Virulence Determinants of Newcastle Disease Virus

Jos Dortmans

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Virulence Determinants of Newcastle Disease Virus

Virulentie Determinanten van het Newcastle Disease Virus

(met een samenvatting in het Nederlands)

Proefschrift

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Johannes Cornelius Franciscus Maria Dortmans

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Promotor: Prof. Dr. P.J.M. Rottier

Co-promotoren: Dr. B.P.H. Peeters

Dr. G. Koch

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Voor Maaike, Evie en Sara

Newcastle disease

Ziekte van Newcastle

Pseudo-fowl pest

Pseudovogel-pest

Atypische Geflügelpest

Pseudo-poultry plague

Avian pest

Avian distemper

Ranikhet disease

Tetelo disease

Korean fowl plague

Avian pneumoencephalitis

…you name it…

Contents

Chapter 1

Introduction

Newcastle disease

Newcastle disease (ND) is one of the most important infectious diseases of poultry. It is distributed worldwide and has the potential to cause large economic losses in the poultry industry (52, 80). Its causative agent is Newcastle disease virus (NDV), a virus that is able to infect over 240 species of birds and which spreads primarily through direct contact between healthy and infected birds (44). The disease is transmitted through infected birds' droppings and secretions from the nose, mouth and eyes. Infection can take place by virus inhalation, ingestion or contact with the conjunctiva (8). The disease may vary from subclinical, with no mortality, to severe infection, with 100% mortality. Clinical signs are dependent on factors such as the virus strain, host species, age of the host, co-infection with other micro-organisms, environmental stress, and immune status (61, 83). In poultry the general symptoms are loss of appetite, listlessness, abnormal thirst, weakness, a drop in egg production and swelling of the tissues around the eyes. Respiratory signs can include sneezing, gasping for air, nasal discharge and coughing, whereas a clear intestinal symptom is a greenish, watery diarrhea. Nervous symptoms may consist of paralysis of wings and/or legs, twisting of head and neck, ataxia, clonic spasms of limbs, and complete paralysis. In acute cases, death is very sudden and birds can die without showing any clinical signs (48, 61).

Based on the severity of the disease in chickens, NDV can be differentiated into three pathotypes: lentogenic, mesogenic and velogenic. Lentogenic NDV strains cause subclinical infection with mild respiratory or enteric disease and are considered to be low-virulent. Mesogenic NDV strains are of intermediate virulence causing respiratory infection with moderate mortality, while velogenic NDV strains are highly virulent causing high mortality (18). Velogenic strains are further classified into viscerotropic velogenic and neurotropic velogenic strains. Viscerotropic velogenic strains produce lethal hemorrhagic lesions in the digestive tract, whereas neurotropic velogenic strains produce neurological and respiratory disorders (6, 52).

The first outbreaks of ND were reported during the mid 1920s in Java, Indonesia and Newcastle-upon-Tyne, England (31, 49), from which the disease received its name (32). Predate indications of the disease have also been reported (6, 52), since the disease was sufficiently different from other highly virulent diseases of poultry. Within a few years ND had spread throughout the world and became endemic in many countries (80). In 2010 outbreaks were reported in Belgium, Belize, France, Germany, Honduras, Israel, Japan, Mongolia, Peru and Spain (88).

Newcastle disease virus

Etiology

NDV is a paramyxovirus and viruses from this family are enveloped, non-segmented, negative-sense RNA viruses, which together with the *Pneumovirinae* constitute the family of *Paramyxoviridae* (60). A number of important human diseases are caused by

paramyxoviruses. These include mumps and measles, and respiratory tract diseases in infants and children caused by respiratory syncytial virus (RSV) and para-influenza viruses (PIV). Paramyxoviruses are also responsible for a range of diseases in mammalian species, for example canine distemper virus (dogs), rinderpest virus (cattle) and Sendai virus (mice). Some paramyxoviruses such as the henipaviruses are zoonotic pathogens, occurring naturally in an animal host but being able to infect humans as well. NDV, or avian paramyxovirus type 1 (APMV-1), is classified in the genus *Avulavirus* of the subfamily *Paramyxovirinae* (60). An overview of the taxonomy of *Paramyxoviridae* and examples of representative viruses are given in Fig. 1.1.

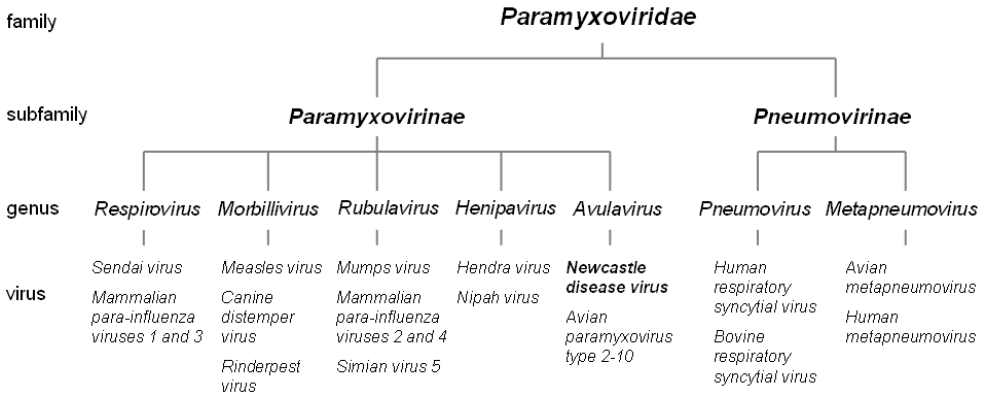

Fig. 1.1. Taxonomic organization of the *Paramyxoviridae*.

Phylogeny and epidemiology

Although only one NDV serotype exists there are 2 major subdivisions, class I and II (28). Class I viruses have been recovered from waterfowl and shore birds (28, 45) and are mostly avirulent in chickens, whereas class II viruses are mainly isolated from poultry and from pet and wild birds (4). The class II NDV's are further categorized into ten genotypes I to X (17, 37, 57, 58, 84). Genotype I consists of avirulent strains of NDV, while viruses of genotypes II, III and IV were responsible for the first panzootic that started in the 1920s (17). Genotype V viruses are thought to be responsible for the second panzootic in the early 1970s (27, 36, 91). These viruses are still the major cause of outbreaks in the USA (70). The third panzootic primarily affected pigeons and was caused by genotype VI viruses (27). It started in the late 1970s in the Middle East (43) and spread to Europe (19) where it caused major outbreaks in the poultry industry (15). Genetic analyses showed that these pigeon-viruses probably emerged as a result of multiple events of chicken-to-pigeon transmission (3, 86). Since the early 1990s outbreaks by the prevalent genotype VII viruses constitutes the fourth panzootic of ND (37, 53, 54, 56, 58, 93) that is still ongoing. Genotype VIII has been found in Southern

Africa (2, 37), and genotype IX viruses have been reported in some regions of China (57). In Taiwan the novel genotype X has been described (84).

Genome organization

The NDV RNA genome consists of 15,186 (29), 15,192 (39) or 15,198 (28) nucleotides (nt). It contains six open reading frames (ORF) which encode the nucleoprotein (NP), the phosphoprotein (P), the matrix protein (M), the fusion protein (F), the hemagglutinin-neuraminidase (HN) and the RNA dependent RNA polymerase (L). Two additional, non-structural proteins, V and W, are generated by RNA editing during P gene transcription (81). The 3' and 5' ends of the genome comprise the leader and trailer regions. The leader sequence is 55 nt long, while the trailer sequence is 114 nt long (29, 50, 85). Exact complementarity is found between the first and the last eight nt of the genome. These nt are identical in all NDV strains of which the complete genome sequence is known, which indicates that these regions have an important role in viral genome transcription and replication (51, 59). Conserved transcriptional control sequences of 10 or 11 nucleotides are preceding and following each gene. The 'gene-start sequence' acts as a transcriptional promoter and the 'gene-end sequence' acts as a transcriptional terminator. The sequences are given in Table 1.1.

Table 1.1. The start and end sequences of each gene

gene	gene-start	gene-end
NP	ACGGGTAGAA	TTAGAAAAAAA
P	ACGGGTAGAA	TTAAGAAAAAA
M	ACGGGTAGAA	TTAGAAAAAA
F	ACGGGTAGAA	TTAAGAAAAAA
HN	ACGGGTAGAA	TTAAGAAAAAA
L	ACGGGTAGGA	TTAGAAAAAA

Between the genes, there are non-coding nucleotide stretches called intergenic regions that vary in length from 1 to 47 nucleotides (50). A schematic representation of the NDV genome organization is depicted in Fig. 1.2.

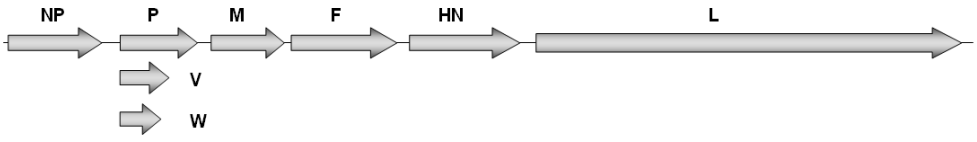

Fig. 1.2. Genomic organization of NDV.

Infection cycle

Virus infection is initiated by attachment of the virion (Fig. 1.3a) to the cell surface of the target cell. Binding of the viral HN glycoprotein to sialic acid-containing cell surface proteins, which serve as receptors, triggers the F protein-promoted fusion of the viral envelope with the plasma membrane of the host cell through a pH-independent mechanism, similar to other paramyxoviruses (51). Recently, it has been reported that NDV may also enter the host cell through receptor-mediated endocytosis by a pH-dependent mechanism similar to togaviruses, rhabdoviruses, orthomyxoviruses and flaviviruses (20, 78). The viral nucleocapsid or ribonucleoprotein complex (RNP) contains the RNA genome encapsidated with NP and associated with the polymerase complex composed of the P and L proteins (Fig. 1.3b). After entry, the viral nucleocapsid dissociates from the M protein and is released into the cytoplasm. The polymerase complex transcribes the viral genomic RNA to produce the mRNAs that are required for the synthesis of the viral proteins. Binding of the polymerase complex to the nucleocapsid is mediated by the P protein, whereas the catalytic activities are functions of the L protein (25, 26, 38, 72, 79).

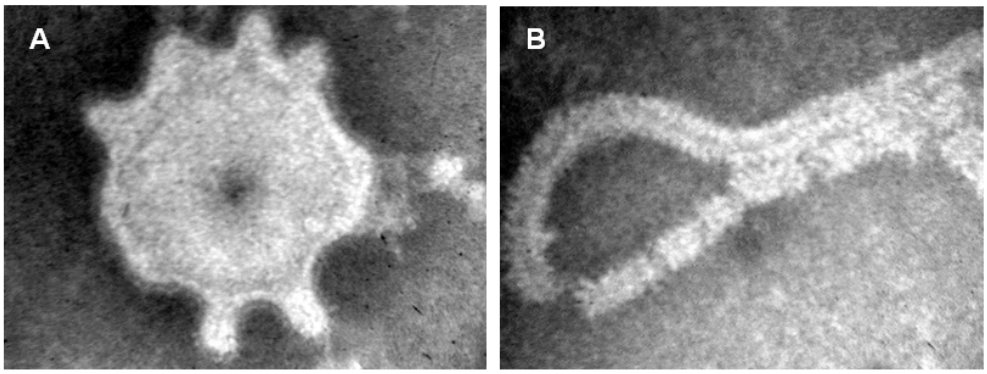

Fig. 1.3. Electron micrographs of particles of the recombinant Newcastle disease virus FL-Herts. a) complete virion. b) The viral nucleocapsid or RNP: genomic RNA encapsidated with NP protein in association with the P and L proteins. Pictures taken by Lisette Ruuls.

Genome replication takes place when sufficient amounts of viral proteins have accumulated. The polymerase complex is also responsible for the synthesis of full-length plus-strand antigenomic RNA, which in turn serves as the template for synthesis of minus-strand genomic RNA. Viral nucleocapsids are then assembled by association of NP with the newly formed genomic RNA and with the polymerase complex. All components of the virus particle are transported to the plasma membrane where they are assembled under the direction of the M protein. Virions are released from the cell by the process of budding. Finally, the neuraminidase activity of the HN protein facilitates the detachment of the virus from the cell and removes the sialic acid from

progeny virus particles to prevent self-aggregation (51, 82). A schematic diagram of the life cycle of Newcastle disease virus is depicted in Fig. 1.4.

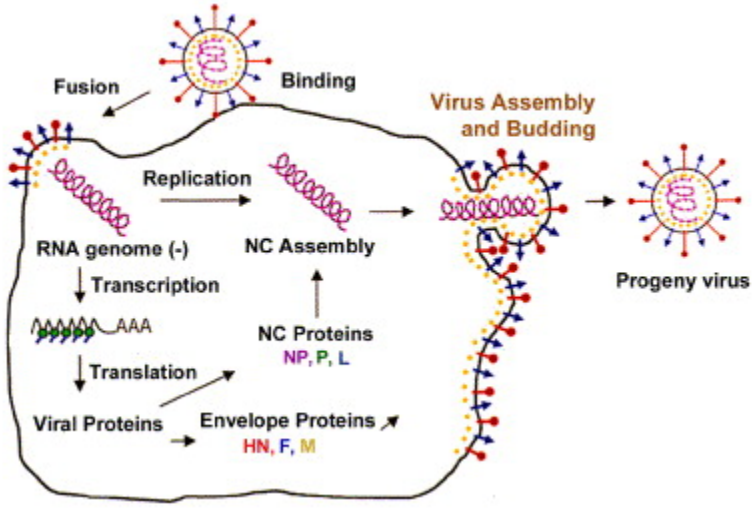

Fig. 1.4. Schematic diagram of the life cycle of Newcastle disease virus; NC refers to the nucleocapsid (Takimoto & Portner, 2004).

