5 and H7 viruses

are thought to emerge from low pathogenic precursors only after the latter have been introduced into domestic poultry. This hypothesis is supported by studies which demonstrated that HPAI viruses do not form separate phylogenetic lineages in waterfowl (Röhm *et al*., 1995).

The precursor of each HA monomer is a single polypeptide chain that is cleaved by host proteases into two disulphide-linked subunits, HA-1 and HA-2, exposing a fusion peptide at the newly formed amino terminal end of HA2 (Rott, 1992). In an acidic environment, this peptide undergoes an irreversible conformational change, enabling it to fuse the viral envelope and host cell membranes, a crucial step in influenza virus infectivity (Rott, 1992). Both the peptide sequence at the cleavage site (H_0), and the availability of corresponding host cell proteases directly correlate with virulence in poultry (Rott, 1992).

Low-pathogenicity notifiable strains isolated from feral birds typically have only two basic amino acids at positions –1 & –3 (H7) and -1 & -4 (H5) from the cleavage site (Wood *et al*, 1993). This sequence is recognised and cleaved by extracellular trypsin-like proteases that are secreted only by cells of the respiratory tract and the avian intestinal tracts. Therefore these viruses, although fully infectious, cause relatively mild symptoms because they only replicate in limited cell types and are restricted in their ability to spread in a host and cause anatomically localised infections. In contrast, HPAI viruses have a connecting peptide sequence of several basic amino acids (Arg or Lys) that is recognised and cleaved by a set of intra-cellular subtisilin-like calcium-dependent proteases, most likely to be furin (Stieneke-Grober *et al*., 1992). Intracellular furin-like proteases have a much broader tissue distribution than the trypsin-like proteases, and therefore HPAI viruses produce lethal systemic infections.

The number of basic amino acids at H_0 of HPAI viruses vary from four to six residues, but cleavability is also influenced by the presence of an adjacent oligosaccharide side chain on the HA molecule (see 1.1.9.4). The proposed sequence requirement for HA cleavage by endogenous proteases, when carbohydrate is nearby, is X-X-R-X-R/K-R (where X=nonbasic residues); otherwise it is R/K-X-R/K-R (Ohuchi *et al*., 1989; Vey *et al*., 1992; Horimoto & Kawaoka, 1994). The emergence of HPAI from LPAI has been proposed to occur by a number of mechanisms, viz. (i) the insertion of basic amino acids at H_0, possibly the result of duplication of purine triplets due to a transcription fault of the polymerase complex (Horimoto *et al*., 1995;

Garcia *et al*., 1996); (ii) the progressive accumulation of basic amino acids at the cleavage site by a stepwise process involving amino acid substitutions (Horimoto *et al*., 1995; Spackman *et al*., 2003); and (iii) non-homologous recombination resulting in the insertion of a foreign nucleotide sequence adjacent to the H_0 (Suarez *et al*., 2004). Only four cases of non-homologous recombination at H_0 have been reported in the literature, however all four cases involved the H7 subtype. In two laboratory cases, passage in chicken embryo cells resulted in the insertion of a 54 nt region derived from the 28s rRNA gene into the H_0 cleavage site of an H7N3 isolate (Khatchikian *et al*., 1989), and a 60 nt fragment from the NA gene inserted into the H_0 cleavage site of an H7N7 virus (Orlich *et al.,* 1994). Increased pathogencicity for chickens was observed in both cases. Two field cases involved the 30 nt insertion of a nucleoptotein gene fragment into H_0 of an H7N3 virus in Chile in 2002 (Suarez *et al*., 2004), and the insertion of a 21 nt segment derived from the matrix protein gene into H_0 of an H7N3 virus in Canada in 2004 (Pasick *et al*., 2005)

1.1.9 Other virulence and host range determinants

The molecular determinants and related mechanisms that make certain AIVs highly pathogenic in poultry and mammals are poorly understood. Both viral factors and host factors may determine virulence. Numerous studies have shown that AIV virulence is a polygenic trait, which may require a critical constellation of genes. Thus, only reassortants with a set of genes that function efficiently together in a particular species of host will emerge as viable viruses, since functional interactions between viral proteins, and host proteins, are essential for replication (Scholtissek, 1987).

1.1.9.1 Neuraminidase

The receptor-binding properties of HA should be functionally compatible with the cleavage specificity of NA. The NA stalk, which holds the active site above the virion envelope, varies in sequence and length (Blok & Air, 1982). Thus a short-stalked NA is inefficient in disaggregating progeny virus because the active site cannot access its substrate efficiently. A shortened NA stalk reduces ability of virus to elute from erythrocytes (Els *et al*., 1985; Baigent *et al*., 1999), can reduce virus growth in MDCK cells (Luo *et al*., 1993) and eggs (Castrucci & Kawaoka, 1993) and can

decrease virulence in mice (Castrucci & Kawaoka, 1993). However, naturally occurring avian viruses having short NA stalks are fully virulent in poultry, showing that a long stalk is not essential for virulence in chickens (Baigent & McCauley, 2003). Incompatibility between HA and NA can restrict the virulence of reassortant viruses (Kobasa *et al*., 1999; Saito & Kawano, 1997; Baum & Paulson, 1991; Matrosovich *et al*., 2001).

1.1.9.2 Host basal body temperatures and pH

In avian species, the primary site of AIV replication is the intestinal tract (termed enterotropism), but in humans, human influenza A viruses replicate in the respiratory tract but not the gut, despite the presence of sialic acid in this system. Human viruses are also unable to replicate in the intestine of experimental ducks, despite the fact that their NA has retained a level of α-2,3 cleavage activity (Kobasa *et al*., 2001). The HK/H5N1 virus, considered to be transmitted directly from chickens to humans without prior adaptation in an intermediate mammalian host was able to replicate in the human intestine, causing severe gastrointestinal symptoms suggesting that a biological difference between human-adapted and avian-adapted viruses determines their ability to replicate in the gut. Studies using avian-human reassortant viruses provide evidence that HA and NA are critical for the enterotropism of avian viruses but are not essential for replication in other avian tissues (Hinshaw *et al*., 1983). This is partly due to the fact that the avian virus NAs function better at higher temperature and lower pH than their mammalian-virus counterparts do (Fiszon *et al*., 1989). The human strains replicate well at 37°C, but poorly at 40°C, while avian strains are still able to replicate efficiently at 40°C consistent with the higher body temperature of birds compared with mammals. More significantly for enterotropism, the NA activity of avian H1N1 influenza viruses is more resistant to acid pH than the NA of human- or swine-adapted H1N1 viruses (probably due to amino acid differences around the active site). Thus, avian NAs are capable of functioning in the low pH values in the upper digestive tract (Baigent & McCauley, 2003).

1.1.9.3 Glycosylation and sialylation

HA molecules are glycosylated at four to eleven sites in the head and stem. Glycosylation and sialylation close to the RBS of HA regulate release of avian viruses from cells (Baigent *et al*., 1999; Ohuchi *et al*., 1997), and thereby contributes to virulence and tissue tropism. These glycans can reduce affinity of HA for its receptor, possibly through simple steric hindrance, while SAs attached to the glycans reduce receptor availability by obscuring or completely filling the RBS. SA removal by a compatible NA is essential for HA to show its full receptor binding capacity. In tissue culture, a readily released glycosylated HA and a long-stalked NA favour virus growth (Wagner *et al*., 2000; Baigent & McCauley, 2001). The combination of HA glycosylation and NA stalk length influences host range and host adaptation. Human H1 influenza viruses show hyperglycosylation compared with those from aquatic birds, or pigs (Inkster *et al*., 1993), during the stable adaptation of an avian virus to human hosts. It has been suggested that hyperglycosylation of HA, combined with a compensating deletion in the NA stalk, modifies the progenitor aquatic bird virus preceding the development of virulence during virus evolution in chickens (Matrosovich *et al*., 1999). Hyperglycosylation of the HA head was associated with increased virulence in chickens of an H7N7 virus in Australia (Perdue *et al*., 1995) and an H7N1 virus in Italy (Banks *et al*., 2001). However, this additional glycosylation does not correlate conclusively with virulence of H7 viruses in chickens (Banks *et al*., 2000). To summarise, virulence requires both effective release of virus from infected target cells, and efficient binding of virus to new target cells. This is achieved by compensating changes in receptor-affinity of HA (regulated by amino acid changes in the RBS, and/or glycosylation/sialylation near the RBS) and effective enzyme activity of NA (regulated by amino acid changes to the active site, and by NA stalk length) to ensure an optimal balance in the activities of these two molecules.

1.1.9.4 Polypeptides of the replication complex

Nucleotide sequence analyses indicate that the genes for the virus internal proteins (PB2, PB1, PA, NP, NS and M proteins) cluster into host-species-specific lineages. The possibility that these lineages correlate with virulence and host range has been analysed *in vitro* and *in vivo*. In both animal models and tissue culture, the PB2 polypeptide confers a strong influence on host range. Residue 627 of PB2 is an

important although not the sole determinant of virulence and host specificity. Viruses with a Lys at this position are able to grow more efficiently at lower temperatures than viruses with a Glu residue (Shinya *et al*., 2006). The other polypeptides in the viral replication complex, PB1, PA and NP, also influence AIV host range, and compatibility between these four polypeptides is vital (Baigent & MaCauley, 2003).

Unique amino acid substitutions are associated with host specificity in both PB1 (Kawaoka *et al*., 1989) and PA (Okazaki *et al*., 1989). The combination of human PA with avian PB1 and PB2 or avian PB1 with human PB2 and PA severely restricts replication of reassortants in mammalian cells and squirrel monkeys (Snyder *et al*., 1987). However, the NP gene may have as broader host range since an avian virus with a temperature-sensitive mutation in NP could be rescued by avian and some swine viruses, but not by human strains (Scholtissek *et al*., 1985). Analysis of replication efficiency of reconstituted RNPs showed that functional compatibility between polypeptides of the replication complex is important for replication in mammalian cells (Naffakh *et al*., 2000). Replication was more efficient when PB2 and NP were both derived from the same avian or human virus, or when PB1 was derived from an avian virus whatever the combination of the other genes. This indicates that avian PB1 could have a higher activity, providing a selective advantage to viruses. In this context, it is interesting that the human pandemic viruses of 1957 and 1968 were reassortants having avian PB1 and HA (and NA for the 1957 virus) genes (Kawaoka *et al*., 1989; Scholtissek *et al*., 1987).

1.1.9.5 Matrix proteins

AIV genome segment 7 encodes two polypeptides: matrix protein 1 (M1) and matrix protein 2 (M2), the proton channel. The M protein was generally associated with the restriction of replication of both an H2N2 avian virus in squirrel monkeys (Buckler-White *et al*., 1986), and an H3N8 avian virus in pigs (Kida *et al*., 1994) but it's unknown whether M1 or M2 contributed to the host range restriction (Buckler-White *et al*., 1986). Functional compatibility between the pH at which HA undergoes conformational change, and the pH at which the M2 ion channel opens, ensures that intracellularly cleaved HAs of HPAI viruses can pass through the Golgi apparatus without undergoing a premature irreversible conformational change during virus budding- a requirement for viability of these viruses. Compared with M proteins of an

early human virus (A/PR8/34, H1N1), M proteins of later human viruses have gradually lost the ability to cooperate with avian virus HAs, being unable to form replication-efficient reassortants *in vitro*. This significant finding suggests that currently circulating human viruses may be unable to successfully recombine with avian viruses to produce the predicted H5 pandemic strain that would be transmissible in humans (Scholtissek *et al*., 2002).

1.1.9.6 Non-structural proteins

The non-structural (NS) genes of influenza viruses can be divided into two subtypes, or alleles, A and B (Suarez & Perdue, 1998). All known mammalian virus NS genes are of the A allele type, with one exception: an avian H3N8 virus that became established in horses in China (A/equine/Jilin/89) (Kawaoka *et al*., 1998). This is supported by evidence that reassortant viruses that have an avian virus NS gene in an otherwise human virus genetic background only replicated in squirrel monkeys if the NS gene was an A allele type (Treanor *et al*., 1989). NS1 shows more variation between alleles than NS2 (Suarez & Perdue, 1998; Kawaoka *et al*., 1998) and it has been speculated that since NS1 is implicated in downregulating the anti-viral response of the host, NS1 of allele A may achieve this more effectively in mammals than the B allele. Allele B genes, which include many avian viruses, have the potential to be pathogenic in mammals. Glutamic acid at position 92 in NS1 is associated with high virulence in pigs upon reassortment of the NS gene of H5N1 with A/Puerto Rico/8/34 (Seo *et al*., 2002).