NDV Virulence

The amino acid sequence at the F protein cleavage site has been shown to be a major determinant of NDV virulence (65, 66). Cleavage of the precursor glycoprotein F0 into F1 and F2 by host cell proteases is essential for progeny virus to become infective (34, 65, 75). Lentogenic viruses have a monobasic amino acid motif at the C-terminus of the F2 protein and a leucine at the N-terminus of the F1 protein, and are cleaved extracellularly by trypsin-like proteases found in the respiratory and intestinal tract. Mesogenic and velogenic strains have a multi-basic motif at the C-terminus of the F2 protein and a phenylalanine at the N-terminus of the F1 protein and can be cleaved intracellularly by ubiquitous furin-like proteases (65, 66). This may result in a systemic infection that is often fatal. Consistently, studies with recombinant NDVs generated by means of reverse genetics showed that the pathogenicity of a virus increased significantly when a lentogenic cleavage site was converted into that of a velogenic strain (70, 73). Pathogenicity is determined by a standardized method called the intracerebral pathogenicity index (ICPI). In this test ten one-day-old chickens are injected intracerebrally with virus, observed every 24 hours for clinical signs, and scored 0 if normal, 1 if sick and 2 if dead. The ICPI is the mean score per bird per observation over an 8-day period and can range from 0.0 to 2.0. In the studies mentioned, the ICPI increased from 0.0 to 1.1-1.3. Interestingly, however, these ICPI values are not as high as those of velogenic strains from which the cleavage site was

derived (which range from 1.6 to 1.9) (67, 71, 74). This suggests that other viral proteins than F also contribute to pathogenicity (47, 67, 71, 74, 89).

By constructing recombinant HN-chimeric viruses or introducing specific amino acid changes in the HN protein, several studies have shown a moderate effect of the HN protein on pathogenicity (30, 41, 73, 90). The mutations most likely affected the attachment activity and fusion promotion activity (62). Furthermore, because of its function in attachment and release of the virus, HN is a key determinant of viral tropism (30, 39, Dortmans et al., unpublished results). In contrast, there are also studies using the same experimental approaches but other NDV strains, that show that HN does not have any effect on virulence (33, 90). This suggests that the involvement of HN in NDV virulence is strain dependent. Although the HN proteins of some strains vary in length due to differences in the sizes of their ORFs, pathogenicity could not be correlated with the size of the HN protein (74).

The V protein, which is generated by RNA editing from the P gene mRNA, is characterized by a cysteine-rich C-terminal region, which is conserved among *Paramyxoviridae*. NDV mutants that completely or partially lack the V protein or contain a mutated V protein show severe growth impairment *in vitro* while their replication in embryonated chicken eggs is age-dependent (40, 63, 68). It has been shown that viruses with mutated V proteins, in contrast to parental viruses, are unable to degrade the STAT1 protein (40), which is an important element of the interferon signaling pathway (35). These results show that the V protein plays an important role in NDV pathogenicity.

The involvement of the NP, P and L proteins in NDV virulence has been examined by generating chimeric viruses in which genes have been exchanged between the lentogenic strain LaSota and the mesogenic strain Beaudette C. Surprisingly, the recombinant Beaudette C virus that contained the L gene of LaSota replicated at a higher level and was slightly more virulent than its parental virus. However, no effect was found for the NP and P proteins (76).

Pigeon paramyxovirus type 1 (PPMV-1)

The first cases of natural NDV infection in racing pigeons were reported in Belgium, Germany, Great Britain and the Netherlands in the early 1970s. Because the isolated viruses were indistinguishable from the epizootic poultry virus, it was assumed that the pigeons had been infected as a result of contact with diseased domestic poultry (87). However, during the 1980s, viruses isolated from diseased pigeons were shown to be distinct from the more classical NDV strains, but to be antigenically and genetically similar to each other. They showed significantly different titres in hemagglutination inhibition tests and an unique binding pattern with polyclonal and monoclonal antibodies directed against the HN and F proteins (13, 15, 21, 77). Nowadays these viruses can be discriminated by phylogenetic analysis of the F gene nucleotide sequence (4). The disease has spread worldwide among racing and show pigeons (10, 14, 69). It arose in the Middle East in and resembled the neurotropic form in chickens but without

respiratory signs (43). Subsequently, the disease reached Europe (19) and spread to all parts of the world as a result of contact between birds at races and exhibitions and during international trade of such birds (6, 52). These variants became known as pigeon paramyxovirus type 1 (PPMV-1). In the past a number of ND outbreaks in poultry have been attributed to PPMV-1, which makes these pigeon-derived viruses a real and continuous threat to the poultry industry worldwide (1, 9, 11, 15, 16, 42, 55, 92).

The F proteins of all PPMV-1 strains examined to date contain a multibasic cleavage site motif, a feature of NDV generally associated with high virulence. However, some PPMV-1 strains cause only minimal disease and have a low ICPI value in chickens (64). Nevertheless, they do have a virulence potential for chickens, which can emerge upon serial passages in these animals (12, 22, 46, 47). Sequence analysis of such passaged viruses has mainly focused on the F gene, and it was concluded that the increase in virulence was not associated with changes in the F protein sequence (22, 23, 47). Thus, upon natural transmission from pigeons to chickens, PPMV-1 strains may evolve into more virulent viruses and lead to major outbreaks.

Management of ND

Because ND can cause severe economic losses, it is a notifiable disease to the World Organization for Animal Health (OIE) (24). To keep ND under control, prophylactic vaccination is applied on a large scale in the EU and elsewhere in the world (5). However, in case of an outbreak, confirmed infected animals are killed in order to eradicate the disease. Furthermore, control measures are taken in suspected herds and in areas around the outbreaks. In these areas a protection zone of 3 km is maintained for at least 21 days, and a surveillance zone of 10 km is kept for at least 30 days, as required by the Council Directive 92/66/EEC (24).

The extreme variation in virulence and clinical signs necessitates the careful definition of ND for the purposes of trade, control measures and policies. By international agreement, the assessment of virus virulence is, therefore, based on the intracerebral pathogenicity index (ICPI). Highly virulent viruses will give indices that approach the maximum score of 2.0, whereas lentogenic strains will give values close to 0.0. Furthermore, it is generally accepted that the molecular basis of pathogenicity relies on the sequence of the cleavage motif in the F protein as described above. Therefore, this has also been incorporated into the definition of ND. The current OIE definition for reporting an outbreak of ND is (7):

"Newcastle disease is defined as an infection of birds caused by a virus of avian paramyxovirus serotype 1 (APMV-1) that meets one of the following criteria for virulence:

a) The virus has an intracerebral pathogenicity index (ICPI) in day-old chicks (Gallus gallus) *of 0.7 or greater.*

or

b) Multiple basic amino acids have been demonstrated in the virus (either directly or by deduction) at the C-terminus of the F2 protein and phenylalanine at residue 117, which is the N-terminus of the F1 protein. The term 'multiple basic amino acids' refers to at least three arginine or lysine residues between residues 113 to 116. Failure to demonstrate the characteristic pattern of amino acid residues as described above would require characterization of the isolated virus by an ICPI test.'

In this definition, amino acid residues are numbered from the N-terminus of the amino acid sequence deduced from the nucleotide sequence of the F0 gene, 113-116 corresponds to residues -4 to -1 from the cleavage site."

Scope of this thesis

Although it appears that the amino acid sequence of the F0 protein cleavage site is the primary factor that determines virulence of NDV, it should be borne in mind that other viral properties may also cause variations in virulence. These other viral determinants are not well understood and may differ between viruses of different genotypes or lineages. Furthermore, the adaptation mechanism by which PPMV-1 becomes more virulent for chickens during passaging remains to be elucidated.

The aim of this thesis is to determine the molecular basis of the low pathogenicity of PPMV-1 isolates in chickens and to identify which other viral factors contribute to NDV virulence besides the cleavage site motif of the F protein. In chapter 2 we describe an approach to elucidate the molecular basis of the large difference in pathogenicity between two PPMV-1 clones containing only four amino acid differences in their entire proteome. In chapter 3 the construction of an infectious full-length cDNA clone of PPMV-1 strain AV324 is described, and the function of the F protein in determining virulence of PPMV-1 strains is investigated. The contribution to virulence of the internal genes NP, P, M and L of the non-virulent PPMV-1 strain AV324 and the highly virulent NDV strain Herts was studied by exchanging these genes using reverse genetics and is described in chapter 4. In chapter 5 the molecular changes responsible for the increase in virulence of a PPMV-1 isolate during passage in chickens are investigated. The pathogenicity for pigeons of a PPMV-1 strain and a NDV strain, that is virulent for chickens, is studied in chapter 6. Finally, this thesis is completed with a general discussion.

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Chapter 2

Two genetically closely related pigeon paramyxovirus type 1 (PPMV-1) variants with identical velogenic fusion protein cleavage sites but with strongly contrasting virulence

Jos C.F.M. Dortmans[1,2], Chad M. Fuller[3], Elizabeth W. Aldous[3], Peter J.M. Rottier[2] and Ben P.H. Peeters[1]

[1]Central Veterinary Institute of Wageningen UR, Lelystad, The Netherlands.
[2]Virology Division, Department of Infectious Diseases & Immunology, Faculty of Veterinary Medicine, Utrecht University, The Netherlands.
[3]Virology Department, Veterinary Laboratories Agency, Addlestone, Surrey, United Kingdom.

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Summary

Two pathogenetically different pigeon paramyxovirus type 1 (PPMV-1) virus clones were recently derived by passage of a single isolate with an intracerebral pathogenicity index (ICPI) of 0.32. The virus clones had an ICPI of 0.025 and 1.3 respectively (Fuller *et al.*, Arch Virol 2007; 152: 1575-82). Remarkably both viruses contained a cleavage site motif in the precursor fusion (F) protein that is usually associated with virulent viruses. In the current study, both viral genomes were completely sequenced and only four amino acid differences were observed. Of these, two were considered irrelevant on theoretical grounds and two amino acid changes were unique for virus 0.025. The latter were introduced into an infectious clone of a virulent Newcastle disease virus strain, individually and combined, and the effects of the mutations on pathogenicity were examined. The results indicate that only the S453P substitution in the F protein had a modest effect on pathogenicity. We were not able to identify the molecular basis for the pathogenicity difference between both viruses. However, our observations emphasize the need to determine both the virulence (ICPI) and the sequence of the cleavage site of the F protein to avoid dismissing of potential virulent PPMV-1 isolates.

Introduction

Newcastle disease (ND) is one of the most severe infectious diseases of birds and the cause of serious economic problems in the poultry industry worldwide. Its causative agent is Newcastle disease virus (NDV) or avian paramyxovirus type 1 (APMV-1) which is taxonomically classified in the genus *Avulavirus* of the subfamily *Paramyxovirinae* (family *Paramyxoviridae*, order *Mononegavirales*) (21). Isolates of NDV are categorized into one of four pathotypes namely apathogenic (avirulent), lentogenic (low virulent), mesogenic (intermediate virulent) or velogenic (highly virulent), based on the results of pathogenicity tests such as the intracerebral pathogenicity index (ICPI) (3).

The pathogenicity of NDV is mainly determined by the amino acid sequence of the precursor F protein protease cleavage site and the cleavability at this site by cellular proteases (23-25). Low virulent viruses have a monobasic cleavage motif and are processed by trypsin-like proteases found in the respiratory and intestinal tract. Virulent strains have a dibasic motif in their F protein and can be cleaved by ubiquitous furin-like proteases resulting in a systemic infection (24, 25).

During the 1980s, a disease caused by an APMV-1 spread worldwide among racing and show pigeons. Viruses isolated from affected birds were shown to be antigenically and genetically similar to each other but distinct from the more classical NDV strains. These APMV-1 variants became known as pigeon paramyxovirus type 1 (PPMV-1) and can be discriminated by monoclonal antibodies (6, 7, 10, 28) and by phylogenetic analysis of the F gene nucleotide sequence (1). Interestingly, PPMV-1 provides some examples of unusual viruses that have a cleavage site motif that is generally associated with virulent viruses and a contrasting ICPI result indicating an avirulent virus. Fuller

and collaborators (17) cloned several viruses originating from a PPMV-1 isolate with an ICPI of 0.32 (avirulent classification) and a velogenic F protein cleavage site by limiting dilution in embryonated specific-pathogen-free (SPF) eggs. They identified two genetically similar PPMV-1 clones that differed significantly in their pathogenicity for one-day-old chickens, despite the fact that they were comparable in *in vitro* infection characteristics such as syncytium formation, plaque size and morphology, and cell-to-cell spread. One virus had an ICPI of 0.025 and the other of 1.3 (17).