1.1.10 Epidemiology

1.1.10 The prevalence of AIV in feral waterfowl

Feral waterfowl (ducks, geese and shorebirds) are the natural hosts and reservoirs of all influenza A subtypes (Webster *et al*., 1992). Waterfowl in the northern hemisphere migrate south in autumn (around September to October), back north in spring (around April to May), and breed in summer (around November to January) in the nesting places in northern territories such as Alaska, Canada or Siberia (Ito *et al*.,1995). Early phylogenetic analysis of viruses isolated from wild aquatic birds has revealed a

geographical separation of avian influenza viruses into the Eurasian and North American lineages (Gorman *et al* 1990; Kawaoka *et al*., 1998). Influenza viruses, that replicate preferentially in the cells lining the intestinal tract of waterfowl, excrete considerable quantities of AIVs (up to $10^{8.7}$ mean egg infectious doses per gram of faeces) into the environment (Webster *et al*., 1978; Hinshaw *et al*. 1979). High titres of AIVs have been isolated from unconcentrated water samples of different lakes in the breeding areas of ducks in northern high latitudes in summer, and furthermore, the viruses remained viable in the lake water after the ducks left for migration to the south (Hinshaw *et al*. 1979; Ito *et al*., 1995; Okazaki *et al*., 2000). Survival of influenza viruses in water is dependent on the virus strain and the salinity, pH, and temperature of the water; at 17°C some strains remain infectious for up to 207 days, and at 4°C they remain infectious for a longer period (Stallknecht *et al*., 1990a and 1990b). It has also been shown that influenza viruses are preserved in frozen lake water during winter when ducks are absent, and that ducks coming back from the south are infected with these viruses in spring (Hinshaw *et al*. 1980; Ito *et al*., 1995; Okazaki *et al*., 2000).

AIV isolation rates and subtypes in waterfowl vary considerably over time, region, between species and between age groups (Kawaoka *et al*., 1988; Sharp *et al*., 1993 Deibel *et al*., 1985). In *Anseriformes*, the greatest prevalence of AIVs has been observed during late autumn and winter (Sinnecker 1982; Halvorson *et al*., 1985). Continuous surveillance of influenza in migratory birds in Canada and USA showed that the frequency of isolation from migratory ducks coming from northern territories in autumn was high (more than 20% of juveniles), whereas those coming from the south in spring was extremely low (less than 0.25%) (Webster *et al*., 1992). In contrast, higher numbers of viruses were isolated from *Charadriiformes* during the spring (Hanson, 2003), where sampling along the Atlantic coast and the Gulf of Mexico revealed 78% of all isolates originated from Ruddy Turnstones (*Arenaria interpres*) in Delaware Bay (Hanson, 2003). Surveillance activities of wild birds across the world have dramatically increased since the emergence of Asian HPAI H5N1. After the Hong Kong influenza outbreak in 1997, monitoring of migratory ducks and shorebirds started in Taiwan. Since 1998, more than two hundred AI viruses were isolated and at least twenty-five LPAI subtypes viz., H1N1, H1N3, H2N2, H3N6, H3N8, H4N2, H4N6, H4N7, H4N8, H5N2, H5N6, H6N1, H6N2, H6N5, H7N1, H7N3, H7N7, H8N4, H10N3, H10N4, H10N6, H10N7, H11N9 and H14N7 were characterised. This suggested that the winter migratory ducks carried

many AI viruses, representing a large antigenic variation (Cheng *et al*., 2006). In France, free-range mule ducks were surveyed for the presence of AIVs, and LPAI H5N1, H5N2, H6N2, H6N8 and H11N9 were isolated. These viruses were all Eurasian lineage types and separate introductions from the wild bird reservoir were suspected. The N1 gene of the H5N1 isolate was phylogenetically very close to an Italian H7N1 virus (Cherbonnel *et al*., 2006). In Italy 3000 samples from wild bird cloacal swabs were tested for AIVs between 2003 and 2005. Approximately 5% of the samples were positive for AIVs, and in approximately 30% of cases it was possible to isolate viruses. H1, H4, H5, H7, H10 and N1, N2, N3, N4, N6 and N7 subtypes were identified (Cattoli *et al*., 2006). In Norway in 2005, 650 hunted wild species including greylag goose, mallard, wigeon, teal, goosander, tufted duck, common scoter and goldeneye were sampled during AIV surveillance. None of the 200 goose samples were positive for AIVs, but a total of 22.4%, 12.8%, 37.9%, 0%, 0% and 16.6% of the other species, respectively, were positive for AIVs. LPAI H5N2, H1N1, H3N2, H3N8, H6N2, H6N8, H8N4 and H9N2 subtypes were identified. The H5N2 viruses were found to be closely-related to viruses isolated in Sweden and the Netherlands in recent years (Jonassen & Handeland, 2006). In the Netherlands and Sweden, cloacal swabs from ducks, geese, gulls and shorebirds were screened for AIVs. Within the duck, geese and gull species, the prevalence of influenza A virus ranged between 0-60% depending on species, location and season. In shorebirds isolations were rare. All hemagglutinin subtypes except H14 and H15 and all nine neuraminidase subtypes were detected, and sequence and phylogenetic analysis revealed a close relationship between the viruses isolated in the study and all recent HPAI outbreaks in Northern Europe (except Asian HPAI H5N1) (Munster *et al*., 2006). In mainland China in 2004 and 2005, 493 serum samples from 15 migratory species were tested for anti-AIV antibodies. Low-level antibodies against influenza subtypes H2, H9 and H10 were detected in the relict gull, little egret, black-crowned night heron, bar-tailed godwit, whimbrel, and common greenshank, but no viruses were isolated from cloacal and orophyrangeal swabs of live bar-tailed geese (Chen *et al*., 2006). In Russia, 53 strains of influenza viruses were isolated from 1750 cloacal swabs (3% prevalence). Subtypes H3, H4, H5, H9 and H13 were isolated from ducks, terns, gulls, snipes, tree sparrows and muskrats (Lvov *et al*., 2004).

1.1.11 Control of AI

1.1.11.1 Vaccination

Maintaining poultry free from HPAI is essential for the continuation in trade in poultry and poultry products between nations, and erradication by stamping out is still the preferred method for dealing with HPAI. This control strategy is based on the application of sanitary restrictions and culling of all animals that are infected, suspected of being infected, or suspected of being contaminated. Inevitably, this results in mass depopulation, and the financial consequences of such a program are severe. The global poultry industry has undergone substantial changes in the past 20 years, mainly resulting in shorter production cycles and in greater animal densities per territorial unit. Consequently, infectious diseases are significantly more difficult to control, and furthermore, the slaughter and destruction of large numbers of animals is increasingly becoming ethically unacceptible to the general public. Therefore, the OIE has become more flexible in recent years regarding the implementation of control strategies involving vaccination for HPAI outbreaks (Alexander, 1996; Capua & Marangon, 2003; Swayne, 2003). Vaccination may be used solely as a strategy to minimize losses and reduce the incidence of disease, or vaccination may be combined with other strategies for the goal of total eradication.

Experimental and field data show that if birds are sufficiently well immunised against the HA subtype corresponding to that of the challenge virus they will be protected from the worst effects of HPAI and the clinical disease and mortalities associated with LPAI. Vaccination also helps to significantly slow the spread of the infection, and reduces the amount of viruses shed into the environment (Capua & Marangon, 2003; Harder *et al*., 2006; Bublot *et al*., 2006).

The existence of a large number of virus subtypes together with the variation of different strains within a subtype poses a challenge when selecting strains to produce influenza vaccines. In addition, some isolates do not grow to a sufficiently high titre to produce adequately potent vaccines without costly prior concentration. Reverse genetics has enabled the recovery of infectious influenza viruses from plasmid DNAs transfected into tissue culture. This technology permits the construction of high yield 6:2 seed viruses by mixing the 6 plasmid DNAs from a good-growing laboratory strain with the HA and NA DNAs obtained by cloning relevant genes from currently circulating viruses. Furthermore, removal of the basic cleavage peptide of HPAI

strains results in a virus that is attenuated for embryonated eggs, thus allowing high yields to be attained, and viruses that are safe to work with (Palese, 2006).

Whole virus AI vaccines are almost always inactivated because of the reassortment risk associated with live vaccines. Vaccines are prepared from infective allantoic fluid inactivated by betapropiolactone or formalin and emulsified with mineral oil. The inactivated vaccines produced have either been autogenous, i.e. prepared from isolates specifically involved in an epizootic (autogenous vaccines are homologous vaccines), or have been heterologous. Heterologous vaccines use the same HA type as the field virus but contain a heterologous NA. This type of vaccine has the advantage over the homologous vaccine of being distinguished from the field infection, because antibodies produced against the NA can be used as a marker, and this approach is commonly known as the DIVA (Differentiating Infected from Vaccinated Animals) strategy (Capua *et al*, 2002). The internal proteins NS1 and M2 have also been used as markers in a DIVA strategy, as both are abundantly expressed during viral replication in infected cells, illiciting specific antibodies that can be detected, whereas this is not the case with an inactivated, non-replicating vaccine (Lambrecht *et al*., 2006; Dundon *et al*., 2006). Recently vaccines have been developed employing new technologies such as baculovirus derived H5 and H7 haemagglutinins (Crawford *et al*., 1999) fowlpox virus recombinants expressing H7 haemagglutinin (Boyle *et al*., 2000), infectious laryngotracheitis virus (ILTV) expressing H5 or H7 antigens (Luschow *et al*., 2001), recombinant Newcastle disease virus expressing H5 antigens (Römer-Oberdoerfer *et al*., 2006) and DNA vaccines expressing HA antigens (Kodihalli *et al*., 2000). Under field conditions, protection afforded by inactivated vaccines could be undermined by improper vaccination technique, improper storage and handling of vaccines and infections that suppress the immune system of the bird (Swayne, 2003).

1.1.11.2 Chemotherapy

Antiviral drug research shows promise as a broad-spectrum means to control influenza, as an alternative to vaccination. Four different chemotherapeutic substances now exist (Lamb & Krug, 2001; Gubareva *et al*., 2000; Hayden 2001; Roberts, 2001; Flemming, 2001). Amantadine and rimantadine (Lamb & Krug, 2001) function by blocking the M2 ion channel protein of the virus, but viruses become resistant to

amantidine through a single amino acid substitution at positions 26, 27, 30, 31 or 34 in the transmembrane region of the M2 protein. Both amantadine and rimantadine may be of substantive benefit in treating influenza A, however side effects on the central nervous system, the liver and kidneys have been reported, and drug-resistant strains have emerged. More useful drugs are zanamivir and osteltamivir which block the action of NA to prevent release of newly formed virus from the infected host cell and its spread within the host (Monto, 2003). However, viruses have been known to become resistant to oseltamivir through a single amino acid substitution at position 119, 152, 274, or 292 in the NA active centre (Gubareva *et al*., 2002). Less is known about the efficacy and safety of the NA inhibitors than the amantadines (Baigent & McCauley, 2003).

1.1.12 The zoonotic potential of avian influenza viruses

1.1.12.1 Historic human influenza epidemics and avian influenza

In humans, influenza is a highly contagious, acute illness for which there are recognisable accounts of epidemics dating back to ancient times, with the earliest documented outbreak of what was probably influenza A occurring in 1173 (Francis, 1953). Seasonal influenza epidemics in man are associated with amino acid changes in the antigenic sites of HA and NA of currently circulating influenza A or B viruses (antigenic drift). The elderly, very young and other immune-compromised groups are particularly susceptible during epidemics, with significant increases in mortalities. Major pandemics are far more serious, but are associated with type A only. They result from introduction of HA and/or NA genes from an animal-derived influenza virus, by reassortment into the genetic background of a currently circulating human virus (antigenic shift). In the 20th century, antigenic shift in human influenza A viruses occurred on four occasions, 1918 (H1N1), 1957 (H2N2), 1968 (H3N2) and 1977 (H1N1), resulting in pandemics. For the worst influenza pandemic, the “Spanish Influenza” of 1918, it has been estimated that between 20 and 40 million people died (Crosby, 1976). Since the entire coding region for the 1918 Spanish influenza strain became available, sequence and phylogenetic analysis suggest that the 1918 Spanish influenza pandemic strain arose from an avian-like influenza virus (Taubenberger & Morens, 2006). Furthermore, reverse genetics was used to regenerate an influenza

virus containing all eight gene segments of the pandemic virus, in order to study the properties associated with its extraordinary virulence (Tumpey *et al*., 2005).