In the present study we report the molecular analysis of these related isolates using reverse genetics techniques. Sequencing the complete genomes identified four nucleotide differences between the two viruses which were analyzed by introducing them into an infectious cDNA clone of the virulent NDV strain Herts (16). Our observations illustrate that pathogenicity is a complex trait determined by multiple genetic factors in addition to the cleavability of the F protein.

Materials & Methods

Cells and viruses

QM5 cells (8) were grown in cell culture plates (Greiner Bio-One) using QT35 medium (Gibco-BRL/LifeTechnologies) supplemented with 5% fetal bovine serum and 1% of an antibiotic stock: penicillin (100 units/ml), streptomycin (100 μg/ml) and fungizone (2.5 μg/ml). The fowlpox recombinant virus fpE-FLT7pol (9) (hereafter called FPV-T7), which expresses the bacteriophage T7 RNA polymerase, was grown in primary chicken embryo liver cells. The virus FL-Herts was derived from the NDV strain Herts/33 as previously described (16). Virus 0.025 and virus 1.3 were parented by PPMV-1 isolate 248VB, which was isolated from dead racing pigeons in 1998 in Belgium (22). As described by Fuller and coauthors (17), virus 0.025 was obtained by passaging the working stock (248VB) in embryonated eggs three times at limiting dilution. Virus 1.3 was obtained by passaging the working stock six times at low dilution (1/10 and 1/100 dilutions) and subsequently three times at limiting dilution.

Construction of pFL-Herts^F

PCR mutagenesis was used to introduce the unique restriction site *Fse*I (at nucleotide position 6347) into the FL-Herts cDNA. Two PCR reactions were performed, one with primers p5380 and pFseIR(H), and one with primers pFseIF(H) and p8106 (Table 2.1) using pFL-Herts as template. The two overlapping PCR fragments were joined in a second PCR using primers p5380 and p8106. The resulting PCR fragment was digested with *Bst*Z17I and *Spe*I and cloned into pFL-Herts, resulting in a plasmid designated pFL-Herts^F.

Construction of pFL-HertsF^{S453P}, pFL-HertsL^{M1378V} and pFL-HertsF^{S453P}L^{M1378V}

The Ser at position 453 in the F protein of FL-Herts was changed to Pro using overlapping PCR with either primers p5380 and p5900R(P) or primers p5900F(P) and HH011 (Table 2.1) using pFL-Herts^{F} as template. The two overlapping PCR fragments were joined in a second PCR using primers p5380 and HH011. The resulting PCR fragment was digested with *Bst*Z17I and *Fse*I and cloned into pFL-Herts^{F}, resulting in a plasmid designated pFL-HertsF^{S453P}.

To change the Met to Val at position 1378 in the L protein, the expression plasmid pCIneo-L^{H} which contains the FL-Herts L gene (16) was digested with *Cla*I and religated resulting in the plasmid pCI-L^{H}. Subsequently, PCR reactions were performed with either primers LH030 and p12512R(V) or primers p12512F(V) and LH045 (Table 2.1) using pCI-L^{H} as template. The two overlapping PCR fragments were joined in a second PCR using primers LH030 and LH045. The resulting PCR fragment was digested wit *Sex*AI and *Stu*I and cloned into pCI-L^{H} obtaining pCI-L$^{H(M1378V)}$. Subsequently, pCI-L$^{H(M1378V)}$ was digested with *Sex*AI and A*vr*II and the purified fragment was cloned into a *Sex*AI and partially A*vr*II digested pFL-Herts^{F} or pFL-HertsF^{S453P}, resulting in pFL-HertsL^{M1378V} and pFL-HertsF^{S453P}L^{M1378V}, respectively.

Table 2.1. Primers used for PCR mutagenesis

primer	sequence	direction	genome position
p3-UIT	ACCAAACAGAGAATCCGTGAGTTA	forward	1
p2599	TAAGTAAAGTTGACTATCAG	forward	2599
pAscIF	CCCGGTTGGCGCGCCCAAGGTGCAA	forward	4518
p4518	CCCGGCTGGCGCGCCCAAAGTGCAAT	forward	4524
pAscIR	TTGCACCTTGGGCGCGCCAACCGGG	reverse	4543
p5380	GACTCAGATCTTGGGTATACAG	forward	5380
HF010	CAGCTTCTCCATAATTTTGC	reverse	5770
p5900F(P)	AATACTAGATCCTCAAGTTAT	forward	5890
p5900R(P)	ATAACTTGAGGATCTAGTATT	reverse	5910
pFseIF(H)	TAGAACGGTCGGGGAGGCCGGCCCTCAATCGGGAAT	forward	6326
pFseIR(H)	ATTCCCGATTGAGGGCCGGCCTCCCCGACCGTTCTA	reverse	6361
HH011	GCGGGAGAGAAATATGAAG	reverse	7664
p8106	AATCTCAACTAGTAAAGGAACGATC	reverse	8106
p8103	CTCAACTAGTAAAGGAACGATTCTGAATTC	reverse	8109
LH030	GTCCGACAGATCCAACCACC	forward	11674
p12512F(V)	CAAATAAGTTTGTGTATGATCC	forward	12501
p12512R(V)	GGATCATACACAAACTTATTTG	reverse	12522
LH045	AGCACCGTGTACAGACAGC	reverse	13149

Construction of FL-Herts^{AF}, FL-Herts(FHN)$^{0.025}$ and FL-Herts(FHN)$^{1.3}$

PCR mutagenesis was used to introduce the unique restriction site *Asc*I in pFL-Herts^{F} (at nucleotide position 4527). PCR reactions were performed either with primers p2599 and pAscIR) or with primers pAscIF and HF010 (Table 2.1) using pFL-Herts^{F} as template. The two overlapping PCR fragments were joined in a second PCR using primers p2599 and HF010. The resulting PCR fragment was digested with *Bst*Z17I and *Pac*I and cloned into a *Bst*Z17I and *Pac*I digested pFL-Herts^{F}, resulting in a plasmid designated pFL-Herts^{AF}.

The F and HN genes of PPMV-1 strains 0.025 and 1.3 were PCR amplified using primers p4518 and p8103 (Table 2.1). The resulting PCR fragment was digested with *Asc*I and *Spe*I and cloned into a *Asc*I and *Spe*I digested pFL-Herts^{AF} resulting in plasmids designated pFL-Herts(FHN)$^{0.025}$ and pFL-Herts(FHN)$^{1.3}$.

Virus rescue

To rescue viable virus from the cDNA clones, QM5 cells were infected with FPV-T7 and co-transfected with full length genome constructs and plasmids expressing NP, P and L as previously described (16, 26). After three days, the culture supernatant was harvested and inoculated into 9-11-day-old embryonated specific-pathogen-free (SPF) eggs.

Hemagglutination (HA) assay, pathogenicity test and re-isolation of virus

The HA assay and determination of the intracerebral pathogenicity index (ICPI) in 1-day-old chickens was performed as described in the European Community Council Directive 92/66/EEC (13). In order to check the sequences of the different recombinant viruses after the ICPI assay, brains, livers and lungs of the birds used in the ICPI were collected and virus was re-isolated using embryonated SPF eggs as previously described (15). The ICPI tests comply with the Dutch law on animal experiments and were reviewed and approved by an ethical committee.

RNA isolation, RT-PCR and sequencing

Isolation of genomic RNA of viruses 0.025 and 1.3 and first-strand cDNA synthesis was performed as previously described (17). The complete genomes were sequenced using primers distributed over the whole genome. Primer sequences are available upon request. The sequences of the 3'- and 5'-terminal ends of the viral RNA were determined by rapid amplification of cDNA ends as described previously (14). The complete sequences of viruses 0.025 and 1.3 have been submitted to GenBank (accession number: EF026579 and EF026583, respectively). Genomic RNA of the recombinant viruses was isolated using a High Pure Viral RNA kit (Roche Diagnostics). First-strand cDNA synthesis was carried out using a Superscript^{TM} II Reverse Transcriptase kit (Invitrogen) with primer p3-UIT (Table 2.1). PCR fragments were purified using a High Pure PCR purification kit (Roche Diagnostics). Nucleotide

sequencing was carried out using a 'Big-Dye' DNA sequencing kit and a PE Applied Biosystems 310 genetic analyzer.

Results

The genomes of the PPMV-1 isolates 0.025 and 1.3 are 15,192 nucleotides long and can be classified as genotype VIb or sublineage 4b, class II isolates. Like other strains with 15,192 nucleotides in their genomes, viruses 0.025 and 1.3 have a six-nucleotide insertion (relative to the 15,186 nucleotides long genomes) located between nucleotides 1647 and 1648 in the intergenic region between NP and P. Both viruses have F proteins with a multibasic amino acid sequence at the cleavage site (^{112}RRQKRF^{117}), which is typical for velogenic viruses, and therefore a surprising feature of the low pathogenic virus 0.025. Only four nucleotide differences between these viruses were detected, each resulting in an amino acid substitution, including a Pro to Ser substitution at position 453 of the F protein, which has been described previously (17). Virus 0.025 had an Asp at position 100 of the M protein, where virus 1.3 had an Asn. The L protein of virus 0.025 contained a Val and an Asn at positions 1378 and 1945, respectively, whereas virus 1.3 contained Met and Ser at these positions. A schematic representation of the amino acid differences and their positions in both genomes is depicted in Fig. 2.1a.

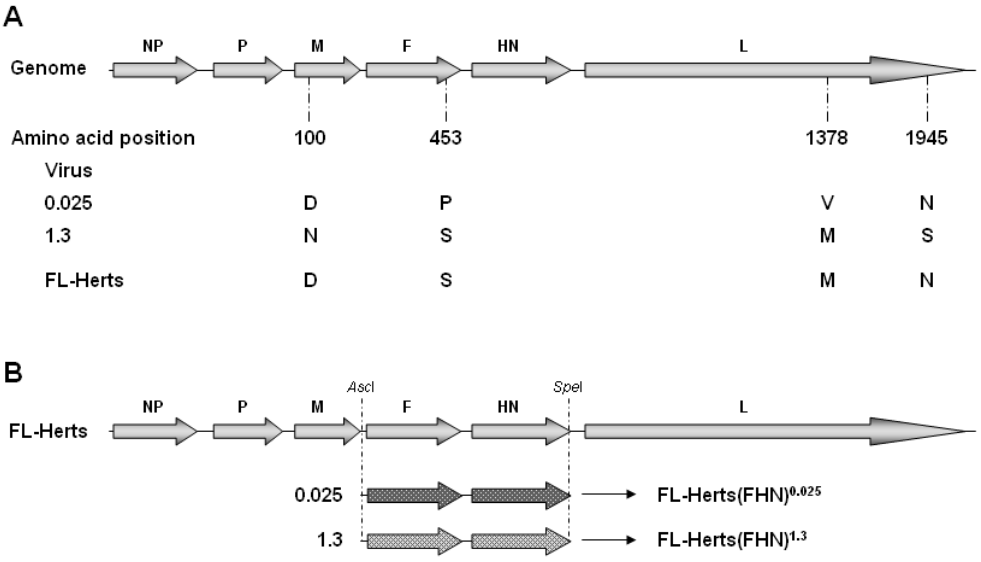

Fig. 2.1. Schematic representation of the NDV genome. (a) the locations of the 4 amino acid differences between viruses 0.025 and 1.3. The identities of the amino acid residues, in comparison with those of FL-Herts, are also shown. D: aspartic acid, P: proline, V: valine, N: asparagine, S: serine, M: methionine. (b) Schematic representation of the cloning strategy for substitution of the combination of F and HN genes of virus 0.025 and 1.3 virus in the FL-Herts backbone.

Because both viruses have an identical velogenic cleavage motif in the F protein, the difference in their pathogenicity must be explained by one or more of the observed genomic differences. An alignment of 105 M protein sequences revealed that the residue at position 100 is not conserved. Furthermore, an Asp at this position is not typical for low pathogenic strains as it also occurs in the velogenic Herts strain (Fig. 2.1a). Hence, this residue was not considered a potential virulence determinant. Alignment of 199 F protein sequences showed that the majority of them (197) contained a Ser at position 453. Strain Sterna/Astr/2755/2001 (AAW62249), which is velogenic, contains a Thr and isolate 01-1108 (AY935489), a lentogenic virus, contains a Phe at this position. The observation that virus 0.025 contained a Pro at this position, which is unique among all strains analyzed, combined with the physicochemical characteristics of this residue, prompted us to study this substitution in more detail. Alignment of 47 L protein sequences revealed that all strains have a conserved Met residue at position 1378 and a conserved Asn at position 1945. Since the Asn at position 1945 did not correlate with pathogenicity (it occurs both in virus 0.025 and in FL-Herts) we focused on residue 1378 since a Val at this position appeared to be unique among all strains analyzed.