Both human and avian viruses are known to infect pigs readily, therefore it was suggested that pigs acted as "mixing vessels" in which reassortment between human and avian influenza viruses could take place. The reassorted virus therefore contained the necessary genome segments(s) from the virus of human origin to allow replication and spread in the human population, but different haemagglutinin surface glycoproteins, so that the human population could be regarded as immunologically naïve (Scholtissek, *et al*., 1985). This theory was also thought to account for the apparent emergence of pandemics in the 20th century in the Far East where agricultural practices result in high concentrations of people, pigs and waterfowl living in close contact (Shortridge & Stuart-Harris, 1982). Direct transmissions of AIV from animals to humans have occurred, without fatal disease. In 1996 an H7N7 virus was isolated in England from the eye of a woman with conjunctivitis who kept ducks. This virus was shown to be genetically closest in all eight genes to viruses of avian origin (Banks *et al*., 1998). During February of 2003, an outbreak of HPAI H7N7 in chickens in Holland was associated with human conjunctivitis, and caused the death of a veterinarian (Fouchier *et al*., 2004). During the second half of the 1990s, widespread outbreaks in poultry due to the H9N2 subtype were reported in Germany, Italy, Ireland, South Africa, USA, Korea, China, the Middle East, Iran and Pakistan (Banks *et al*., 2000b). In March 1999, two independent isolations of influenza virus subtype H9N2 were made from girls aged one and four who recovered from flu-like illnesses in Hong Kong. Subsequently, five isolations of H9N2 virus from humans on mainland China in August 1998 were reported (Peiris *et al*., 1999a; 1999b).

1.1.12.2 The emergence and spread of Asian HPAI H5N1 (genotype Z)

In May 1997 in Hong Kong, an H5N1 subtype was isolated from a young child who died and by December the same strain had infected 18 people, six of whom died (Shortridge *et al*., 2000). The viruses isolated from the human cases appeared to be identical to viruses first isolated from chickens in Hong Kong in March 1997 following an outbreak of HPAI, and they possessed the typical multi-basic H_0 cleavage sites (Suarez *et al*., 1998). The precursor of this virus was first detected in Guangdong, China, in 1996, when it caused a moderate number of deaths in geese

(Tang *et al*., 1998). This goose virus acquired internal gene segments from influenza viruses later found in quail (A/Quail/HK/G1/97(H9N2)) and the neuraminidase gene segment from a duck virus (A/Teal/HK/W312/97(H6N1)). The newly-assorted virus, A/Goose/Guangdong/96(H5N1), rapidly became widespread in live poultry markets in Hong Kong (de Jongh *et al*., 1997; Sims *et al*., 2003). The HPAI H5N1strain that infected the humans was eradicated from Hong Kong by stamping out, but the goose precursor viruses continued to circulate in geese in southeastern China (Cauthen *et al*., 2000; Webster *et al*., 2002). These strains reassorted with other avian H5N1 viruses that were highly pathogenic in chickens, but not ducks, and again emerged in Hong Kong in 2001 and 2002, and were eradicated on both occasions (Guan *et al*., 2002). Then, in late 2002 a single genotype was responsible for killing most wild, domestic, and exotic waterfowl in two of Hong Kong's nature parks (Guan *et al*., 2004; Sturm-Ramirez *et al*., 2004). This high pathogenicity in ducks was a rare property of AIV, and had not been observed in strains isolated during previous years. An antigenically and molecularly similar virus caused the two confirmed human cases in early 2003 in a Hong Kong family, and this virus was the precursor of the so-called Z genotype that became dominant (Guan *et al*., 2004; Peiris *et al*., 2004). HPAI H5N1 spread in an unprecedented fashion across South East Asia, affecting Vietnam, Thailand, Indonesia, Cambodia, Laos, Korea, Japan, Thailand and later Malaysia, although phylogenetic analysis showed that the viruses that caused the poultry outbreaks in Japan and Korea were of a different lineage, the V genotype (Mase *et al*., 2005; Li *et al*., 2004). After mass die-offs of H5N1-infected bar-headed geese (a migratory species) at Qinghai Lake in western China, an important staging area for migratory waterfowl, the scientific community was greatly concerned that HPAI H5N1 could be spread from southeastern Asia to other regions through migration movements (Webster *et al*., 2006). Worst fears were realised when Russia confirmed H5N1 in poultry in western Siberia. The outbreak spread to affect six administrative regions in Siberia, and dead migratory birds were reported in the vicinity of outbreaks. By August 2005 the infection had spread to neighboring Khazakstan's poultry flocks. Later that month, Mongolia confirmed the deaths of migratory birds at two lakes, caused by HPAI H5N1. By October 2005, H5N1 had caused outbreaks in Turkish turkey flocks, before breaking out in poultry in Romania. By April 2006, the infection had spread to poultry flocks, in order of appearance, in the Ukraine, Cyprus, Iraq, Nigeria, Egypt, India, France, Niger, Bosnia, Azerbaijan, Albania, Cameroon, Myanmar, Afghanistan, Israel, Pakistan, Jordan, Burkina Faso, Germany, Sudan and the Ivory Coast. H5N1 has also been found in dead wild waterfowl in Austria,

Bulgaria, Czech Republic, Denmark, Greece, Hungary, Iran, Italy, Kuwait, Poland, Serbia and Montenegro, Croatia, Slovakia, Slovenia, Sweden, Switzerland and the United Kingdom (www.oie.int.org). The most commonly affected wild birds in Europe have been swans, representing 62.8% of the total, followed by ducks (16.3%), geese (4.5 percent), birds of prey (3.9%) and others (13%) (ProMED, 31 May 2006). Research on viruses isolated from dead birds at Qinghai Lake demonstrated transmission of the virus among migratory geese and confirmed that the virus was indeed carried out of south east Asia by migratory birds (Chen *et al*., 2005). The spread of H5N1 from Eastern Europe to the rest of Europe has been attributed to unusual migration patterns of swans, after a particularly cold spell froze over wintering grounds around the Black Sea region (Brown *et al*., 2006). In contrast, the spread of Asian HPAI H5N1 into and within Africa is thought to be via the importation or illegal movement of infected poultry or poultry products from infected countries in the Middle East/Eastern Europe (FO Fasina, personal communication), rather than via migratory bird movements.

Since the emergence of HPAI H5N1 in 2003 and up to the end of November 2006, 258 human cases had been reported, resulting in 154 human deaths. The human fatalities have occurred in Azerbaijan, Cambodia, China, Djubouti, Egypt, Indonesia, Iraq, Thailand, Turkey and Vietnam (WHO). Hundreds of millions of poultry have been culled since the start of the outbreak in 2003. Most human cases have thought to have arisen via direct contact with the excretions of infected or dead poultry (eating of raw meat, drinking of duck blood, keeping chickens in the house etc). However, increasing reports of clusters of human infections (for example, health workers who treat infected patients, or family clusters) suggest that the extent of person-to-person transmission of HPAI H5N1 has been underestimated (WHO). The evolution of HPAI H5N1 viruses in South East Asia since 1997 has been associated with increasing virulence and an expanding host range. These viruses acquired the unprecedented capability to directly infect humans, to cause neurotropic disease and high mortalities in waterfowl, to be transmitted among felid species, including domestic cats (Kuiken *et al*., 2004), and to cause neurotropic disease and death in ferrets and mice (Govorkova *et al*., 2005). These incremental changes intensified concerns over the pandemic potential of this H5N1 virus. For the human population as a whole, the main danger appears to be if people infected with an avian virus are infected simultaneously with a human influenza virus. In such circumstances reassortment could occur with the potential emergence of a virus fully capable of spread in the

human population, but with an HA for which the human population was immunologically naive. Presumably this represents a very rare coincidence, but one which could result in a true influenza pandemic (Webster *et al*., 2006).

1.1.13 History of avian influenza in South Africa

The first report of avian influenza in South Africa was also the first international report of avian influenza occurring in wild birds. 1300 common terns died along the Western Cape coast between Port Elizabeth and Lamberts Bay in late April 1961. The orthomyxovirus that was isolated from the dead birds was initially named "Tern virus", and later determined to be an HPAI H5N3 strain (Becker, 1966). For thirty years thereafter no isolations of AIV were made in South Africa, but in the early 1980s ostrich farmers in the Oudtshoorn area first observed a syndrome, which they described as "green urine" with respiratory signs (Allwright *et al*., 1993). The green urine syndrome appeared to be a seasonal occurrence, peaking in the autumn and winter months, but was also associated with periods of drought. Then, in 1991, an LPAI H7N1 virus was isolated during an outbreak in young ostriches with symptoms of green urine in Oudtshoorn. The LPAI H7N1 virus had a low pathogenicity for chickens, and the increased mortalities in ostriches were ascribed to factors such as concomittent infections with other pathogens, high population densities, inadequate ventilation and bad hygiene resulting from poor management practises (Allwright *et al*., 1993). Since then, LPAI has been periodically isolated from the Western Cape ostriches. In 1994 an LPAI H5N9 virus was recovered, in 1995 an H9N2 virus, in 1998 an H6N8 virus, and in 2001 an H10N1 virus (Olivier, 2006). Furthermore, an H10N9 virus was isolated from wild birds in the Oudtshoorn region in 1998 (Pfitzer *et al*., 2000). Some literature cites the isolation of an LPAI H5N2 virus from ostriches in Zimbabwe in 1995 and 1996 (Pfitzer *et al*., 2000; Olivier 2006), however this was later determined to be a laboratory contamination (R Horner, personal communication). AI viruses isolated from 2001 onwards are the focus of this study.

1.2 NEWCASTLE DISEASE

1.2.1 Introduction

The earliest description of a poultry disease with symptoms that closely resembled Newcastle disease (ND) originated from an island of Java in 1926 (Kranevald, 1926), but ND may have been present in Korea since as early as 1924 (Konno *et al*., 1929; Alexander, 1997). Doyle (1927) was the first to identify a filterable aetiological agent as the cause of disease in a poultry flock near the town Newcastle on Tyne (UK), and named it accordingly. In the USA, a similar disease called pneumoencephalitis with notable respiratory and neurological symptoms was later discovered to be Newcastle disease. ND has also been known as fowl pest, pseudofowl pest and Ranikhet disease (Lancaster & Alexander, 1975) and like notifiable AIV strains, is notifiable to the OIE.

1.2.2 Aetiology

The etiological agent of Newcastle disease, Newcastle disease virus (NDV), is classified in the *Avulavirus* genus within the family *Paramyxoviridae* in the order *Mononegavirales* (Mayo *et al*., 2002a; 2002b). NDV is also referred to as avian paramyxovirus-1 (APMV-1), one of nine identified serotypes of paramyxoviruses known to infect birds. There is some cross-reaction, albeit low, between the APMV serotypes, especially between APMV-1 and APMV-3 (Box *et al*., 1988). Strains of NDV have been distinguished on the basis of symptoms produced in infected chickens. The following five groups or pathotypes were defined:

Viscerotropic velogenic virus	high mortality with hemorrhagic lesions in the intestines
Neurotropic velogenic virus	high mortality following respiratory and nervous signs, gut lesions usually absent
Mesogenic	moderate (50%) to low mortality with respiratory signs and occasional nervous signs
Lentogenic	no mortality with only mild respiratory signs
Asymptomatic enteric	no mortality with subclinical infection of the intestine, where replication primarily occurs

1.2.3 Disease

The incubation period for NDV infection is four to six days. The symptoms seen in infected birds vary widely and are dependent on factors such as the pathogenicity of the virus, host species, age of the host, infection with other organisms, environmental stress, and immune status. Symptoms may include respiratory and/or nervous signs such as gasping and coughing, drooping wings, dragging legs, twisting of the head and neck (torticollis), circling, depression, inappetence, and complete paralysis. Egg production partially or totally ceases. Eggs are often misshapen, rough-shelled, thin-shelled and contain watery albumen. Birds develop greenish watery diarrhoea, and there is some swelling of the tissues around the eyes and in the neck. Virulent panzootic NDVs usually cause haemorrhagic lesions of the intestinal tract, although there are no pathognomonic gross lesions. Lesions are sometimes seen in other tissues and organs, depending on the tropism of the particular strain. Differential diagnoses include fowl cholera, avian influenza, laryngotracheitis, fowl pox (diphtheritic form), psittacosis (chlamydiosis in psittacine birds), mycoplasmosis, infectious bronchitis, Pacheco's parrot disease (psittacine birds) and also management errors such as deprivation of water, air or feed.

Virulence differences between NDV strains are determined by chicken and chicken embryo inoculation. Four pathogenicity tests are utilized for this differentiation (Alexander, 1997; Pearson *et al*., 1975):

- Intracerebral pathogenicity index (ICPI) test in 1-day-old chicks from specific-pathogen-free (SPF) parents: birds are inoculated intracerebrally and then examined every 24h for eight days. At each observation, the birds are scored: (0) if normal, (1) if sick and (2) if dead. The ICPI is the mean score per bird per observation over the eight-day period. According to the OIE, an isolate with an ICPI equal to or greater than 0.7, or having a dibasic cleavage motif at the F_0 protein cleavage site (see 1.2.7) will be classified as a virulent virus, and its presence is notifiable to the OIE (OIE Manual, 2004).
- Intravenous pathogencity index (IVPI) in 6-week-old SPF chickens.
- Intracloacal inoculation test in 6-to-8-week-old chickens, and
- Mean death time (MDT) in 9-to-10-day-old embryonating eggs. Viruses are characterised as low (lentogens), moderate (mesogens) or high virulence (velogens) based on clinical signs and mortality in chickens along with time to

embryo death postinoculation. Strains are considered to be velogenic if they take <60 hours; mesogenic taking between 60-90 hours; and lentogenic if > 90 hours to kill.

1.2.4 Host Range

ND infections have been established in at least 241 species of birds representing 27 of the 50 bird orders. Domestic fowl, turkeys, pheasants, pigeons, quail and guinea fowl are highly susceptible to infection. Ostriches are less susceptible (Verwoerd, 1995b). Ducks and geese are susceptible but severe disease is rare. Psittacines (parrots) are highly susceptible and can excrete virus for long periods (Kaleta and Baldauf 1988), and many species of wild birds are also susceptible. Newcastle disease viruses have also been reported to infect animals other than birds, ranging from reptiles to humans (Lancaster, 1966).

1.2.5 Morphology and genome structure

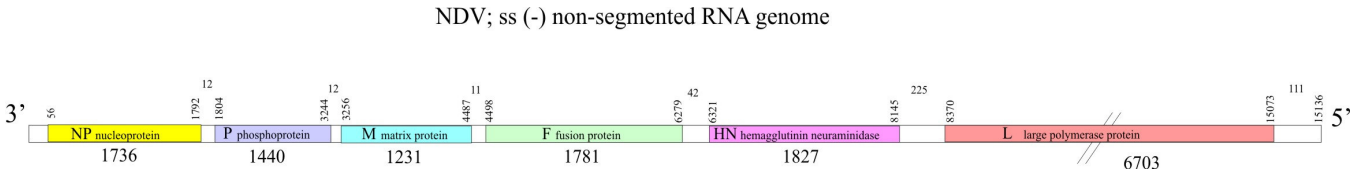

Figure 1.3. Genomic organization of Newcastle disease virus, with relative gene sizes indicated below.

Paramyxovirus virions are pleiomorphic, enveloped particles. Under the electron microscope (EM), NDV appears very similar in size and appearance to avian influenza virus. The virions vary considerably in size; most are 150-350 nm in diameter and spherical in shape, but larger particles (500-600 nm) as well as long filamentous forms are occasionally observed. The glycoproteins are inserted into the lipid envelope (which is derived from the host cell where the virion is assembled), appearing as spikes that protrude from the envelope surface (Bang, 1948; Cunha *et al*., 1947; Elford *et al*., 1947; Horne & Waterson, 1960; Horne *et al*., 1960; Hosaka *et al*., 1966).

NDV has a non-segmented, negative-sense, single stranded RNA genome of 15,186, 15,192 or 15,198 nucleotides (Phillips *et al*., 1998; Huang *et al*., 2004; Czeglédi *et al*., 2006). The genome encodes six proteins, viz. the nucleoprotein (NP), phosphoprotein (P), matrix protein (M), fusion protein (F), haemagglutinin-neuraminidase protein (HN) and the large polymerase protein (L), in the order 3'-NP-P-M-F-HN-L-5' (Fig. 1.3). Apart from these six proteins, NDV extends its genome capacity by the use of two overlapping ORFs in the P gene, V and W. V and W are transcribed as alternative mRNAs that are generated by RNA editing (Steward *et al*., 1993). In NDV, insertion of one nontemplate G residue gives rise to a V-encoding mRNA, while insertion of two nontemplate G residues generates a W-encoding mRNA. Analysis of mRNAs produced from the P gene showed that 68% are P-encoding mRNA, 29% are V-encoding mRNA, and 2% are W-encoding mRNA (Mebatsion *et al*., 2001).

The 3' termini of all non-segmented negative strand RNA virus genomes and antigenomes (including NDV) contain cis-acting sequences (promotors) that allow the viral RNA-dependent RNA polymerase to initiate RNA synthesis (Kolakofsky *et al*., 2005). In paramyxoviruses, the 3'-terminal 12 nucleotides are identical. The 3' end of the genome itself is not an essential part of this replication promotor, but a second promoter element lies downstream of the 3' terminal element, and spacing between the two elements is critical for promoter function: genome analogues of members of the paramyxovirus genus replicate efficiently only if the total genome length is a multiple of six, a requirement which is called the 'rule of six' (Calain & Roux, 1993; reviewed by Kolakofsky *et al*., 1998). At each turn, the helix contains 13 nucleocapsid subunits, each associated with precisely six nucleotides. The terminal and internal promoter elements are thereby aligned on the same face of the helix, thus both promoter elements can simultaneously interact with RNA-dependent RNA polymerase (vRNAP) to initiate RNA synthesis at the 3' end of the nucleocapsid (Lamb & Kolakofsky, 2001). All NDV genomes analysed to date are subject to the rule of six (Krishnamurthy & Samal, 1998; de Leeuw & Peeters, 1999; Phillips *et al*., 1998).

The ND virion can be viewed as consisting of two structural units: the nucleocapsid (also known as the ribonucleoprotein), which completely covers the RNA genome, and the envelope with its surface projections. The nucleocapsidprotein (NP) is the structural subunit of the nucleocapsid and serves several functions in virus replication, including encapsidation of the genome RNA into an RNase–resistant nucleocapsid,

association with the P-L polymerase during transcription and replication, and interaction with the M protein during particle assembly. When nucleocapsids of the *Paramyxovirinae* are viewed under an EM, successive turns of the helical assembly packed closely together are observed, creating the herringbone pattern that is characteristic of this family (Kolakofsky *et al*., 2005). From the number of turns in the helix and the number of NP subunits per turn, it is calculated that a single nucleocapsid contains 2,200-2,600 subunit molecules (Finch, 1970). Two other proteins, the phosphoprotein (P) and the large (L) protein, are associated with the nucleocapsids, but are recognisable only by immune electron microscopy (Portner & Murti, 1986).

The phosphoprotein (P) plays the central role in viral replication and transcription and has multiple functions. It has been shown that the P protein stabilizes the L protein in the P:L complex which acts as the viral RNA-dependent RNA polymerase (Smallwood *et al*., 1994). A P tetramer (Tabouriech *et al*., 2000) mediates the interaction between the L protein and the N:RNA template (Curran, 1998). The P protein also acts as a chaperone to prevent uncontrolled encapsidation of non-viral RNA by the NP protein (Errington & Emmerson, 1997). The V open reading frame (ORF) is found in the middle of the P gene, it is cysteine-rich and is translated from an mRNA with a single G insertion. The C-terminal contains seven conserved cystein residues and this domain binds two atoms of Zn^{2-}. The V domains of some paramyxoviruses specifically interact with the large subunit of the cellular damage-specific DNA-binding protein (Lin *et al*., 1998), but the consequence of this interaction and the function of V protein cysteine-rich domain remain to be elucidated (Lamb & Kolakofsky, 2001).

The large (L) protein, with a molecular weight of approximately 250 kDa, is the least abundant of the structural proteins (about 50 copies per virion). L is believed to be responsible for all of the catalytic activities of the viral polymerase associated with transcription, including initiation and elongation of transcripts, as well as co-transcriptional modification of RNAs such as capping, methylation, and polyadenylation. Polyadenylation is thought to result from polymerase stuttering on a short stretch of uridylate (U) residues, but the capping step requires both guanylyl and methyl transferase activities, both of which are thought to be provided by the L protein. The function of the RNA polymerase is dictated by the start, stop and restart

template's *cis* signals at the borders of each transcription unit (Bannerjee 1987; Smallwood *et al*., 1999).

1.2.6 The infection cycle

1.2.6.1 Adsorption and penetration

Virion- and infected cell surfaces are characterised by two types of glycoproteins, which mediate early interactions with the target cell: the hemagglutinin-neuraminidase (HN) and the fusion (F) proteins (Scheid & Choppin, 1974). HN proteins are type II integral membrane proteins. Type II proteins possess a sequence that serves both as a signal sequence and as a membrane anchor and are positioned with the amino terminus in the cytoplasm and the carboxyl terminus in the ectodomain. HNs span the membrane once and protrudes from the surfaces of virions and infected cells as tetrameric spikes. The orientation of HN in the membrane is analogous to that of the influenza virus NA. The ectodomain of the HN spike consists of a stalk that supports a terminal globular head in which receptor recognition, NA activity, and all of the known antigenic residues reside (Mirza *et al*., 1993; Thompson *et al*., 1988) (this is in contrast to avian influenza virus in which the receptor-recognition and NA functions reside on independent spike structures). For some strains of NDV, HN is synthesized as a biologically inactive precursor (H_0), and ~90 residues are removed from the C-terminus to activate the molecule (Nagai & Klenk, 1977; Nagai *et al*., 1976a). The HN glycoprotein mediates attachment to sialic-acid containing receptor(s) on the target cell surfaces. NDV recognises both N-acetyl- and N-glycolylneuraminic acid residues (Suzuki *et al*., 1985). The virus, however, elutes rapidly by the action of the neuraminidase even at low temperatures (4°C), and thus the pattern of hemagglutination, which is caused by the virus binding to the receptor of red blood cells, is unstable. Thermostability of the HN glycoprotein is sometimes used as a pathogenicity marker in epidemiological studies (Hanson & Spalatin, 1978). The thermostability of HN is assessed at 56ºC, and hemagglutinating activity is measured at time intervals: the longer the HN protein retains HA activity, the more thermostable it is. Generally, lentogenic field isolates are thermolabile, whereas velogenic viruses have thermostable hemagglutinins (Werner *et al*., 1999). There are several exceptions, however, as thermostable lentogenic field strains have been isolated, and thermostable progeny of vaccine strains have been selected for in the

laboratory (Kim & Spradbrow, 1978; Rosenberger *et al*., 1975; King, 2001). The molecular determinant(s) of HN thermostability have not yet been elucidated.

The complete mechanism of NDV-induced membrane fusion remains unknown. As with many other paramyxoviruses, NDV requires type-specific HN-F interactions that must be present in the same bilayer to induce fusion (Hu *et al*., 1992; Lamb & Kolakofsky; 2001). Since the F proteins of most paramyxoviruses cannot mediate membrane fusion alone and they require the coexpression of the homologues HN protein (Lamb & Kolakofsky, 2001). The fusion-complementing domain in the HN protein has been shown to reside in the HN stalk region, and residues 89, 90 and 94 are likely candidates (Melanson & Iorio, 2004). It has been proposed that the interaction of HN with the cellular receptor induces conformational changes in the HN protein that activates the F protein (Lamb & Kolakofsky; 2001), although the nature of such changes is obscure. The second step of infection is virus penetration, mediated by the fusion of the viral envelope with the lipid bilayer of target cells and resulting in the delivery of the nucleocapsid into cells. The F protein is involved in this process.

The F proteins are type I integral membrane proteins, i.e. the amino-terminal signal sequences are positioned so that the carboxyl terminus is located in the cytoplasm and the amino terminus is in the ectodomain (Chen *et al*., 2001; Russell *et al*., 1994). However, recent evidence suggests that a second topological form may be present and that both exist as a mixed population on the virion surface (McGinnes *et al*., 2003). The NDV F protein is synthesised as a precursor, F_0, the activation of which requires proteolytic cleavage into the disulphide-linked polypeptides F_1 and F_2. This cleavage exposes the hydrophobic amino terminus of F_1, called the fusion peptide, which is inserted into the target cell membrane, thereby disordering the lipid bilayer and preparing it for merger of the membranes (Lamb & Kolakofsky, 2001). Unlike the orthomyxoviruses (AIV), paramyxovirus fusion occurs at neutral pH. Thus, there is no requirement for a low pH-mediated conformational change of their glycoproteins to induce fusion, and paramyxoviruses are not internalised into endosomes. Instead, vRNP complexes are released directly into the cytoplasm, and the cytoplasm is the site for viral transcription and genome replication, as well as viral protein synthesis and processing.