To date there is no infectious clone available for either strain 0.025 or strain 1.3 that could be used to examine the genetic differences found between the two viruses. Therefore we used the infectious cDNA clone pFL-Herts, which was constructed from the velogenic NDV strain Herts/33 by de Leeuw and collaborators (16), to investigate the S453P substitution in the F protein and the M1378V substitution in the L protein. The two mutations were engineered into pFL-Herts either separately or in combination and viruses were rescued as described in the Materials and Methods section. Subsequently, the pathogenicity (ICPI) of these strains was determined and compared with that of the parent strain FL-Herts^{F}. Of the newly generated strains, FL-HertsL^{M1378V} was found to have an ICPI of 1.6, that of FL-HertsF^{S453P} was 1.3 and that of FL-HertsF^{S453P}L^{M1378V} was 1.4 (Table 2.2). Sequence analysis of the recombinant viruses re-isolated after the ICPI confirmed the specific amino acid substitutions. The S453P substitution in the F protein did cause some decrease in virulence, but not to an extent comparable to the decrease in ICPI from 1.3 to 0.025 observed with the PPMV-1 strains. Also the combination of the S453P and M1378V substitutions resulted only in a slight decrease in pathogenicity compared to the parent strain. These results indicate that the M1378V substitution in the L protein does not determine the difference in pathogenicity between viruses 0.025 and 1.3, but that the S453P substitution in the F protein may have an influence.

Previous work indicated that the HN protein may also be an important virulence factor (16, 18, 27). Because the F protein interacts with the HN protein during entry it is conceivable that the proline mutation in the F protein of virus 0.025 affects the interaction with its cognate HN protein in a different way than with a ‘foreign’ (e.g. Herts) HN protein. To test this possibility, we replaced both the F and HN genes of FL-Herts by those of viruses 0.025 and 1.3 (Fig. 2.1b). Of the resulting viruses, FL-Herts(FHN)$^{0.025}$ showed an ICPI of 0.9 whereas FL-Herts(FHN)$^{1.3}$ showed an ICPI of 1.0 (Table 2) and sequence analysis of the recombinant viruses re-isolated after the ICPI

confirmed the specific gene exchange. Although both recombinants have a velogenic cleavage site originating from 0.025 or 1.3, virulence is significantly decreased compared to FL-Herts. The F + HN gene cluster is known to play an important role in determining the virulence and differs significantly among APMV-1 strains. However, no significant difference in ICPI value was found between the viruses carrying either the F+HN proteins of the 0.025 or those of the 1.3 virus. This observation leads us to conclude that the S453P substitution in the F protein apparently does not change its interaction with HN in such a way that it significantly affects pathogenicity, leaving the difference in pathogenicity between viruses 0.025 and 1.3 unexplained.

Table 2.2. Virus titres and virulence

virus	HA[a]	EID_{50}/ml[b]	ICPI[c]
FL-Herts^{F}	7	9.20	1.6
FL-HertsL^{M1378V}	7	nd[d]	1.6
FL-HertsF^{S453P}	6	nd	1.3
FL-HertsF^{S453P}L^{M1378V}	8	nd	1.4
FL-Herts(FHN)$^{0.025}$	7	10.07	0.9
FL-Herts(FHN)$^{1.3}$	8	9.20	1.0

[a] Hemagglutination titre expressed as log 2.

[b] Embryo infectious dose per ml expressed as log 10.

[c] Intracerebral pathogenicity index.

[d] Not determined.

Discussion

Newcastle disease (ND) is a serious and hence notifiable disease of poultry. The current definition of ND of the World Organization for Animal Health (OIE) relies on the ICPI value determined in day-old chickens or on the amino acid sequence at the F protein's cleavage site. These are the two parameters the OIE uses to differentiate between virulent isolates (notifiable disease), and avirulent isolates (not notifiable). An isolate with an ICPI equal to or greater than 0.7 or having a multiple basic amino acid motif at the F protein cleavage site is classified as virulent and its presence should be reported to the OIE (2). However, a number of recent reports start to cast serious doubts on the reliability of the OIE virulence criteria by revealing biological (i.e. ICPI) phenotypes not consistent with nucleotide sequence data. Some field strains have been described exhibiting a low pathogenicity index but a velogenic F protein cleavage site. So far this has only been reported for PPMV-1 isolates (11, 22). In one of these studies

Meulemans and collaborators (22) investigated the characteristics of 27 PPMV-1 isolates. While all of them appeared to have the velogenic cleavage site motif ^{112}RR(Q/K/R)KRF^{117}, 14 isolates had an ICPI value of below 0.7, including also the 248VB isolate (ICPI 0.32), which parented viruses 0.025 and 1.3 (17, 22).

In order to shed light on these observations we wanted to take advantage of the availability of the latter viruses to try and identify additional determinants apparently contributing to their virulence. Thus, the viral genomes were sequenced and found to differ at only 4 nucleotide positions. Of these 4 differences two were present in both virus 0.025 and FL-Herts and therefore deduced to be irrelevant for changing the pathotype from 1.3 to 0.025. To study the significance of the remaining two, we used an infectious cDNA clone of the velogenic NDV strain Herts to introduce, both individually and together, the S453P and M1378V mutations as they occur in the F and L protein of the lentogenic 0.025 virus, respectively. Furthermore, for reasons explained, we additionally substituted the entire F + HN gene cluster of the Herts strain by that of the 0.025 and of the 1.3 virus. Although these changes in all cases except for the M1378V mutation reduced the ICPI value of the Herts virus, the effects were small to moderate and did not allow us to identify the mutation(s) responsible for the pathogenicity difference of the two PPMV-1's. That the combined F+HN replacement resulted in a virulence reduction of the Herts virus was not completely surprising as these two proteins are known to act cooperatively (16, 18, 27). If the F gene is only changed by a single point mutation the effect on virulence will probably be much milder, especially when this mutation is not critical, as apparently is the case in this study.

There are several possible reasons for our inability to elucidate the molecular basis of the pathogenicity difference of the two PPMV-1's. One relates to the quasispecies nature of our virus stocks. Viruses 0.025 and 1.3 were obtained from the original low pathogenic (ICPI 0.32) PPMV-1 isolate by limiting dilution in embryonated chicken eggs, not by plaque purification. It is thus possible that the 1.3 virus stock might contain a spurious genotype not picked up by consensus sequencing that is responsible for the observed phenotype. This phenomenon has been observed in foot-and-mouth disease viruses (29). In that study it was shown that populations with different pathogenicity could not be distinguished either by the consensus sequence or by the average complexity of the mutant spectrum. Furthermore, it has been shown for poliovirus that pathogenicity is not determined by a single genotype but by multiple different genotypes (i.e. quasispecies) that act cooperatively (30). A potential new method for the detection of minor sequence variants in complex genetic populations - not available to us yet - is ultra-deep pyrosequencing, a technique expected to greatly facilitate the characterization of RNA virus populations (31). Another possible explanation for our results is that the mutations were not studied in an appropriate genetic background. We tested the differences observed among pigeon-derived isolates in the background of a chicken-derived virus, simply because no infectious PPMV-1 cDNA clones have been established yet while the APMV-1 Herts clone was available. It is hence quite conceivable that the mutations were not phenotypically expressed in the context of the Herts virus genome but will require the use of a PPMV-1 genotype.

We would like to stress that the cleavability of the F protein is not a reliable predictor of virulence for PPMV-1 as it is for classical NDV. Compelling examples are the 14 isolates described by Meulemans and collaborators which carried a velogenic cleavage motif but exhibited ICPI values below 0.7 (22). If not used for meat production, pigeons are not considered as poultry and notification to the OIE is thus not required. However, chickens are easily infected by PPMV-1 and previous work has shown that the virulence of PPMV-1 isolates can increase by serial passage in chickens and that the differences in virulence were not related to the primary structure of the F protein (4, 12, 19). Such isolates may infect poultry, circulate sub-clinically and evolve into more virulent derivatives capable of causing serious outbreaks (20). An example of this were the more than 20 outbreaks during an epizootic in poultry in the UK, most of them caused by feed that was contaminated with PPMV-1 infected pigeon faeces (5). Because the atypical PPMV-1 isolates have the potential of becoming more virulent, the F protein cleavage site of field isolates should always be determined in addition to the ICPI.

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Chapter 3

Virulence of pigeon paramyxovirus type 1 (PPMV-1) does not always correlate with the cleavability of its fusion protein

J.C.F.M. Dortmans[1,2], G. Koch[1], P.J.M. Rottier[2] and B.P.H. Peeters[1]

[1]Central Veterinary Institute of Wageningen UR, Lelystad, The Netherlands.
[2]Virology Division, Department of Infectious Diseases & Immunology, Faculty of Veterinary Medicine, Utrecht University, The Netherlands.

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Summary

Cleavage of the fusion (F) protein of Newcastle disease virus (NDV, also known as avian paramyxovirus type 1; APMV-1) is required for the initiation of infection and acts as a major determinant of virulence. Virulent NDV strains have at least one pair of basic amino acid residues at the proteolytic cleavage site of the F protein, whereas non-virulent strains have single basic amino acids at the cleavage site. Remarkably, some pigeon paramyxovirus type 1 (PPMV-1) strains exhibit low virulence in chickens, despite their F proteins' multibasic cleavage site. To elucidate the molecular basis of the low pathogenicity of these strains in chickens, we constructed an infectious full-length cDNA clone of PPMV-1 strain AV324. This strain is non-virulent for chickens although its F protein contains the typical virulence motif ^{112}RRKKRF^{117}. By using reverse genetics, we exchanged the F genes of the AV324 and of a virulent NDV strain (Herts) and evaluated the recovered chimeric viruses for their pathogenicity in day-old chickens and in embryonated eggs. Our results show that the F protein of AV324, and probably those of similar PPMV-1 strains, are functionally not different from those of virulent NDV strains and that the difference in pathogenicity must be determined by other factors.

Introduction

Newcastle disease virus (NDV), or avian paramyxovirus type 1 (APMV-1), is the causative agent of one of the most severe infectious diseases in birds. NDV is classified in the genus *Avulavirus* of the subfamily *Paramyxovirinae* (family *Paramyxoviridae*, order *Mononegavirales*) (16) and contains a single-stranded negative-sense RNA genome consisting of 15,186 (7), 15,192 (12) or 15,198 (6) nucleotides. The genome contains six genes encoding the nucleoprotein (NP), the phosphoprotein (P), the matrix protein (M), the fusion protein (F), the hemagglutinin-neuraminidase (HN) and the RNA dependent RNA polymerase (L). Two additional, non-structural proteins, V and W, are generated by RNA editing during P gene transcription (27). NDV strains can be differentiated into three different pathotypes i.e., lentogenic (low virulent), mesogenic (intermediate) or velogenic (highly virulent) based on the intracerebral pathogenicity index (ICPI) in day-old chickens (1).

The amino acid sequence at the F protein cleavage site has been shown to be a major determinant of NDV virulence (19, 20). Cleavage of the precursor glycoprotein F0 into F1 and F2 by host cell proteases is essential for progeny virus to become infective (10, 19, 25). Lentogenic viruses have a monobasic amino acid motif at the C-terminus of the F2 protein and a leucine at the N-terminus of the F1 protein and are cleaved extracellularly by trypsin-like proteases found in the respiratory and intestinal tract. Mesogenic and velogenic strains have a dibasic motif at the C-terminus of the F2 protein and a phenylalanine at the N-terminus of the F1 protein and can be cleaved intracellularly by ubiquitous furin-like proteases resulting in a systemic infection (19,

20). *In vitro* plaque formation, plaque size and plaque morphology have been used to characterize NDV strains (11). Lentogenic strains need the addition of exogenous trypsin to spread and form syncytia in cell culture monolayers, whereas mesogenic and velogenic strains do not. Several reverse genetics studies have shown that the virulence of a lentogenic NDV strain increases significantly when the cleavage site of its F protein is converted into that of a velogenic strain (21, 22, 24). Interestingly, however, despite the fact that many NDV strains have the same cleavage site, they can still differ significantly in their degree of virulence, suggesting that viral proteins other than F contribute to pathogenicity (15, 21, 22, 24, 30). Knowledge about these determinants and about the mechanisms by which they exert their effects is obviously of great importance. Yet, little is still known about the underlying processes (8, 13, 14, 17, 26).

Variant strains of APMV-1 associated with infections of pigeons, known as pigeon paramyxovirus type 1 (PPMV-1), sometimes behave as lentogenic viruses. Although these viruses are virulent for pigeons, they show a low ICPI in chickens, despite the presence of an F protein cleavage site motif that is generally associated with virulent viruses (18). In a recent study, we investigated two genetically closely related PPMV-1 variants with strongly contrasting virulence patterns (chapter 2). We tried to identify the molecular basis for phenotypic differences between both variants by introducing mutations in an infectious cDNA clone of the virulent NDV strain Herts, since at that time an infectious PPMV-1 clone was not available. This did not, however, allow us to identify the responsible mutation(s), most likely because they were not studied in an appropriate genetic background. In this chapter we report the construction of an infectious cDNA clone of PPMV-1 strain AV324, a strain that is non-virulent for chickens although it carries a cleavage site motif typical for mesogenic or velogenic strains in its F protein. This AV324 clone can be used not only to investigate the compatibility between NDV and PPMV-1 proteins but, more importantly, it will allow us to identify determinants that affect the host range and pathogenicity of the virus. With this tool the remarkable features of PPMV-1 strains can finally be studied.

In view of the established role of the F protein in avian paramyxovirus virulence, this protein was the obvious first focus of our studies. Thus, we replaced the F gene in the cDNA clone of the non-virulent AV324 virus by that of the virulent NDV Herts strain and *vice versa*. Surprisingly, neither replacement affected the virulence phenotype, implying that, apparently, not the F protein but features contributed by other regions in the genome play critical roles in the pathogenicity of these viruses.