A hallmark cytopathic effect of cell infection by paramyxoviruses occurs later in infection: when proteolytically-cleaved F proteins are expressed at the plasma membrane of infected cells, neighboring cells fuse to form syncytia (giant cell formation), a cytopathic effect that can lead to tissue necrosis *in vivo* and might also be a mechanism of virus spread (Choppin & Scheid, 1980).

1.2.6.2 Transcription and replication

All events of transcription, replication and assembly of paramyxoviruses occur in the cytoplasm. Paramyxovirus ribonucleocapsids, the active template for transcription and replication of the viral genome, function without disassembling (Lamb & Kolakofsky, 2001). Intracellular replication begins with the viral RNA-dependent RNA polymerase (RNAP) (minimally a homotetramer of P and a single L protein) transcribing the N-encapsidated genome RNA (N:RNA) into 5' capped and 3' polyadenylated mRNAs. The viral RNAP begins all RNA synthesis at the 3' end of the genome, and it transcribes the genes into mRNAs in a sequential (and polar) manner by terminating and reinitiating at each of the gene junctions. The junctions consist of a gene-end sequence, at which polyadenylation occurs by the reiterative copying of four to seven U's (followed by release of the mRNA), a short non-transcribed intergenic region, and a gene-start sequence that specifies capping as well as mRNA initiation. The RNAP occasionally fails to reinitiate the downstream RNA at each junction, leading to the loss of transcription of further-downstream sequences. Therefore, paramyxovirus genomes exhibit transcriptional polarity, i.e. transcripts of genes at the 3' end of the virus genome are more abundant than those of genes at the 5' end of the genome, permitting the regulation of the relative amounts of structural (3' genes) and non-structural (5' genes) proteins produced (Lamb & Kolakofsky, 2001).

Later in infection, when viral proteins accumulate, RNA synthesis switches from transcription to replication. This switch is mediated by the binding of NP to the nascent leader RNA. Newly-synthesized NP molecules are recruited by the polymerase complex and are sequentially added to the growing RNA chain. As a result of binding of NP, the polymerase complex ignores the transcription start and stop signals, resulting in the synthesis of full-length antigenomic RNA. The antigenomic RNA, in turn, serves as template for the synthesis of full-length genomic

RNA. Newly synthesized RNP complexes are assembled and transported with viral structural proteins to the plasma membrane (Lamb and Kolakofsky, 2001).

1.2.6.3 Virus assembly and release

Assembly of the virus occurs at the cell surface. The viral intergral membrane proteins are synthesized in the ER and undergo a step-wise conformational maturation before transport through the secretory pathway. Folding and conformational maturation of glycoproteins are not spontaneous events that occur in the cell but instead are assisted by numerous folding enzymes and molecular chaperones. Only correctly folded and assembled proteins are generally transported out of the ER. In the Golgi apparatus, the carbohydrate chains may be modified from the high-mannose to the complex form, and for those F_0 proteins with multiple basic cleavage sites, cleavage occurs in the trans-Golgi network. Finally, the glycoproteins are transported to the plasma membrane (Lamb & Kolakofsky, 2001).

The M protein is the most abundant protein in the virion, and is peripherally associated with the plasma membrane that already contains the glycoproteins (Nagai *et al*., 1976; 1989), although it does not appear to be an intergral membrane protein. The M protein interacts with the plasma membrane, the cytoplasmic domain of a viral glycoprotein(s), and the nucleocapsid (Peeples, 1991). Thus, its role appears to be the central organizer of virus assembly and budding, althought the exact mechanism by which the virus particle is assembled at the plasma membrane is unknown. The protein-protein interactions involved in assembling a virion presumably must be specific, as cellular membrane proteins are largely excluded from the virions. The virus is released from the cell by budding, and the neuraminidase activity of the HN protein hydrolyzes the surface sialic acid residues, preventing virus self-aggregation and enhancing virus spread (Lamb & Kolakofsky 2001).

1.2.7 F_0, the primary molecular determinant of virulence

In order for the NDV particle to be infectious, the F_0 precursor protein must be cleaved into disulphide-linked F_2 and F_1 polypeptides, so that the fusion peptide on F_1 is exposed. This post translation cleavage is mediated by host cell proteases, and the

cellular site of this cleavage depends upon the sequence at the cleavage site, as for *Orthomyxoviridae*. Some F_0 proteins have a furin recognition site (R-X-K/R-R) and are consequently cleaved in the trans-Golgi membranes by intracellular furin-like enzymes. In this case, the majority of F proteins delivered to the plasma membrane are potentially active F proteins. Thus, cleavage of F_0 requires a double pair of basic amino acids (e.g. Arg and Lys) at residues 112 and 113, and 115 and 116, plus a phenylalanine at residue 117 (Collins *et al*., 1993). Virulent viruses can therefore replicate in a wide range of tissues and organs, resulting in a fatal systemic infection (Rott, 1979). In contrast, F proteins of avirulent viruses have single basic residues at the cleavage site and are delivered to the plasma membrane in an inactive, uncleaved form. In order to direct fusion, these F proteins must be cleaved by an extracellular host cell enzyme (trypsin-like, which recognize a single Arg residue), usually found exclusively in the respiratory and intestinal tracts, thereby restricting replication to these tissues. As with AIV, avirulent NDVs require the addition of exogenous trypsin to facilitiate replication in cell culture (Nagai *et al*., 1976a).

1.2.8 Other virulence determinants

The F_0 protein cleavage site is the primary determinant of virulence, however, comparison of the amino acid sequence of the F_0 protein cleavage site with the intracerebral pathogenicity indices (ICPI) for several NDV viruses has shown that significant differences in virulence can exist between strains with the same velogenic consensus sequence. For instance, virulent field strain Herts/33 with the cleavage site ^{112}RRQRRF^{117} has an ICPI value of 1.88 whereas strains Beaudette C/45 and Komarov with the same sequence showed ICPI values between 1.4 and 1.5 (Collins *et al*., 1993). In addition, NDV isolates recovered from many different bird species with the same velogenic F protein cleavage site were found to exhibit different virulence levels in chickens. These NDV isolates were either mesogenic strains that produced mild pathogenesis or velogenic strains causing severe pathogenesis and death (de Leeuw *et al.*, 2005). Moreover, using reverse genetics Peeters and colleagues (1999) established an infectious clone of the non-virulent La Sota strain (NDFL), and demonstrated that modification of the cleavage site ^{112}GRQGRL^{117} to ^{112}RRQRRF^{117} (NDFLtag) increased the ICPI from 0.00 to 1.28. Although NDFLtag contains exactly the same F_0 protein cleavage site as Herts/33, the ICPI did not reach the level of 1.88, indicating that other factors contribute to virulence. The most likely candidate would

be the HN protein, since it is also involved in viral entry (de Leeuw *et al*., 2005). The V protein is another candidate since it is an interferon antagonist (Park *et al*., 2003; Huang *et al*., 2003) and therefore likely to be involved in pathogenesis and host-range restriction (Mebatsion *et al*., 2001; Park *et al*., 2003).

1.2.9 Zoonotic potential of NDV and the use in cancer treatment for humans

Human infections with NDV have usually resulted from direct contact with the virus, infected birds or carcasses of diseased birds. Human-to-human spread has never been reported, although it is theoretically possible. The most frequently reported clinical signs are conjunctivitis and flu-like symptoms, but infections have never been lethal. Most cases have been reported by laboratory workers, veterinarians, workers in broiler processing plants, and vaccination crews, especially when live vaccines are administered as aerosols or fine dust (Alexander, 2000b).

NDV is one of at least a dozen virus types that are being investigated as alternative therapies for people in whom conventional cancer therapy has failed to stabilize or shrink existing tumors. The observation that tumor regressions occurred during natural virus infections or immunizations were the basis of preliminary studies (Csatary *et al*., 1993; Kirn *et al*., 2001). A particular case history involved a poultry farmer whose metastatic gastric carcinoma underwent regression, coinciding with an outbreak of ND in his chickens. This led to the application of an attenuated NDV vaccine for treatment of a few terminal cancer patients, with favorable results (Csatary *et al*., 1993). Various NDV strains have been used in cancer treatment, because antitumor responses have differed among the isolates used. The mechanism for NDV oncolytic activity is unknown, but NDV has been shown to be a potent inducer of tumor necrosis factor α (TNFα) production by human peripheral blood mononuclear cells, and enhances sensitivity of the tumor cells to the cytolytic effect of TNFα (Lorence *et al*., 1988). Furthermore, NDV may provide anticancer therapy through pleiotrophic modification of the patient's own immune response against the tumor, rather than direct oncolytic activity (Schirrmacher *et al*., 1999).

1.2.10 Genotypes and global epidemiology

Binding pattern analysis of a panel of monoclonal antibodies (mAbs) has been used to separate NDV isolates into specific antigenic groupings (Alexander *et al*., 1997). This system has proved useful for rapidly sorting viruses into broad groups, and the mAb groups described by Alexander and colleagues (1997) have been used as a descriptive basis (Aldous *et al*., 2003). A shortcoming of mAb binding tests is that they fail to differentiate viruses that are antigenically similar, although not genetically identical (Alexander *et al*., 1999; Aldous *et al*., 2003). Restriction enzyme analysis defined six lineages (I to VI) of NDV (Ballagi-Pordany *et al*., 1996) and nucleotide sequencing confirmed these six groups, adding two more lineages, VII and VIII. Several sub-lineages have been described within these eight lineages (Lomniczi *et al*., 1998; Seal *et al*., 1998; Herczeg *et al*., 1999; Yang *et al*., 1999; Herczeg *et al*., 2001; Ke *et al*., 2001; Yu *et al*., 2001). Although considerable genetic diversity has been detected in NDV, viruses sharing temporal, geographic, antigenic or epidemiological parameters tend to fall into specific lineages or clades and this has proven valuable in assessing both the global epidemiology and local spread of ND (Alexander, 2000b, Aldous *et al*., 2003). Thus, three main panzootics of ND have been defined (Alexander, 1997; Lomniczi *et al*., 1998; Sakaguchi *et al*., 1989; Toyoda *et al*., 1989; Ballagi-Pordany *et al*., 1996; Lomniczi *et al*., 1998). Recently, the genetic groupings of ND viruses were revised, and six lineages (1 to 6) were described (Aldous *et al*., 2003):

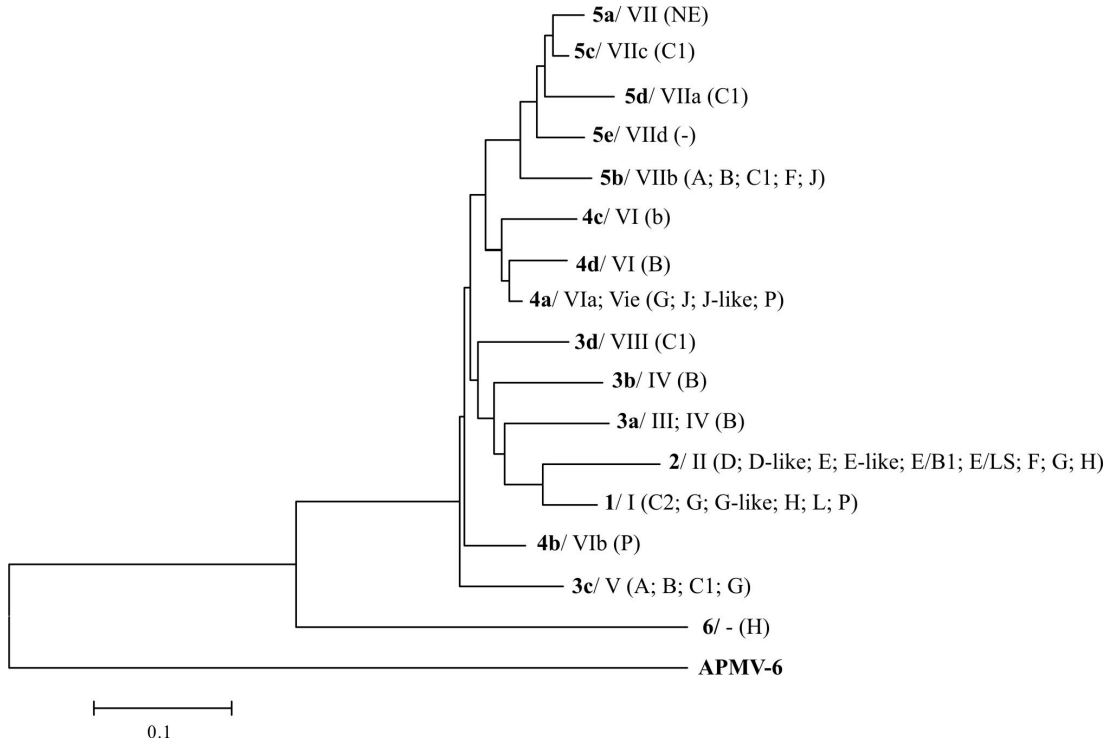

Figure 1.4. Classification of NDV lineages (adapted from Aldous *et al*., 2003). The original genogroups (Lomniczi *et al*., 1998; Alexander *et al*., 1999; Herczeg *et al*., 1999; Yang *et al*., 1999; Herczeg *et al*., 2001, Ke *et al*., 2001; Yu *et al*., 2001, Liang *et al*., 2002) are indicated in Roman numerals, and the corresponding monoclonal antibody-binding groups (Alexander *et al*., 1997) in brackets thereafter. The Neighbour-joining tree is based on a 374 nt region of the F protein and is rooted with avian paramyxovirus 6 (APMV-6)