Materials & Methods

Cells and viruses

QM5 cells (2) were grown in cell culture plates (Greiner Bio-One) at 37ºC in a 5% CO_2 incubator in QT35 medium (Gibco-BRL/LifeTechnologies) supplemented with 5% fetal bovine serum (FBS) and 1% of an antibiotic stock consisting of penicillin (100 units/ml), streptomycin (100 μg/ml) and fungizone (2.5 μg/ml). The PPMV-1 isolate AV324/96 which had been passaged once in embryonated specific-pathogen-free (SPF) eggs

(designated AV324/96p1) was received from Dr. M.S. Collins (Virology Department, Veterinary Laboratories Agency, Addlestone, Surrey, United Kingdom) and was originally isolated by H. de Geus (PV 17/96) from a racing pigeon loft in Ireland. An intracerebral pathogenicity index (ICPI) of 0.44 and an intravenous pathogenicity index (IVPI) of 0.00 (Collins, pers comm.) were determined for this isolate at the Veterinary Laboratories Agency, Addlestone, Surrey, UK. The fowlpox recombinant virus fpE-FLT7pol (3) (hereafter called FPV-T7), which expresses the bacteriophage T7 RNA polymerase, was used as recently described (chapter 2). The full length infectious cDNA clone FL-Herts was derived from virulent NDV strain Herts/33 as previously described (8). Strain Dove/Italy/2736/00 was provided by Istituto Zooprofilattico Sperimentale delle Venezie, Legnaro, Italy.

Limiting dilution and plaque purification

Serial ten-fold dilutions of working stock (AV324/96p1) were prepared and 0.2 ml of each dilution was inoculated into each of five 9- to 11-day-old embryonated SPF eggs. Following three to four days of incubation, eggs were chilled o/n at 4-7ºC and the allantoic fluid from one egg inoculated with the highest dilution that tested positive in the hemagglutination test (5) was used for subsequent titration. After three limiting dilutions (AV324/96p4), three plaque purifications were performed on QM5 cells. After 1 h of infection, cells were washed with phosphate-buffered saline (PBS) and incubated 2 days under an overlay of GMEM/EMEM (ASG-Lelystad) medium containing 1% methylcellulose, 5% FBS and 1% antibiotics. Virus from the third round of plaque purification (designated AV324/96p4pp) was grown in 9- to 11-day-old embryonated SPF eggs to prepare a virus stock.

Phylogenetic tree

Phylogenetic analyses was performed using the complete genomic sequences of the following NDV isolates: N.Ireland/Ulster/67, AY562991; Clone 30, Y18898; LaSota/46, AF077761; Mukteswar, EF201805; Herts/33, AY741404; U.S./Largo/71, AY562990; U.S.(FL)/44083/93, AY562986; AV324/96, GQ429292; 0.025, EF026579; Dove/Italy/2736/00, GQ429293; IT-227/82, AJ880277; Goose Paramyxovirus SF02, NC005036; ZJ1, AF431744. The sequences of the complete genomes were aligned by the MegAlign program in the Lasergene package (DNASTAR), using the CLUSTALW multiple alignment algorithm. Phylogenetic analyses were performed using the ClustalW2 software of the EMBL-EBI website (http://www.ebi.ac.uk/Tools/clustalw2/index.html). The results are presented as an unrooted maximum likelihood phylogenetic tree created by the neighbour-joining method in which the branch lengths are proportional to the predicted number of substitutions.

Sequencing and cloning of full-length AV324 cDNA

Genomic RNA of AV324/96p4pp was isolated with a High Pure Viral RNA kit (Roche Diagnostics). First-strand DNA synthesis was carried out using a Superscript^{TM} III Reverse Transcriptase kit (Invitrogen). Five overlapping sub-genomic cDNA fragments were generated, purified using a High Pure PCR purification kit (Roche Diagnostics) and subsequently sequenced (BaseClear, Leiden, the Netherlands). The sequences of the 3'- and 5'-terminal ends of the viral RNA were determined by rapid amplification of cDNA ends as described previously (7). Primer sequences used for the generation of the overlapping sub-genomic cDNA fragments and for genome sequencing are available upon request. To establish a reverse-genetics system for AV324/96p4pp, we constructed the full-size cDNA clone, designated rgAV324, and the NP, P and L expression plasmids as described previously (8, 22). Details of this construction are available upon request.

Construction of rgAV324^{AF}, rgAV324(F)^{Herts} and FL-Herts(F)^{AV324}

PCR mutagenesis was used to introduce the unique restriction sites *Asc*I (position 4533) and *Fse*I (position 6353) into the rgAV324 cDNA, resulting in a plasmid designated rgAV324^{AF}. All PCR-generated regions were sequenced in order to check for unintended mutations. The F genes of FL-Herts and AV324 were exchanged by using restriction sites *Asc*I and *Fse*I and reciprocally cloned into rgAV324^{AF} and pFL-Herts^{AF} (chapter 2) resulting in plasmids rgAV324(F)^{Herts} and pFL-Herts(F)^{AV324}, respectively.

Virus rescue

To rescue virus from cDNA, QM5 cells (2) were infected with FPV-T7 for 1 h and subsequently co-transfected with full length cDNA constructs and helper plasmids expressing P and L as previously described (8, 22). We could rescue virus without adding expression plasmid pCI-NP, suggesting that sufficient NP is synthesized from full-length anti-genomic RNA. After three days, the culture supernatant was harvested and inoculated into 9-11-day-old embryonated specific-pathogen-free (SPF) eggs.

Plaque formation

To determine whether viruses FL-Herts^{AF}, FL-Herts(F)^{AV324}, rgAV324^{AF} and rgAV324(F)^{Herts} were able to form plaques, QM5 cells in 6 well cell culture plates (Greiner Bio-One) were infected with ten-fold serial dilutions as described above. Plaques were visualized 30 h post infection by immunological staining (31) using monoclonal antibody (mAb) 8E12A8C3 (CVI of Wageningen UR) against the F protein and HRPO-conjugated polyclonal rabbit anti-mouse Ig (Dako). The low-virulent NDV strain NDFL+ (22), was used to confirm that lentogenic strains do not form plaques in the absence of exogenous trypsin.

Hemagglutination inhibition (HI) assay and pathogenicity tests

The HI assay, the mean death time (MDT) in 9-day-old embryonated chicken eggs, and the determination of the intracerebral pathogenicity index (ICPI) in 1-day-old chickens was performed as described in the European Community Council Directive 92/66/EEC (5). Of the chickens that survived the ICPI test sera were tested in a HI assay to verify that the animals became infected. Animal experiments complied with the Dutch law on animal experiments and were reviewed and approved by an ethical committee.

RNA isolation, RT-PCR and Sequencing

Viral RNA isolation and first-strand DNA synthesis was carried out as described above. PCR fragments were purified using a High Pure PCR purification kit (Roche Diagnostics). Nucleotide sequencing was carried out using a BigDye Terminator v1.1 cycle sequencing kit (Applied Biosystems) and an ABI 3130 genetic analyzer (Applied Biosystems).

Growth kinetics

The growth kinetics of rgAV324(F)^{Herts} and pFL-Herts(F)^{AV324} along with their respective parental viruses, were determined by using multi-cycle and single-step growth curves in QM5 cells. QM5 cells in triplex wells of six-well plates were infected with virus at a multiplicity of infection (m.o.i.) of 0.001 or 10 $TCID_{50}$/ml. After 1 h of adsorption, the cells were washed with PBS and then covered with QT35 medium supplemented as described above. Supernatant samples were collected and replaced with an equal volume of fresh medium at 8, 16, 24, 32, 40 and 48 hours post infection. Virus titres of samples were determined by serial end-point dilution in 96-well plates using QM5 cells and an immuno-peroxidase monolayer assay as described above. Virus titres ($TCID_{50}$/ml) were calculated using the Reed and Muench method (23).

Results

To study the molecular basis of virulence of PPMV-1 we obtained isolate AV324/96p1 from the European Reference Laboratory for Newcastle disease virus (Veterinary Laboratories Agency, Weybridge, UK). This isolate is low-virulent for chickens (ICPI=0.44; ICPI values range from 0.0 for non-virulent viruses to 2.0 for highly virulent viruses), but contains an F protein cleavage site motif typically associated with virulent viruses (^{112}RRKKRF^{117}) and is able to form plaques in tissue culture cells without the addition of trypsin. To ascertain that our virus stock did not consist of a mixture of low- and high-virulent viruses, the original sample was passaged 3 times by limiting dilution in SPF eggs. Subsequently, this virus was plaque purified three times in QM5 cells resulting in a virus (AV324/96p4pp) with an ICPI of 0.00. Sequence analysis of the cleavage site of the F protein of virus AV324/96p4pp as well as of the parental virus (AV324/96p1) showed the presence of the same multibasic cleavage site ^{112}RRKKRF^{117}, explaining why these viruses formed plaques in tissue culture cells

without the addition of exogenous trypsin. The complete genome sequence of AV324/96p4pp was determined and the assembled sequence consists of 15,192 nt (accession number GQ429292), which puts it into class II according to the classification based on genome size. Phylogenetic analyses showed that AV324 can be classified as a genotype VI or sublineage 4b virus, similar to previously studied PPMV-1 strains such as IT-227/82 (29) and 0.025 (9) (Fig. 3.1).

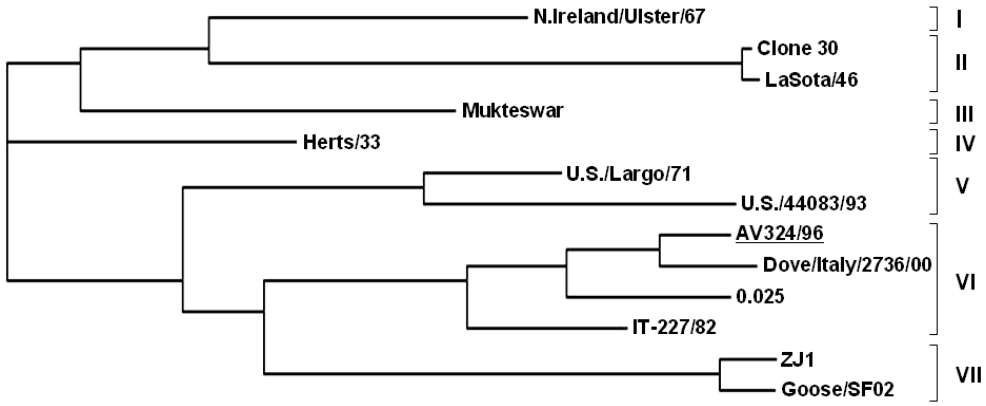

Fig. 3.1. Phylogenetic analysis of NDV strains based on complete genome nucleotide sequences. Branch lengths represent the predicted number of substitutions and are proportional to the differences between the isolates. Brackets represent the different genotypes.

Comparison of the genome sequence of AV324 with those of all APMV-1 and PPMV-1 strains for which full-length genome sequences are available in GenBank showed that the top 15 hits were of mesogenic or velogenic origin. Highest similarity (96%) was found with the mesogenic PPMV-1 strain Dove/Italy/2736/00 (accession number AY562989), of which an ICPI of 1.2-1.3 has been documented (28, 32). Hence we reasoned that a detailed comparison of the two strains might provide indications of the amino acid or gene sequences that are responsible for their difference in virulence. However, our observation that a particular region (nt 2491-3892) of the published sequence of Dove/Italy/2736/00 showed a high similarity (98-99%) with vaccine strains B1 (AF375823), Clone 30 (Y18898) and LaSota (AF077761; AY845400) but not with other known PPMV-1 sequences, made us decide to re-sequence the complete genome of Dove/Italy/2736/00 ourselves (kindly provided by Ilaria Capua, Istituto Zooprofilattico Sperimentale delle Venezie, Italy). The new sequence (accession number GQ429293) lacked the vaccine resembling sequences and had 97% overall homology with our AV324/96p4pp isolate. The 292 nucleotide and 53 amino acid differences between AV324/96p4pp and Dove/Italy/2736/00 were distributed all over the genome: ten amino acids differing in NP, nine in P, nine in M, seven in F, seven in

HN, and eleven in L. Thus, the comparison did not allow us to pinpoint unambiguously the molecular basis of the non-virulent phenotype of AV324/96p4pp.

To allow us to introduce specific mutations or replacements that would result in a change in pathogenicity, we decided to construct an infectious cDNA clone of AV324/96p4pp. Viable virus (designated rgAV324) could be rescued and was completely non-virulent, similar to the parent virus AV324/96p4pp, as evidenced by an ICPI value of 0.00. Interestingly, rescue of the recombinant virus was achieved without the necessary inclusion of the helper plasmid expressing NP to the transfection mix, which is in contrast with the established requirement of this protein needing to be provided *in trans* to initiate the replication/transcription process. To verify this observation we repeated the experiment now including also the infectious cDNA clones pNDFL+ (LaSota) and pFL-Herts (Herts). Transfections without adding plasmids expressing P or L protein did not result in the rescue of viable virus but all three viruses could be rescued without the plasmid expressing NP (data not shown). Since NP is the 5' proximal gene in the anti-genomic RNA expressed from the full-length cDNA by T7 RNA polymerase, our explanation for this observation is that apparently sufficient NP is expressed from the anti-genomic RNA to start the replication process, without the need for additional NP.