1.2.10.1 Lineage 1

Lineage 1 was isolated worldwide from 1967 to 2000, the main pathotypes are assymptomatic enteric, but some velogenic viruses are included. The main hosts are ducks and chickens. In recent years, Queensland/V4 and Ulster2C/67 derivatives have been used as live vaccines in many countries. These viruses, with few exceptions, usually cause little or no disease in chickens, replicate primarily in the gut, and are often isolated from feral waterfowl throughout the world (Aldous *et al*., 2003). Two isolates in this group are virulent, apparently derived from an avirulent precursor strain (Gould *et al*., 2001).

1.2.10.2 Lineage 2

Lineage 2 (II) was isolated worldwide from chickens and ostriches from 1945 to 2000, and consists of mixed pathotypes. This lineage contains viruses that had origins in North America, including the virulent Texas GB/48 and the avirulent B1/47 and La Sota/46 strains. B1/47, LaSota and to a lesser extent strain F are widely used as live vaccines throughout the world. Komarov and Roakin, both mesogenic strains, are also used as vaccines in some parts of the world where ND is endemic (Aldous *et al*., 2003).

1.2.10.3 Lineage 3

Sublineage 3a (III; IV) viruses were isolated from 1932 to 1999 in Asia, Australasia and Europe. They are velogenic viruses, isolated mainly from chickens. These include the 'ancient' isolates from Australia (1932) and Japan (1951, Miyadera/51), the highly virulent UK isolate from a field pheasant (1962), and the mesogenic vaccine strain Mukteswar used widely in south east Asia. Sublineage 3b (IV) was isolated from 1933 to 1989 in Asia, Africa and Europe and these viruses are velogenic in quail and chicken, their main hosts. This sublineage includes the Herts/33 virus, one of several reference strains. Sublineage 3c (V) velogenic viruses were isolated from 1970 to 1994 from Africa, America and Europe. Their hosts are chickens, pheasants, turkeys and cormorants. Sublineage 3c is largely composed of isolates derived from or considered to be from the 'second' NDV panzootic in the 1970s, which was influenced by trade in exotic birds (Alexander, 2001). Sublineage 3d (VIII) is composed of velogenic viruses isolated from 1965 to 1994 in South Africa, Asia and Europe from chickens and turkeys (Aldous *et al*., 2003).

1.2.10.4 Lineage 4

Sublineage 4a (VIa; VIe) viruses are velogenic, isolated from 1968 to 1996 in Africa, Europe and the Middle East from falcons and chickens. Sublineage 4b's (VIb) velogenic viruses were isolated from 1984 to 2000 from European and Middle Eastern pigeons, and this sublineage is comprised solely of viruses associated with the ongoing panzootic in pigeons. Sublineage 4c contains velogenic viruses isolated from 1989 to 1999 in Europe and the Middle East from chickens and falcons, whereas

sublineage 4d comprises velogenic viruses isolated from 1989 to 1999 in Europe from chickens, pigeons and ostriches (Aldous *et al*., 2003).

1.2.10.5 Lineage 5

Lineage 5 consists exclusively of velogenic viruses. Sublineage 5a (VII) viruses were isolated in Europe from 1988 to 1996 from chickens and pheasants. Lineage 5b (VIIb) viruses were isolated from 1982 to 2000 from Africa, Asia and Europe from chickens, pheasants, falcons and turkeys. Lineage 5c (VIIc) viruses were isolated from 1984 to 1999 from Asia and Europe. Sublineage 5d (VIId) viruses have been isolated since 1997 from Asia and the Middle East from chickens and ostriches, and sublineage 5e (VIIe) viruses were isolated in Taiwan from 1994 to 1997 from chickens and finches (Aldous *et al*., 2003).

1.2.10.6 Lineage 6

Lineage 6 viruses were isolated worldwide from 1977 to 1998 from chickens and ducks. The majority are lentogenic, but the group does include some velogenic strains. Although their mAb binding pattern is unusual, they react readily with polyclonal sera to NDV strains and share neutralising epitopes on the fusion protein with vaccinal NDVs (Collins *et al*., 1993; Alexander *et al*., 1997). All except one isolate are avirulent. On average, lineage 6 viruses have 50% sequence divergence from other isolates, and with the high degree of genetic heterogeneity, it has been suggested that this group of viruses should be regarded as an APMV subtype distinct from APMV-1 (ie not NDVs). However, the presence of a virulent virus in lineage 6 complicates control and statutory regulations, therefore the *status quo* remains (Aldous *et al*., 2003).

1.2.11 Vaccination and control

There is no treatment for Newcastle disease, therefore a stamping out policy (based on slaughter of infected and potentially infected birds, quarantine procedures, cleaning and disinfection) or vaccination, or a combination may be used to control ND, depending on circumstances. In emergency situations in previously ND-free countries, eradication by stamping out is usually the preferred option. New Zealand,

Papua New Guinea, Fiji and a number of Pacific island countries have non-pathogenic ND strains, but are free of pathogenic strains. Most other countries have experienced outbreaks of virulent strains, and many countries, including those of Africa, Asia, Central and South America are regarded as NDV enzootic (Copland 1987; Spradbrow 1988; Rweyemamu *et al*., 1991). In countries where virulent ND is considered to be enzootic, vaccination is routinely practised. Types of vaccines and vaccination schedules used vary depending on the potential threat, virulence of the field challenge virus, type of production, and production schedules. Both live attenuated and inactivated whole-virus vaccines have been developed, as well as recombinant vaccines. Historically, the first attenuated NDV strain, Hertfordshire (H), was obtained by serial passage in eggs of a virulent field virus isolated in England in 1933. Later, this virulent strain was referred to as Herts'33 and became a standard challenge and reference virus (OIE, 1996).

1.2.11.1 Live attenuated vaccines: Lentogenic strains

The live vaccines used to control NDV around the world are, with very few exceptions, of the lentogenic type and include the strains La Sota/46 (also known as La Sota), B1/47 (also known as B1 or Hitchner/B1), F (Asplin), V4, Ulster and VG/GA. B1/47 and La Sota/46, isolated and developed in the 1940s in the USA (Hitchner & Johnson, 1948; Goldhaft, 1979), are the most commonly used lentogenic live ND vaccines today. Both replicate in the respiratory and alimentary tracts with induction of local as well as humoral immunity. Generally, La Sota vaccines are more virulent and induce a stronger immune response than B1 vaccines (Glisson & Kleven, 1993), but both can be administered in drinking water or in spray, sometimes via the nares or eye. Healthy chicks are vaccinated as early as day 1-4 of life, however if vaccination is delayed until the second or third week, interference with an active immune response by maternal antibody can be avoided.

In 1966, a mild enteric lentogenic strain of NDV was isolated from a healthy chicken in Queensland (Simmons, 1967) and soon after it was discovered that the strain was widely distributed throughout Australia (Anonymous, Australian Veterinary Journal, 1966). The strain (Queensland/66), designated “V4”, was relatively thermostable, and it was demonstrated that it would respond rapidly to selection for enhanced

thermostability. V4 was soon being used as a vaccine. The heat-resistant variant (NDV4-HR) is now the basis of thermostable vaccines for commercial chickens. From a panel of 45 contemporary apathogenic isolates of Australian viruses, a second vaccinal strain, I-2, was selected (Spradbrow *et al*. 1995). The NDV4-HR and I-2 ND vaccines proved to perform well under the adverse conditions in developing countries of Africa and Asia (Alders and Spradbrow, 1998). The NDV4-HR vaccine is a commercial vaccine and can be purchased when foreign exchange is available. For countries where foreign exchange is not readily available, the Australian Centre of International Agricultural Research (ACIAR) has provided the I-2 ND vaccine master seed free of charge to enable a ND vaccine suitable for use in village chickens to be produced locally (Alders and Spradbrow 2001a).

The VG/GA strain was isolated in the early 1990s from commercial turkeys in Georgia in the USA by Pedro Villegas (Beard *et al*., 1993). It also replicated in the respiratory and gastrointestinal tracts of chickens, producing an immune response similar to that induced by B1, but without any recognisable respiratory symptoms (Glisson & Kleven, 1993). It is marketed under the name *AVINEW* in South Africa.

1.2.11.2 Live attenuated vaccines: Mesogenic strains

Mesogenic strains are avirulent for chickens older than eight weeks of age but retain virulence for 3 day-old chickens (ICPI 1.0-1.5). A distinct mesogenic vaccine virus, Mukteswar, was independently developed in India by egg passages. Other mesogenic NDV strains include Roakin, isolated from a field case in the USA, and the Komarov strain. The Komarov strain (also sometimes called the K strain) was obtained by the intracerebral passages through ducklings of a virulent virus isolated in Palestine in 1945 (Komarov & Goldsmit, 1946). The so-called Onderstepoort (OP) strain of Newcastle disease, sold as a live mesogenic vaccine in South Africa, was developed in the late 1970s at Onderstepoort by passaging the imported Komarov strain on cell cultures of monkey kidneys (Anonymous, 1974, Prof L Coetzee, personal communication). Mesogenic vaccines are now very seldom used. Instead, the tendency of the poultry industry is to use vaccines of low reactivity that still provide a high level of protection (Villegas, 1998).

1.2.11.3 Inactivated vaccines

Mycoplasma and some other bacteria, if present, may act synergistically with some live vaccines to aggravate the vaccine reaction after spray administration. When other infections are present in the flock, and where required by law, inactivated (killed) vaccines are used. Furthermore, inactivated vaccines with oil adjuvants give the longest protection. A combination of live and inactivated ND vaccine administered simultaneously is shown to provide the best protection against virulent NDV and has been successfully used in control programmes in areas of intense poultry production (OIE, 1996).

1.2.11.4 Limitations of vaccination

Whether inactivated vaccines, lentogenic mass vaccines, or intramuscularly-injected mesogenic strains are used, repeated vaccination is required to protect chickens throughout life because vaccine-induced immunity is short-lived (8–10 weeks). The frequency of re-vaccination largely depends on the risk of exposure and virulence of the field virus. Proper administration of a high-titered vaccine is essential for induction of a good immune response, as failure to follow instructions (e.g. use of sprays in open or windy houses, or use of chemically treated water to dilute the virus) may result in incomplete or no protection after vaccination. In practice, vaccination of poultry against NDV does not always prevent periodic outbreaks of the disease (Khalafalla *et al*., 1992; Oncel *et al*., 1997; Yang *et al*., 1997, 1999; Roy *et al*., 2000). The factors promoting disease occurrence in vaccinated birds are firstly factors decreasing host resistance to the infection; and secondly changes of the virus allowing it to overcome the 'immunological barrier' (antigenic drift). Panshin and colleagues (2002) investigated the factors affecting antigenic drift in NDV and reminded us that although the AMPV-1 represents a serologically homogeneous group, antigenic variations have been detected by monoclonal antibody studies (Alexander, 1990). So far there is no evidence that antigenic differences between virulent and vaccine viruses have been sufficient to result in failure of the vaccines to provide protection against challenge (Roy *et al*., 2000; Alexander & Parsons, 1986). However, in other studies, it has been demonstrated that protection activity of a mixture of monoclonal antibodies (MAbs) directed against different epitopes of NDV is higher than the protection caused by a single MAb (Umino *et al*., 1990). As far as the immune

response in natural conditions is polyclonal, it is reasonable to expect that just after vaccination, there is an induction of antibodies of a wide spectrum, i.e. directed against various epitopes including those common to both the vaccine and challenging virus. At the period of the maximal antibody induction, the level of those antibodies is sufficient for the protective effect. Afterwards, and especially under immunosuppressive influence, the level of these antibodies may drop below the protective level, enabling the virulent virus to overcome the vaccine barrier, with initiation of the disease (Panshin *et al*., 2002). The most important drawback of vaccination however is that it has a "masking" effect. Vaccinated birds challenged with virulent NDV may become infected and excrete virus, although in relatively small amounts, while remaining apparently healthy, thus enabling the low-level the circulation of a virulent virus in the population (Guittet *et al*., 1993; Parede & Young, 1990; Utterback & Schwarts, 1973; Alexander, 2000b).