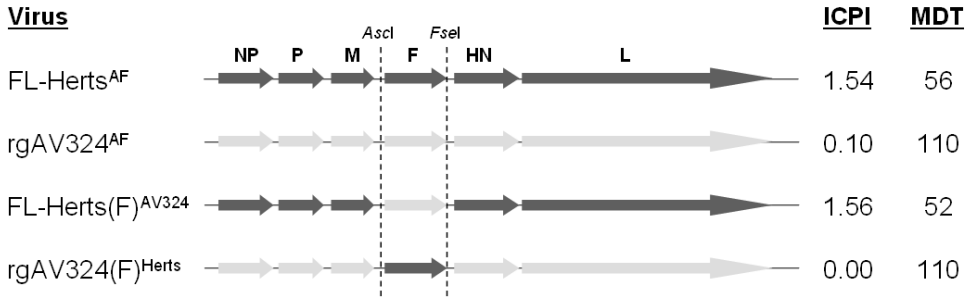

Fig. 3.2. Schematic illustration of the cloning strategy used to exchange the F gene between FL-Herts^{AF} and rgAV324^{AF} resulting in FL-Herts(F)^{AV324} and rgAV324(F)^{Herts} and the virus virulence determined by the ICPI and the MDT. ICPI values range from 0.0 for non-virulent viruses to 2.0 for highly virulent viruses and the MDT duration is more than 90 h for lentogenic strains, 60 to 90 h for mesogenic strains, and under 60 h for velogenic strains.

The proteolytic cleavage site of the F protein is the main determinant of NDV virulence. In order to determine whether the lack of cleavage *in vivo*, or other properties of the F protein are responsible for the non-virulent phenotype of AV324, we used our infectious cDNA clone and that of the virulent Herts virus to exchange the F genes, as described in the Material and Methods section and illustrated in Fig. 3.2. Both chimeric viruses could be rescued, indicating that the F proteins of these strains are compatible. The virulence of the F-chimeric viruses along with their respective parental viruses, was

evaluated by determining the ICPI in 1-day-old chickens and the mean death time (MDT) in 9-day-old embryonated chicken eggs. FL-Herts^{AF} and FL-Herts(F)^{AV324} had an ICPI value of 1.54 and 1.56, respectively, whereas both rgAV324^{AF} and rgAV324(F)^{Herts} were low virulent (ICPI values of 0.10 and 0.00, respectively; Fig. 3.2). In embryonated eggs strains FL-Herts^{AF} and FL-Herts(F)^{AV324} had MDT values of 56 h and 52 h, respectively, compared with 110 h for both rgAV324^{AF} and rgAV324(F)^{Herts} (Fig. 3.2). These observations indicate that the exchange of the F gene between the non-virulent PPMV-1 strain AV324 and the highly virulent strain Herts did not significantly affect the pathogenicity of the chimeric viruses relative to their respective parental viruses. Taken together, these results show that the F protein of AV324 is not functionally different from that of a virulent NDV strain.

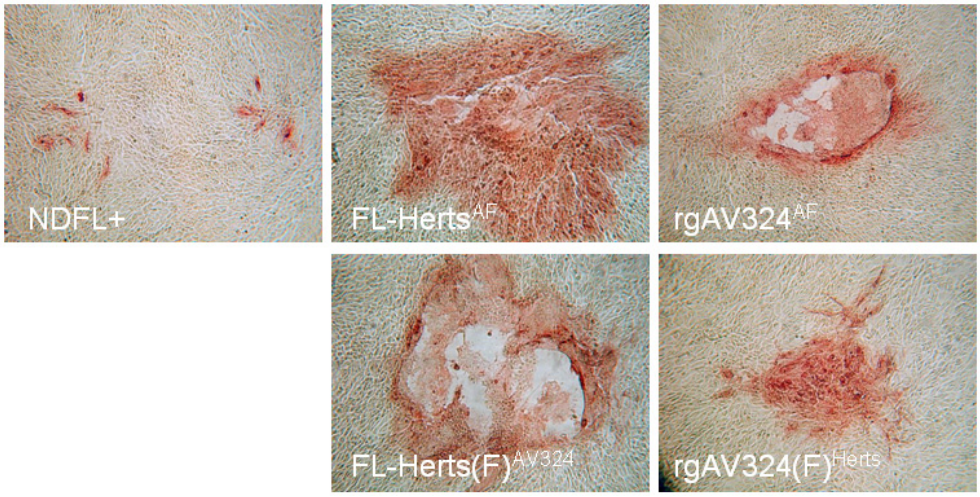

Fig. 3.3. Plaque formation in QM5 cells in the absence of exogenous trypsin in medium containing 1% methylcellulose at 30 hours post infection. Plaques were visualized by immunological staining using a mAb against the NDV F protein.

We examined whether the F-chimeric viruses were able to form plaques in QM5 cell cultures without the addition of exogenous trypsin. All viruses produced plaques 30 h post infection, whereas the lentogenic NDV strain LaSota, derived from the infectious cDNA clone NDFL+, only gave rise to single-cell infections (Fig. 3.3). The viruses FL-Herts^{AF} and FL-Herts(F)^{AV324} showed larger plaques than rgAV324^{AF} and rgAV324(F)^{Herts}, suggesting the involvement of viral replication features in determining this phenotype. Furthermore, the viruses rgAV324^{AF} and FL-Herts(F)^{AV324} showed more syncytia and a more pronounced cytopathic effect (CPE) than the viruses FL-Herts^{AF} and rgAV324(F)^{Herts}.

To try and understand the plaque phenotypes, growth kinetics of the F-chimeric viruses along with their respective parental viruses were studied by determining multi-cycle (m.o.i. of 0.001) and single-step (m.o.i. of 10) growth curves in QM5 cells. The results showed that rgAV324^{AF} replicated with slower kinetics than FL-Herts^{AF} irrespective of the m.o.i. used, but that these viruses eventually reached similar titres (Fig. 3.4). Exchange of the F genes affected the productive capacities of both viruses.

It appeared that FL-Herts(F)^{AV324} replicated with slower kinetics than FL-Herts^{AF} and rgAV324(F)^{Herts} reached higher titres than rgAV324^{AF}. It is noteworthy that rgAV324^{AF} and particularly FL-Herts(F)^{AV324} again developed more extensive CPE compared to FL-Herts^{AF} and rgAV324(F)^{Herts}, which may explain why FL-Herts(F)^{AV324} grew to lower titres than FL-Herts^{AF}.

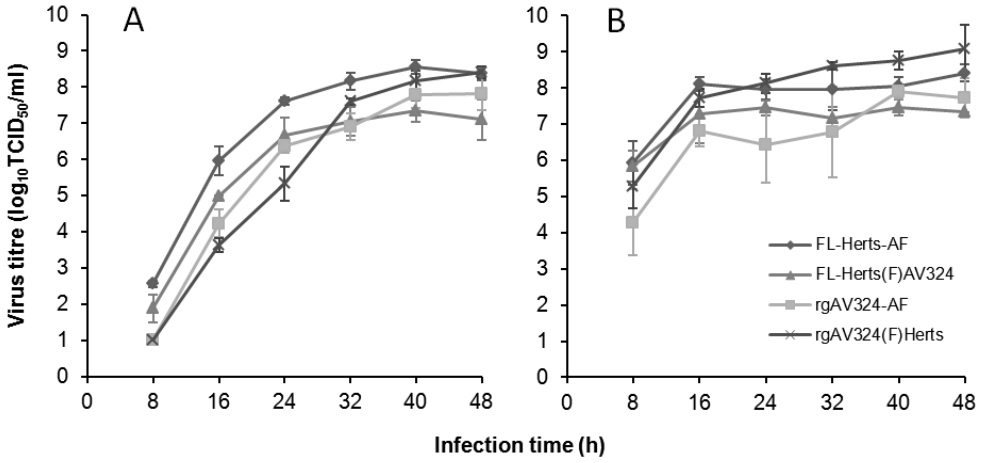

Fig 3.4. Multi-cycle (a) and the single-step (b) growth kinetics of the F-chimeric viruses along with their respective parental viruses in QM5 cells. Six-well plates of QM5 cell monolayers were infected with virus at an m.o.i of 0.001 (a) or 10 (b) $TCID_{50}$ per cell for 1 h. The cells were washed with PBS and then incubated in QT35 medium at 37ºC and 5% CO_2. Supernatant samples were collected at 8, 16, 24, 32, 40, and 48 h post infection and replaced with equal volumes of fresh medium. Virus yields were determined by $TCID_{50}$. Error bars show standard deviations.

Discussion

In this study we report the rescue of a PPMV-1 strain, AV324, that carries a typical velogenic cleavage site motif in its F protein, from cloned full-length cDNA. We showed that, although AV324 is non-virulent in chickens, its F protein is not functionally different from that of other virulent NDV strains.

All PPMV-1 isolates described so far, have the typical velogenic cleavage site motif in their F protein and most isolates are therefore at least mildly-virulent for chickens.

However, some PPMV-1 isolates, although isolated from pigeons that succumbed to the infection, are low- or non-virulent for chickens (18). We studied the PPMV-1 isolate AV324/96 which was isolated from a dead racing pigeon loft in Ireland in 1996. The virus that we rescued, rgAV324, is non-virulent for chickens despite the fact that its F protein contains a typical velogenic cleavage site. This clone can be used to finally study the a-typical properties of PPMV-1 and investigate which specific host factors are needed for adaptation upon becoming virulent for chickens.

Since the fusion (F) protein is strongly associated with virulence, we focused on the role of this protein using a reverse genetics approach. Exchanging the F proteins of AV324 and Herts did not affect virulence as determined by the ICPI or the MDT assay. Thus, the non-virulent character of AV324 cannot be explained by its F protein being functionally different from that of virulent strains. While the multibasic amino acid cleavage motif is an absolute prerequisite, other decisive factors are critically involved in determining the virulent phenotype of at least some PPMV-1 isolates. This is consistent with the increasing virulence observed upon passaging PPMV-1 strains through chickens without changing the primary structure of the F protein (4).

To study replication kinetics of the viruses we first looked at plaque formation. Like the parental viruses, also the F-chimeric viruses induced the formation of plaques in cell monolayers without the addition of exogenous trypsin. This indicates that the F protein of AV324 is cleaved both in the AV324 and in the Herts background (Fig. 3.3). To form plaques in cell culture, lentogenic NDV strains need the addition of exogenous trypsin, whereas mesogenic and velogenic strains do not. This feature has often been used to characterize NDV strains (11). However, our observations and those of others (9) show that plaque formation and thus F protein cleavage does not always correlate with virulence of NDV.

The observation that Herts-based viruses produced larger plaques than AV324-based viruses, irrespective of the F protein (Fig. 3.3), implicates the involvement of other mechanisms that are responsible for the differences in virulence. The observation that viruses containing the F protein of AV324 produce more syncytia and CPE than viruses containing the F protein of Herts (Fig. 3.3) may explain why FL-Herts(F)^{AV324} grew to lower titres than FL-Herts (Fig. 3.4). However, this phenomenon does not seem to correlate with differences in virulence in embryonated eggs or day-old chickens.

The current definition of the World Organization for Animal Health (OIE) of an ND outbreak is the isolation of an NDV strain that either has an ICPI value larger than 0.7 or carries a typical velogenic amino acid motif at the F protein cleavage site. Infections of poultry by strains with similar characteristics as AV324 will probably be dismissed if only an ICPI is performed. Our results underline the importance of sequencing viruses isolated from suspected infections to differentiate between virulent and low-virulent strains or to detect low-virulent strains that may potentially become virulent. These and other studies should shed light on the potential high risk for poultry represented by some NDV-infected non-poultry species

Future work will need to address the question which viral or cellular factors are responsible for the differences in pathogenicity of AV324 in pigeons and chickens. Using the available infectious AV324 and NDV cDNA clones we will initially focus on

viral genes that have previously been shown to contribute to pathogenicity of avian paramyxoviruses in chickens (V/P, L, HN). In addition, comparative analyses of virus growth in (primary) cells from chickens and pigeons might be performed focusing on aspects such as receptor binding, fusion activity, F protein cleavage, interferon antagonism and host range adaptation.

Acknowledgements

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Chapter 4

The viral replication complex is associated with virulence of Newcastle disease virus

J.C.F.M. Dortmans[1,2], P.J.M. Rottier[2], G. Koch[1] and B.P.H. Peeters[1]

[1]Central Veterinary Institute of Wageningen UR, Lelystad, The Netherlands.
[2]Virology Division, Department of Infectious Diseases & Immunology, Faculty of Veterinary Medicine, Utrecht University, The Netherlands.

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Summary

Virulent strains of Newcastle disease virus (NDV, also known as avian paramyxovirus type 1) can be discriminated from low-virulence strains by the presence of multiple basic amino acid residues at the proteolytic cleavage site of the fusion (F) protein. However, some NDV variants isolated from pigeons (pigeon paramyxovirus type 1; PPMV-1) have low levels of virulence, despite their F protein cleavage site containing a multibasic amino acid sequence and have the same functionality as that of virulent strains. To determine the molecular basis of this discrepancy, we examined the role of the internal proteins in NDV virulence. Using reverse genetics, the genes encoding the nucleoprotein (NP), phosphoprotein (P), matrix protein (M) and large polymerase protein (L) were exchanged between the nonvirulent PPMV-1 strain AV324 and the highly virulent NDV strain Herts. Recombinant viruses were evaluated for their pathogenicities and replication levels in day-old chickens, and viral genome replication and plaque size was examined in cell culture monolayers. We also tested the contribution of the individual NP, P and L proteins to the activity of the viral replication complex in an *in vitro* replication assay. The results showed that the replication proteins of Herts are more active than those of AV324 and that the activity of the viral replication complex is directly related to virulence. Although the M protein affected viral replication *in vitro*, it had only a minor effect on virulence.

Introduction

Newcastle disease is a severe infectious disease of birds caused by Newcastle disease virus (NDV), or avian paramyxovirus type 1 (APMV-1). NDV is classified in the genus *Avulavirus* of the family *Paramyxoviridae* (30) and has a single-stranded, negative-sense RNA genome consisting of six genes in the order 3'-NP-P-M-F-HN-L-5' (26) that encode at least seven proteins: the nucleocapsid protein (NP), the phosphoprotein (P), the matrix protein (M), the fusion protein (F), the hemagglutinin-neuraminidase (HN) and the polymerase (L) protein. During P gene transcription, an additional, non-structural protein (V) is produced by means of mRNA editing (53).

The M, F, and HN proteins are associated with the viral envelope. The F and HN proteins mediate entry and release, whereas the M protein is involved in morphogenesis and budding of NDV (26). The V protein is involved in interferon antagonism (40). The NP protein encapsidates the RNA genome to form the nucleocapsid which serves as the template for viral transcription and replication. The P protein is essential for viral RNA synthesis and has multiple roles (10, 12). It forms separate complexes with the NP and L proteins and the nucleocapsid (20). Transcription of the viral genomic RNA occurs by way of the viral polymerase (P-L complex) in which the catalytic activities of the polymerase are functions of the L protein, whereas the P protein is responsible for the binding of the P-L complex to the nucleocapsid. Once sufficient viral proteins are generated, NP starts to bind to the

leader chain, a process in which the P protein acts as a chaperone to deliver NP to the nascent RNA (11). The NP-P complex is believed to regulate the switch from transcription to replication (57), but several findings also show an important role for the M protein in this process. Because the M protein associates with the nucleocapsid (17, 27, 54) it may also affect transcription and/or replication (15, 17, 23, 36). The P-L complex is responsible for genome replication, i.e., the synthesis of full-length plus-strand antigenomic RNA, which in turn serves as the template for synthesis of minus-strand genomic RNA that is ultimately packaged into progeny virions. The L protein performs posttranscriptional modification activities such as capping, methylation, and polyadenylation of mRNAs (45, 51). The NP, P and L proteins together constitute the viral replication complex (26).

Based on the mean time to kill inoculated chicken embryos and their virulences for day-old chickens, NDV strains can be categorized into four pathotypes, i.e., non-virulent, lentogenic (low-virulent), mesogenic (intermediate) or velogenic (highly virulent) (3). Cleavage of the F protein is required for the initiation of infection and is the major virulence determinant. The cleavage site of the F protein of virulent NDV strains contains multiple basic amino acid residues and is recognized by ubiquitous intracellular furin-like proteases resulting in a systemic infection. The cleavage site of the F protein of low virulent strains does not contain these multiple basic amino acids and is recognized by extracellular trypsin-like proteases found in a limited number of tissues, predominantly in the respiratory and intestinal tracts (34, 35), thereby limiting replication of low-virulence strains to these tissues.

Pigeon paramyxovirus type 1 (PPMV-1) strains are variant strains of NDV associated with infections of pigeons. Some PPMV-1 strains behave as lentogenic viruses, i.e., they show a low intracerebral pathogenicity index (ICPI) in chickens, despite the presence of an F protein cleavage site motif that is generally associated with virulent viruses (32). In a previous study we showed that the exchange of the F gene between a low-virulence PPMV-1 virus and a highly virulent virus did not significantly affect virulence of the chimeric viruses relative to their respective parental viruses (chapter 3). Thus, the low virulence of some PPMV-1 strains must be determined by other factors. The V, HN and L proteins of NDV have all been shown to be involved in virulence of NDV (14, 21, 22, 31, 39, 40, 48, 49). However, little is still known about the mechanisms underlying their function as virulence determinants in NDV strains and especially in PPMV-1 strains. In this study, we examined the contribution of the NP, P, M and L proteins to the virulence of NDV. Using reverse genetics, the genes encoding these internal proteins were exchanged between the low-virulence PPMV-1 strain AV324 and the highly virulent NDV strain Herts. The pathogenicity and the level of *in vivo* replication of the chimeric viruses were determined in day-old chickens. Furthermore, we investigated the replication kinetics and the plaque size of the different chimeric viruses in cell culture monolayers, and we developed an *in vitro* replication assay using cotransfection of plasmids encoding a minigenome that expresses luciferase in the presence of the NP, P and L proteins. Our results indicate that the virulence of NDV is directly related to the activity of the viral replication complex.

Materials & Methods

Cells, viruses and animals

QM5 cells (4) were grown in QT35 medium (Gibco-BRL/LifeTechnologies) and DF-1 cells were grown in DMEM + glutaMAX (Invitrogen) in cell culture plates (Greiner Bio-One). Both media were supplemented with 5% fetal bovine serum and 1% of an antibiotic stock consisting of penicillin (100 units/ml), streptomycin (100 μg/ml) and fungizone (2.5 μg/ml). Both cell lines were grown at 37ºC in a 5% CO_2 incubator. The fowlpox recombinant virus fpE-FLT7pol (6) (hereafter called FPV-T7), which expresses the bacteriophage T7 RNA polymerase, was used as recently described (chapter 2). The cDNA clone rgAV324 was derived from the low-virulent PPMV-1 strain AV324/96 (chapter 3) and the cDNA clone FL-Herts was derived from virulent NDV strain Herts/33 as previously described (14). In this study specific-pathogen-free (SPF) chickens were used. Animal experiments were approved by the Ethics Committee for animal Experiments of the Central Veterinary Institute of Wageningen UR, and comply with Dutch law on animal experiments.

Construction of full-length chimeric AV324/Herts antigenomic cDNAs

Chimeric viruses in which either the individual NP, P, M or L genes, or combinations thereof, were exchanged between strains AV324 and Herts were generated (Fig. 4.1). The published nucleotide sequences of strains Herts/33 (GenBank accession no. AY741404) and AV324/96 (GenBank accession no. GQ429292) were used as guidance for the construction of the chimeric viruses.

In order to swap the replication genes between the plasmids FL-Herts and rgAV324, the unique restriction sites *Sfi*I, *Pac*I, *Asc*I, *Spe*I and *Sgf*I were used (Fig. 4.1). To introduce a unique restriction site for *Spe*I (position 8101) in the rgAV324^{AF} cDNA (chapter 3), site directed mutagenesis was performed resulting in rgAV324^{AFS}. The superscript “AFS” represents the introduced restriction sites *Asc*I, *Fse*I and *Spe*I. Plasmids FL-Herts^{AF} and rgAV324^{AFS} were digested with *Sgf*I and *Pac*I to simultaneously exchange the NP and P genes, resulting in rgAV324(NP-P’)^{Herts} and FL-Herts(NP-P’)^{AV324}. Because the *Pac*I site is positioned at nucleotide 2900 in Herts and 2906 in AV324, 57 amino acids of the C-terminus of the P proteins were not exchanged. Of these 57 amino acids, 7 differ between both viruses. To exchange the L gene, both full-length cDNA clones were digested with *Spe*I and *Sgf*I and reciprocally cloned resulting in rgAV324(L)^{Herts} and FL-Herts(L)^{AV324}. The *Pac*I and *Spe*I sites were used to simultaneously exchange the NP, P and L genes, resulting in rgAV324(NP-P’-L)^{Herts} and FL-Herts(NP-P’-L)^{AV324}. To exchange the NP and P genes individually, site directed mutagenesis was performed to introduce a unique *Sfi*I site in FL-Herts^{AF} (this site is present in AV324 but is lacking in Herts). To exchange the NP gene, the *Sgf*I and *Sfi*I sites were used, resulting in rgAV324(NP)^{Herts} and FL-Herts(NP)^{AV324}, and to exchange the P gene, the *Sfi*I and *Pac*I sites were used, resulting in rgAV324(P’)^{Herts} and FL-Herts(P’)^{AV324}. Finally, the M genes were exchanged using the *Pac*I and *Asc*I sites, resulting in rgAV324(M)^{Herts} and FL-Herts(M)^{AV324}.

Rescue of virus from cDNA

QM5 cells were infected with FPV-T7 for 1 h and subsequently cotransfected with full length cDNA constructs and helper plasmids expressing P and L as previously described (chapter 3). The respective helper plasmids of either rgAV324 or FL-Herts were used along with their respective full-length cDNAs to prevent potential heterologous recombination in the transfected cells. After three days, the culture supernatant was harvested and inoculated into 9-11-day-old embryonated SPF eggs to obtain a virus stock.

Pathogenicity test, hemagglutination inhibition (HI) assay and re-isolation of virus

The determination of the intracerebral pathogenicity index (ICPI) in one-day-old chickens and the HI assay were performed as described in the European Community Council Directive 92/66/EEC (8). Sera of the chickens that survived the ICPI test were tested in a HI assay to verify that the animals had been infected. In order to check the sequences of the different recombinant viruses after the ICPI tests, brains, livers and lungs of dead chickens were collected and virus re-isolation on embryonated SPF eggs was performed as previously described (13).

RNA isolation, RT-PCR and sequencing

RNA of the recombinant viruses was isolated using a High Pure Viral RNA kit (Roche Diagnostics). First-strand DNA synthesis was carried out using a Superscript^{TM} III Reverse Transcriptase kit (Invitrogen) and PCR fragments were purified using a High Pure PCR purification kit (Roche Diagnostics). Nucleotide sequencing (primer sequences are available upon request) was carried out using a BigDye Terminator v1.1 cycle sequencing kit and a 3130 genetic analyzer (Applied Biosystems).

Virus quantitation in organs of infected day-old chickens

To compare the replication kinetics of the NP-P'-L-chimeric and parental viruses in chickens, 15 one-day-old SPF chicks were intracerebrally inoculated with $2x10^3$ $TCID_{50}$ virus per chicken. Three birds were sacrificed daily until 5 days post infection. Brain, spleen, liver and lungs were collected and homogenized in PBS, and the virus titres were determined on QM5 cells using 10-fold serial dilutions of the cleared homogenates. Mean virus titres were calculated using the Reed and Muench method (46) and are expressed as $log_{10}TCID_{50}$ per gram tissue. The dotted line in Fig. 4.2 indicates the virus detection limit in QM5 cells. Because undiluted homogenized tissue samples were toxic for the cells, these "QM5-negative" samples were tested for the presence of virus by inoculation of undiluted tissue homogenates in embryonated SPF eggs. Open symbols indicate that inoculated eggs remained virus negative, whereas closed symbols indicate that inoculated eggs had become virus positive (Fig. 4.2).

For statistical analysis a non-parametric approach based on rank numbers was used, because numbers of animals are modest and some observations fall below the detection limit. Tests over times were performed and the group infected with virus A

was compared with the group infected with virus B, employing Wilcoxon's rank sum test (the Mann-Whitney test) (9). Per time point, observations were replaced by ranks. The sum of the ranks over time for the group infected with virus A was used as a test statistic, large or small values being critical. The distribution under the null hypothesis of no difference between both infected groups of the test statistic was obtained by simulation, by randomly reshuffling the data over the groups. A 0.05 significance level was used. All calculations were performed with GenStat (42).

Replication assay

A synthetic DNA containing the T7 RNA polymerase promoter, the Gaussia luciferase (GLuc) gene (in an anti-sense orientation) flanked at the 5' side by the trailer region (AV324: nt 14999-15192 or Herts: nt 14993-15186) and at the 3' side by the leader region (AV324 or Herts: nt 1-121), followed by the Hepatitis Delta virus ribozyme and the transcription termination signal from bacteriophage T7 was synthesized (GenScript Corporation, USA) and cloned between the *Lgu*I and *Bam*HI sites of transcription plasmid pOLTV5 (43) resulting in the minigenome plasmids designated pAV324-GLuc and pHerts-GLuc. The size of the minigenomes complied to the rule-of-six (44). Transcription, using T7 RNA polymerase, of plasmid pAV324-GLuc or pHerts-GLuc gives rise to genomic (negative-sense) RNA as has been described previously (44).

QM5 cells were infected at an m.o.i. of 1 with FPV-T7 for 1 h and subsequently cotransfected with either pAV324-GLuc or pHerts-GLuc and helper plasmids expressing NP, P and L originating either from the AV324 strain (chapter 3) or from the Herts strain (14). The minigenome and its expression plasmids containing NP, P and L were cotransfected at a ratio of: 1.0:1.6:0.8:0.8, respectively, by using FuGENE HD (Roche). For normalization, a plasmid containing the firefly luciferase gene under control of the human cytomegalovirus (hCMV) immediate-early promoter was cotransfected (a kind gift of Erik de Vries and Xander de Haan, Faculty of Veterinary Medicine, Utrecht, The Netherlands). After 24 h the expression levels of the secreted (Gaussia) and internal (Firefly) luciferase activities were measured using a luciferase assay kit (Promega) and a GloMax luminometer (Promega). One experiment comprises a triplicate measurement of the luciferase expression. In total six separate experiments were performed. Differences in luciferase expression were statistically analyzed using the Wilcoxon test. Mean differences were considered significant when the *p* value was less than 0.05.