1.2.12 Role of wild birds in the perpetuation and spread of Newcastle disease

Newcastle disease virus isolates have frequently been obtained from migratory feral waterfowl and other aquatic birds (Alexander *et al*., 1979; Bannerjee *et al*., 1994; Kellerher *et al*., 1985; Spalatin & Hanson, 1975; Stallknecht *et al*., 1991). These strains are usually of the 'asymptomatic enteric' pathotype but differ from lentogenic strains prevalent in chickens by being thermostable (Spalatin and Hanson, 1974, Kida *et al*., 1980). For example, in a survey of mallard ducks (*Anas platyrhyncos*) trapped in New Zealand in 1997, ten APMV-1 viruses (NDV) were isolated from 321 cloacal and tracheal swabs. All isolates had low pathogenicity indices (0.00-0.16). Phylogenetic analysis showed that nine of the isolates were most similar to a reference strain, D26/76 originally isolated in Japan and also to the Queensland/66 virus isolated in Australia. The tenth isolate was very similar to a virus obtained from a shelduck in France. In these isolates, F_0 cleavage sites were of the sequence ^{112}GKQGRL^{117}, ^{112}ERQGRL^{117} or ^{112}EKQGRL^{117} (Stanislawek *et al*., 2002). In a similar study, five lentogenic NDVs were isolated from white-fronted geese in 2000 over wintering in the San-in district of western Japan (Shenqing *et al*. 2002a), and in recent survey of wild birds in Australia, two non-pathogenic APMV-1 isolates were obtained from cloacal and tracheal swabs from ducks. One of the viruses was V4-like, and the other belonged to monoclonal antibody group H (lineage 6) (Peroulis & O'Riley, 2004). In a similar study, viruses were isolated from faecal samples of

waterfowls in Alaska and Siberia collected from 1991 to 1996. Eight of the avirulent viruses isolated in Siberia and Alaska were closely related to the D26/76 and Queensland/66 viruses. In a South African survey conducted in the Oudshoorn region in 1998, antibodies to NDV were detected in 34 out of 46 serum samples from 14 mostly-aquatic species although no NDVs could be isolated (Pfitzer *et al*., 2000).

Müller *et al*. (1999) not only showed that species of wild geese were serologically positive for NDV, but also that the main resting and wintering areas of migratory waterfowl are likely to be important sites for the inter- and intraspecies transmission of avian diseases. Waterfowl in the northern hemisphere migrate south in autumn, back north in spring, and breed in summer in the nesting places in northern territories such as Alaska, Canada or Siberia. NDVs are preserved in the freezing water during winter when ducks are absent, and ducks returning from the south in spring are reinfected (Takakuwa *et al*., 1998).

The majority of NDVs isolated from wild birds, and especially waterfowl are lentogenic, causing no clinical diseases, but recent genetic comparisons between NDVs in domestic poultry and feral waterfowl suggested that velogenic viruses arose from avirulent strains originating from wild birds. The two most well-known cases are the following: in Ireland in 1990, two outbreaks of ND occurred in poultry. Not only were the viruses isolated highly virulent and apparently identical, but they were also very closely related antigenically and genetically to viruses of low virulence normally isolated from feral waterfowl (Alexander *et al*., 1992; Collins *et al*., 1993). Moreover, the genetic distinctiveness of this group of viruses (lineage 6) compared to all other NDVs suggested that the velogenic viruses that arose from the avirulent ones originated from wild birds (Collins *et al*., 1993). At the molecular level, the virulent viruses had four nucleotide differences at the site encoding the F_0 cleavage site compared to the related viruses of low virulence, which would explain the higher virulence for chickens (Collins *et al*., 1993). Genetic analysis of the viruses isolated during the second case, the 1998-2000 outbreaks in Australia, supported this hypothesis. In the 1960s it was recognised that Australia had its own endemic avirulent strains of NDV, characterised by the Queensland/66 or V4 strain (Simmons, 1967). Molecular characterization has shown that these viruses have avirulent F_0 cleavage site sequences of ^{112}GKQGRL^{117}, and HN extensions of 45 amino acids (Sato *et al*., 1987; Toyoda *et al*., 1989). Certain strains of this particular type are involved with a respiratory form of the disease (Hooper *et al*., 1999). From December

1998 to 1999, outbreaks of virulent ND occurred at Dean Park, Mangrove Mountain, and Tamworth in New South Wales, Australia. Prior to the outbreak, in September 1998, samples were collected from chickens showing respiratory distress on properties close to Peat's Ridge and Mangrove Mountain. Two viruses with the F_0 cleavage site ^{112}RRQGRL^{117} were isolated. It was noted that just two nucleotide substitutions ($G^{4888}\rightarrow A$ and $C^{4894}\rightarrow T$) in either of these sequences would generate a virulent motif (^{112}RRQRRF^{117}) at this site. The Peat's Ridge virus therefore appeared to be the progenitor of the virulent strains, additionally the virulent viruses had only 1.4% sequence diversion from the avirulent progenitor strain (Gould *et al*., 2001). There is mounting evidence that the presence of an unstable virus genome, represented by the Peat's Ridge strain, occasionally generates virulent variant strains in Australia (Kirkland, 2000).

In vivo studies have been performed to investigate the mechanism of mutation of avirulent strains into virulent ones as it was not clear whether the acquisition of virulence takes place in feral bird populations, with subsequent introduction of the virulent mutant into poultry, or whether an avirulent virus is transmitted directly to chickens and then becomes virulent. To investigate this, Shengqing *et al* (2002b) passaged an avirulent wild goose isolate in chickens. After nine consecutive passages by air-sac inoculation, followed by five passages in chick brain, the virus became highly virulent in chickens, producing a 100% mortality rate, and demonstrating typical velogenic properties in pathogenicity tests (MDT >120h → 56h; ICPI 0.00 → 1.88; IVPI 0h→2.67h). Sequence analysis at the F_0 cleavage site showed that the original isolate containing the typical avirulent type sequence ^{112}ERQERL^{117}, progressed incrementally to a typical virulent type, ^{112}KRQKRF^{117}, during the repeated passages in chickens. These results clearly demonstrated that avirulent viruses, maintained in wild waterfowl in nature and bearing the consensus avirulent type sequence, have the potential to become velogenic after transmission to and circulation in chicken populations. These results also suggested that chickens provide a mechanism for the selection of virulent viruses from an avirulent background (Shengqing *et al*., 2002b).

Virulent NDVs have also been recovered from wild birds. The most significant outbreaks of virulent ND in feral birds were those reported in double-crested cormorants (*Phalacrocorax auritus*) in North America during the 1990s. Virulent

viruses isolated from dead cormorants appeared to be closely related to the strain that later caused an outbreak in domestic turkeys (Kuiken, 1998). Isolations of virulent viruses from wild aquatic birds that did not develop clinical symptoms have also been reported (Majiyagbe & Nawathe, 1981; Takakuwa *et al*., 1998). In one study, 47 ND viruses were isolated from faecal samples of waterfowls in Alaska and Siberia collected from 1991 to 1996. 29 of these viruses were virulent according to their MDTs, and five of the eleven viruses sequenced, contained the dibasic pair of amino acids at the F_0 cleavage site (^{112}RRQKRF^{117} or ^{112}RRQRRF^{117}), which indicated virulence (Takakuwa *et al*., 1998). In a recent survey of migratory birds in Brazil, five virulent ND viruses were isolated from shorebirds, with ICPIs ranging from 1.68 to 1.72 (Cavalléro, 2004). Therefore, both virulent and potentially virulent strains of NDV are maintained in migratory waterfowl populations in nature (Takakuwa *et al* 1998).

1.2.13 Transmission and spread

Wild birds, especially migratory waterfowl, are therefore often regarded as a reservoir of NDV infection, and are considered a potential risk for the introduction of NDV into NDV-free countries (Kouwenhoven, 1993; Müller *et al*., 1999). This concern is supported by reports of ND outbreaks having occurred in 1991 in Finland, United Kingdom, The Netherlands, Czech Republic and also Austria, where wild birds were presumed to be the primary source of infection (OIE, 1996; 1997). Although the role of wild birds in the epidemiology of NDV is not sufficiently understood, it seems a realistic assumption that infected birds have the potential of spreading the disease very rapidly over long distances (Qureshi, 1988). Like AIV, NDV is spread primarily through direct contact between healthy birds and the bodily discharges of infected birds. The disease is transmitted through infected birds' droppings and secretions from the nose, mouth, and eyes. NDV is highly contagious and spreads rapidly among birds kept in confinement, such as commercially raised chickens. During the active respiratory stage, it can be transmitted through the air, however, the virus is not thought to travel any great distance by this method. All birds in a flock usually become infected within three to four days. Once introduced into poultry, the virus spreads farm-to-farm by the movement of inapparently infected poultry species; on contaminated objects such as boots, clothing, sacks, egg trays, and crates; in manure; or by flies, free-flying birds or mice. NDV can survive for several weeks in a warm

and humid environment, and can survive indefinitely in frozen material. However, the virus is destroyed rapidly by dehydration and by the ultraviolet rays in sunlight (Bram *et al*., 1974; OIE Terrestrial Manual, 2004)

1.2.14 History of Newcastle disease in South Africa

ND was officially diagnosed for the first time in South Africa in 1945, after a severe disease with respiratory, nervous and intestinal symptoms, and high mortalities swept through poultry in the Natal (KwaZulu Natal) province. The diagnosis was made in England, by serum neutralization tests performed at Weybridge (Kaschula *et al.*, 1945). Kashscula believed that ND was already present in Durban in September 1944, and everything seemed to point to the possible introduction of the disease through the Port of Durban. Because the symptoms and autopsy findings in Natal so closely resembled those described by Hudson in Mombassa, Kenya in 1935, it was "almost certain" that the disease had been brought by ship from some harbour on the East Coast of Africa. Hudson considered that the infection had spread south to Lindi, and there were persistent rumours suggesting that the entire African East Coast had been affected (Kaschula *et al.*, 1945). In retrospect, this outbreak appears to have been part of a panzootic that occurred during the Second World War that swept through Italy, Palestine, and the whole of central Europe. It is interesting to note that the rapid spread and devastation caused by ND during the Second World War was partly ascribed to the scarcity of wire, that lead to the absence of efficient barriers between adjacent areas or across water routes (Kluge, 1964).

The 1944/45 outbreak in South Africa was confined almost entirely to the sugar-cane belt of Natal and the free-ranging flocks of the Indian population suffered the heaviest losses. The disease was eventually stamped out, but 100 000 fowl are estimated to have perished. Kaschula (1945) suspected that the Mombassa outbreak in 1935 was not the first African case of ND. His suspicion is corroborated by the following letter that I came across, extracted from an 1892 South African agricultural journal:

Query No. 221- Fowl-sickness (in Fort England, Grahamstown)
Can any of your readers give me some information as to the causes, prevention, and treatment of a disease that from time to time attacks my fowls, and seems to resist all known treatment.The disease apparently begins with a cough, and gasping breathing, the fowl trembles all over, and when it sits down one leg is stretched in front the other behind the body. There seems to be a gradual paralysis of both legs. The patient has all along an excellent appetite, which is maintained to the very end of the disease-invariably death. Some of the cases make gurgling noise when breathing, and they generally live in this dying state for months. Aloes, rusty water, lime, and paraffin-remedies recommended by "fowl" authorities have all been tried-as well as homoeopathic doses of aconite, without benefit. I have two English imported canaries ill, and the symptoms are very similar. As the canaries are valuable birds I am anxious to know of a cure (Greenlees, 1892).

Apart from the prolonged "dying state" described, the symptoms and particularly the neurological ones are consistent with Newcastle disease. The spread from chickens to canaries suggests that the disease was caused by a highly infectious avian pathogen with a wide host range, like APMV-1. Thereafter and up to 1944 there were regular reports of disease outbreaks in poultry in various parts of South Africa. In some cases the diseases were confirmed to be bacterial in origin, but in other cases the etiological agent was never defined (Martinaglia, 1926). For example, another letter written in 1903 described the following symptoms in a flock of mixed-breed, free-ranging fowl at Hamans Kraal (Hammanskraal) near Pretoria:

The fowl becomes unable to walk, and appears to have paralysis of the legs, the wings droop, the foeces [sic] are loose, weakness sets in and the bird dies. We give paraffin externally and caster oil internally. This treatment is no good. What is the right treatment, please? (Rosenbloom, 1903)

The symptoms described here are typical of ND and it seems likely that NDV was present in South Africa long before 1945. Therefore, the account given by Mr Greenlees of Grahamstown in 1892 could be the earliest known description of ND anywhere in the world.

Confirmed ND reappeared in the Windermere Township of the Cape Peninsula in July, 1949, and in the Port Elizabeth area of the Eastern Cape in September. By January 1950, the disease had spread to Natal (KZN) and was also confirmed in Johannesburg. Another outbreak was diagnosed in Durban during January, 1951, and

a smaller outbreak in 1953. With the application of strict control measures, the disease was successfully eliminated until early October, 1954, when an outbreak occurred in Johannesburg. By the end of December 1954, the disease had been stamped out. It is estimated that more than 300 000 fowls died in the outbreaks of the early 1950's, but by then vaccines had been developed, and more than a million birds were vaccinated with an attenuated live virus (De Kock, 1954). ND re-emerged in the northern outskirts of the Pretoria district and the Pretoria Municipal area in August 1961. An intensive control campaign was successfully concluded at the end of November 1961, and no further outbreaks occurred. During November 1961, the disease was diagnosed in Evaton near Vereeniging and again in the same area in June 1962 (Kluge, 1964). All viruses isolated in the outbreaks from the 1940's to the 1960's were of the velogenic type, and caused high mortalities in both small lots of household fowl, and the larger poultry farms which became infected. In July 1960, the mild lentogenic type of Newcastle disease was diagnosed in South Africa for the first time. The strain of virus was so mild that symptoms of infection were apparent only to the careful observer (Kluge, 1964). The distribution of the 1944, 1950 and 1961 outbreaks were localised and mainly confined to small holding and backyard poultry farms, except in the Western Cape areas where the infection spread to 13 larger poultry farms (Kluge 1964). At that time, is was believed that the disease was not enzootic in South Africa and the localised nature of the outbreaks indicated that the infections were probably introduced from some unknown external sources (Kluge, 1964). These outbreaks caused serious losses while they lasted, but the control measures instituted by the State (quarantine and other zoo-sanitary measures, in conjunction with immunization of all birds in the surrounding and proclaimed areas) seemed to effectively control the disease, and eliminated the infection in each instance. The live mesogenic Komarov strain was used as a vaccine at the time, and no immunization against ND was practised or allowed at any time except during outbreaks of the disease and then always done under State supervision. Therefore, ND was not considered to be a disease of any importance for South Africa, especially since there was no evidence, serological or otherwise, to suggest the presence of even lentogenic strains of ND in South Africa (Coetzee & Abrams, 1965).

South Africa experienced one of its most severe ND epidemics from 1970 to 1972. The rapid expansion of the poultry industry since 1960 had created large concentrations of susceptible poultry, and poultry farms accommodating hundreds of thousands of chickens were established in close proximity to one another and near the

urban markets. The method of erradication previously employed to control ND had become impractical because of the rapid dissemination of the infection in various regions of the country (L Coetzee, 1980). The industry suffered great economic losses, and by 1971 ND was believed to be enzootic in the country. By 1974, outbreaks of ND had subsided and occurred only in unvaccinated birds on small poultry farms and among unvaccinated poultry kept in back-yards. Outbreaks were reported fairly frequently in consignments of imported birds at the quarantine station at the Jan Smuts (Johannesburg International) airport. Evidently, these consignments were infected while they were handled by dealers prior to shipment because all birds developed ND virtually without exception from 3-7 days after their arrival in South Africa. Vaccination against ND became a common practise at the airport quarantine station (Anonymous, SAPV-Pluimveebulletin Februarie 1974). Subsequent to the severe outbreak of the 1970's, only small sporadic outbreaks occurred until June 1993. The outbreaks since the early 1990s are the topics of Chapters Four, Five and Six.

1.2.15 The threat of Newcastle disease to sustainable livelihoods

Poultry production in South Africa may be seen as a gradient between two extremes, with highly integrated commercial enterprises with world-class facilities on one hand, and unimproved rural chickens kept by subsistence farmers on the other. During outbreaks, spillover into commercial operations result in severe economic losses, such as the 1993/94 outbreak, that caused an estimated loss of a million broilers a week during its peak (Coetzee, 1994). Because ND is notifiable to the OIE, outbreaks usually result in trade restrictions on poultry and poultry products, with the consequent threat of loss of valuable export markets. Although it is a serious threat to the broiler and layer industries, NDV is not considered an important production disease for ostrich farming, due to its very slow mode of spread (only faecal/oral and drinking water, not respiratory) in ostrich rearing systems (Verwoerd, 1995a).

There has been increasing acknowledgement of the potential of small-scale poultry production to stimulate the socio-economic growth of resource-poor households, and ND is the single largest threat to poultry production in South Africa. The greatest impact, however, is probably on the traditional small-scale production. This is well-illustrated by Kaschula's account of the 1944/1945 outbreak in Natal. As a result of the diminished supplies of mutton during the war, the Indian community (the

workforce at the sugar mills of KZN) were compelled to rely on poultry as a protein source, and as most could not afford to buy birds, they took to raising them on a greater scale than before. In certain districts, the Indian homes were built very close to one another, and the six or twelve fowls belonging to each family roamed through two or three backyards, generally roosting in the highest tree in the neighbourhood. Under these circumstances, there was nothing to check the outbreak. Moreover, in contrast to what happened in the 'native reserves', Indians bought and sold fowls freely and transported them considerable distances. At the sugar mills the Indians usually lived in barracks, and often thousands of birds of all ages were kept there. No barriers existed to prevent the disease killing all the fowls owned by any one group of families once ND was introduced. Kaschula relates how it was a common experience to visit a barracks, to find only the ducks (who were resistant) walking around after a ND outbreak. In contrast, the flocks owned by Europeans were well housed and fed, and losses were limited to those who had purchased table poultry from Indian and African sources. In one such case, a farmer was left with 22 survivors out of a flock of 850 white leghorn chickens. Another lost over 600 leghorns (100% of his flock), but bantams running with these fowls somehow escaped, as did a dozen turkeys on the same farm. The Africans did not suffer as heavy losses as the Indians, only a few very minor outbreaks were reported, apparently because Africans seldom bought fowls to take to their homes and limited movement of fowl between kraals occurred. Kaschula writes that when their birds were moved, they were carried in baskets and crates to trading stores and railway stations for transport to Durban, where an excellent market was established. Very often large numbers were taken at the weekend for sale to the Indians at the sugar mills. From the locations, the poultry moved predominantly in a one-way stream to Durban and the sugar mills, with practically no traffic in the reverse direction.

Nowadays, with the exception of urban areas in northern and southern Africa, most poultry production in Africa is still undertaken through the extensive system at village or family level. Almost every village household keeps domestic fowl (on average between 5 and 20 birds) and it is estimated that village fowl make up more than 80% of the total domestic fowl population in Africa estimated at 1068 million in 1995 (Guèye, 1998). Poultry provides a good source of protein and ready cash for villagers. Moreover, poultry helps to sustain the village economy and contributes to the prevention of urban migration. The benefits of family poultry production go directly to the rural poor, in most cases to the women being most active as caretakers.

Constraints on backyard poultry production include poor nutrition, particularly during the rearing phase, and losses due to predators because of insufficient housing, but ND is frequently responsible for devastating losses in village poultry. For example, Spradbrow (1992) estimated that 90% of the village chickens in Nepal die each year from ND. The extremely high mortalities reported for ND is a major factor that discourages the resource-poor from investing much of their time and scarce resources in expanding flock size. The survivors have high antibody levels and are resistant for a while, but as the levels of antibodies and the level of protection fall, the population becomes susceptible again, and the cycle is repeated. The disease is spread by contact between birds, which is exacerbated by the practise of taking birds to the market from a flock where disease is incubating. However, many aspects of the epidemiology of the disease in the village situation are not fully understood (Awan *et al*., 1994). Although vaccines do exist, even thermostable vaccines which are suited to use in remote rural areas, vaccination is not an accepted method of control in rural chickens. The reasons for this are cost (vaccination in South Africa is not subsidized by the government), large dosage formulations, difficulties associated with catching essentially wild chickens, lack of trained extension staff to administer vaccines and general misconceptions or superstitions about vaccination. Another fear is that vaccinated village flocks, or the small number of survivors of epidemics, could mask the circulation of virulent or potentially virulent viruses therein. Therefore, it has been speculated that village chickens could serve as reservoirs of virulent NDVs, from which spillover into the commercial sector poses a serious threat (Verwoerd, 1995b).

1.3 OBJECTIVES OF THE INVESTIGATION

Highly pathogenic avian influenza (HPAI) and velogenic Newcastle disease are the two most feared diseases of poultry in the world. South Africa's valuable poultry industry, consisting of broiler, layer and ostrich-producing sectors suffer heavy losses due to mortalities, drops in egg production, loss of export markets, and trade restrictions during periodic outbreaks of avian influenza and velogenic Newcastle disease. Newcastle disease is also cited as the biggest threat to sustainable backyard poultry production in developing countries, including South Africa. Whereas Newcastle disease outbreaks occur on average every four years in South Africa and vaccination is widely practised to control the disease, avian influenza is a relatively recent threat to the local poultry industry. LPAI viruses have been periodically isolated from ostriches since the 1990s, but the first outbreak in chickens was recorded in the early 2000's and HPAI broke out for the first time in forty three years in South Africa in ostriches of the Eastern Cape Province in 2004. Global awareness surrounding avian influenza has intensified since Asian HPAI H5N1 arose and spread westwards, and consequently a new emphasis has been placed on surveillance of wild migratory waterfowl, the primary hosts of both diseases. Prior to the work described in this thesis, very little sequence data existed for South African strains of AI and Newcastle disease viruses, and therefore little was known about the true status of these two diseases in South Africa, or what the chances were of introduction of Asian HPAI H5N1 by migratory birds. For decades it has been assumed that Newcastle disease is enzootic in South Africa, periodically spilling over into the commercial sector from an unknown reservoir, which some have suggested to be village chickens. The Newcastle disease project at OVI was initiated on the premise that a novel field strain of Newcastle disease existed in South Africa, with the potential to mutate to the velogenic form, as was reported in Australia in the late 1990s. For avian influenza, the origins of the LPAI strain in ostriches were unknown, although the farmers and veterinarians suspected that wild birds were somehow involved in the periodic outbreaks of disease.

The objectives of this study were to:

- determine the origins of the first outbreak of AIV in chickens (LPAI H6N2) in South Africa in the early 1990s (Chapter 2)
- characterise the 2004 HPAI H5N2 Eastern Cape ostrich outbreak strain and compare it to LPAI H5N1, H5N2, H3N8 and H4N8 viruses isolated from wild

ducks in 2004, determine their origins, the relationship of the H5N2 and H5N1 viruses to the Asian HPAI H5N1 strain, and to characterise any virulence determinants (Chapter 3)

- clarify the status of NDV in South Africa during the 1990s, with incorporation of new sequence data (Chapter 4)
- determine the origins of Pigeon paramyxovirus (PPMV-1) infections in South Africa (Chapter 5) and to
- determine the source of the current outbreak of NDV in South Africa, caused by a "Goose Paramyxovirus" strain and to describe the epidemiology of the disease in South Africa (Chapter 6).

This was the first time that molecular and bioinformatical techniques were applied in South Africa to analyse AIV and NDV strains. Although a strong tradition of serological diagnosis existed in South Africa for both diseases, previously viruses had to be sent to the international reference laboratory at VLA Weybridge (UK) for further characterization.