Analysis of genome replication by quantitation of negative-sense genomic RNA

To investigate the onset and kinetics of virus replication, the relative amount of negative-sense genomic RNA was determined by quantitative real-time RT-PCR (qRRT-PCR) at different time-points post infection (pi). To set up a qRRT-PCR the sequences of Herts and AV324 were aligned using the web-based software of EMBL-EBI ClustalW2 (http://www.ebi.ac.uk/Tools/clustalw2/index.html). A PCR primer pair was selected in a homologous region of the L genes, such that the primers had no mismatches in either virus. The sequence of the forward primer is 5'-

CCCGACCGACTGTGATCTAT-3' and of the reverse primer is 5'-GCAGCAAGTTGGATTGCAG-3'. Subsequently, a perfectly matching probe within the same region was chosen and labeled at the 5'end with FAM and at the 3'end with BHQ1: 5'-FAM-TGCTAGAGGGGGCATTGAGGGA-BHQ1-3' (TIB MOBIOL).

DF-1 cells were seeded in 24-well plates (Greiner Bio-One) and infected in duplicate with virus at an m.o.i. of 10. At 2, 4, 6, 8, and 10 h pi plates were frozen at -70ºC. RNA was extracted from the combined infected cells and supernatant using the MagNA Pure LC Total Nucleic Acid Isolation Kit and the MagNA Pure LC Instrument (Roche Applied Science) according to the manufacturer's instructions. Cycling conditions for the qRRT-PCR using the MX3005P (Stratagene) were one cycle at 50ºC for 30 min, one cycle at 95ºC for 15 min, 45 cycles at 95ºC for 15 s, 55ºC for 30 s and 72ºC for 30 s. For data analysis, the MxPro QPCR software version 4.10 (Stratagene) was used. The correlation coefficients (R^2) of the standard curves were 0.999 (FL-Herts) and 0.993 (rgAV324), respectively. All PCR amplification efficiencies (E) were >0.95. Details of the PCR protocol are available upon request.

Determination of plaque size

Monolayers of QM5 cells and DF-1 cells were infected with the parental and chimeric viruses and incubated for 2 days under an overlay of Glasgow modification of Eagle medium/Eagle's minimal essential medium (ASG-Lelystad) containing 1% methylcellulose, without the addition of exogenous trypsin. Plaques were visualized by immunological staining using monoclonal antibody (mAb) Fusie 133 8E12A8C3 (CVI of Wageningen UR) against the NDV F protein and HRPO-conjugated polyclonal rabbit anti-mouse Ig (Dako). The mean plaque size was determined by measuring the area of digital images of 12-16 discrete plaques per virus (photographed at a magnification of 6.3) using the Image-Pro Plus software (Media Cybernetics, Inc.). Differences in plaque size were statistically analyzed using the Wilcoxon test. Mean differences were considered significant when the *p* value was less than 0.05.

Results

Construction and recovery of recombinant viruses

To study the molecular basis of the low pathogenicities of PPMV-1 strains that contain a fully functional multiple basic amino acid motif in their F protein cleavage site, we exchanged the genes encoding the internal viral proteins NP, P, M and L between the nonvirulent PPMV-1 strain AV324 and the highly virulent NDV strain Herts. The amino acid sequence identity of these proteins is 94% for NP, 84% for P, 93% for M and 95% for L. The cloning strategy that was used to construct the chimeric cDNAs is described in the Materials and Methods section and is illustrated in Fig. 4.1. Sequence analysis of the chimeric cDNAs confirmed the intended gene exchanges and the absence of any undesired mutations. For virus recovery by means of cotransfection, helper plasmids expressing P and L of either rgAV324 or FL-Herts were used along with their respective

full-length cDNA. All chimeric viruses could be rescued, indicating that the biological functions of these proteins of strains Herts and AV324 are compatible.

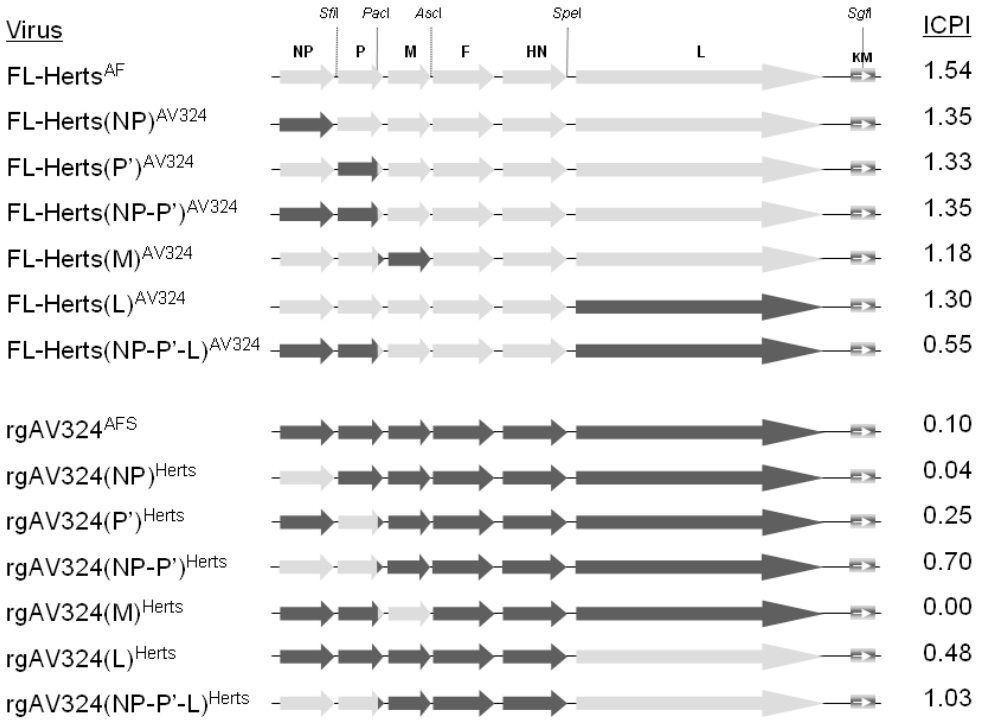

Fig. 4.1. Schematic illustration of the cloning strategy used to exchange the NP, P, M and L genes between FL-Herts^{AF} and rgAV324^{AFS}. The virulence of the different viruses was determined by measuring the intracerebral pathogenicity index in day-old chickens (ICPI score: max 2.0). KM, kanamycine resistance gene positioned in the vector plasmid.

Pathogenicity in day-old chickens

The virulences of the chimeric viruses was assessed by a standard intracerebral pathogenicity test (ICPI) in 1-day-old chickens. Replacement of the internal genes of the Herts strain, either individually or in combination, by those of the AV324 strain resulted in a decrease in virulence. Especially the simultaneous replacement of the genes encoding the complete NP-P'-L replication complex had a significant effect on virulence as shown by a decrease in ICPI value from 1.54 for FL-Herts to 0.55 for FL-Herts(NP-P'-L)^{AV324} (Fig. 4.1). By contrast, although not all replacements of the internal genes of AV324 by those of Herts resulted in an increase in virulence, again the simultaneous replacement of the NP, P and L genes had a large effect. The exchange

resulted in a significant increase in ICPI value from 0.10 for rgAV324 to 1.03 for rgAV324(NP-P'-L)^{Herts} (Fig. 4.1). These results show that the origin of the complete replication complex from either AV324 or Herts has a major effect on determining the virulence of the chimeric viruses.

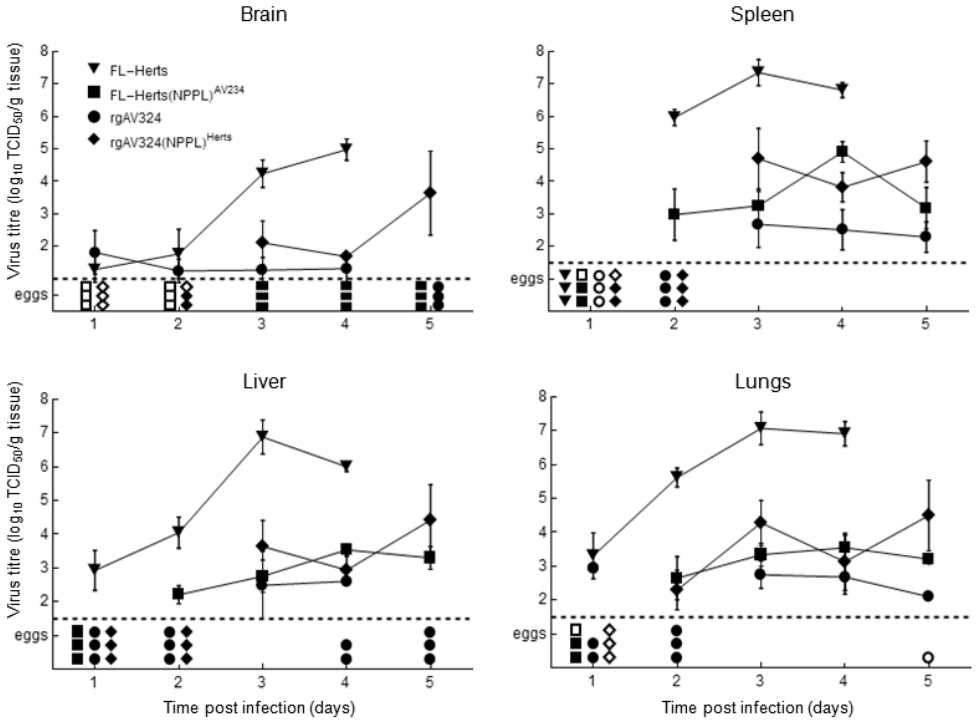

Fig. 4.2. Viral titres of parental and NP-P'-L chimeric viruses in different organs after intracerebral inoculation of one-day-old chickens. Each day three chickens were sacrificed and brain, spleen, liver and lungs were collected. Virus titres were determined using QM5 cells and are presented as the mean virus titre ($log_{10}TCID_{50}$/g tissue). The dotted line indicates the detection limit of the virus detection assay in QM5 cells. "QM5-negative" samples were additionally tested for the presence of virus by inoculating embryonated eggs. Open symbols represent that inoculated eggs remained virus negative, whereas closed symbols indicate that inoculated eggs had become virus positive. Because three FL-Herts infected chickens died 3 days post infection, there are only four time points for this group. Error bars show standard deviations.

In vivo replication in day-old chickens

Because exchanging the complete viral replication complex had the largest effect on virulence, the levels of *in vivo* replication of these chimeric viruses and their parental viruses were determined in day-old chickens after intracerebral inoculation (Fig. 4.2). The results showed that the AV324 strain replicated to much lower levels than strain Herts in all organs examined. In brain tissue no significant difference in replication of the AV324 recombinant that expresses the replication complex of strain Herts (rgAV324(NP-P'-L)^{Herts}) and the parental AV324 strain was observed ($p > 0.05$). However, in liver, lungs and spleen, replication of rgAV324(NP-P'-L)^{Herts} was significantly enhanced compared to rgAV324. Conversely, providing the Herts virus with the AV324 replication complex (FL-Herts(NP-P'-L)^{AV324}), significantly reduced the replicative abilities of the chimeric virus in all tissues examined (Fig. 4.2). These results strongly suggest that the pathogenicity of avian paramyxoviruses is directly correlated with the level of virus replication in organs of infected animals.

In vitro replication assay

To test whether *in vivo* virus replication correlated with the intrinsic activity of the viral replication complex, we developed an *in vitro* replication assay using a minigenome that expresses the Gaussia luciferase (GLuc) gene in the presence of the NP, P and L proteins. Two slightly different minigenomes were used, one based on the leader and trailer sequences of the Herts strain (pHerts-GLuc) and the other on the leader and trailer sequences of the AV324 strain (pAV324-GLuc). Transcription of pAV324-GLuc or pHerts-GLuc using T7 RNA polymerase generates genomic (negative-sense) minigenome RNA. Hence, expression of the GLuc gene is dependent on conversion of the negative-sense RNA into positive-sense RNA. This process is completely dependent on the viral NP, P and L proteins, providing a suitable assay to test the roles of the individual proteins in minigenome replication activity.

The *in vitro* replication assay revealed that the two minigenomes behaved similarly, exhibiting comparable levels of reporter gene expression both when driven by the AV324 replication proteins, and by those of the Herts virus (data not shown). This indicates that the nucleotide differences in the leader and trailer sequences that code for the genomic (3 nt difference) and antigenomic (4 nt difference) promoter (29) of AV324 and Herts did not affect the efficiency of viral replication. Therefore, only the results obtained with the pHerts-GLuc minigenome are shown. The results revealed that the activity of the AV324 replication complex is significantly lower than that of the Herts replication complex (Fig. 4.3). Furthermore, none of the possible combinations of the NP, P and L proteins of AV324 and Herts reached the 100% level obtained with the Herts replication proteins only. These observations suggest that optimal replication is not determined by one or two proteins but by the combined action of all three replication proteins.

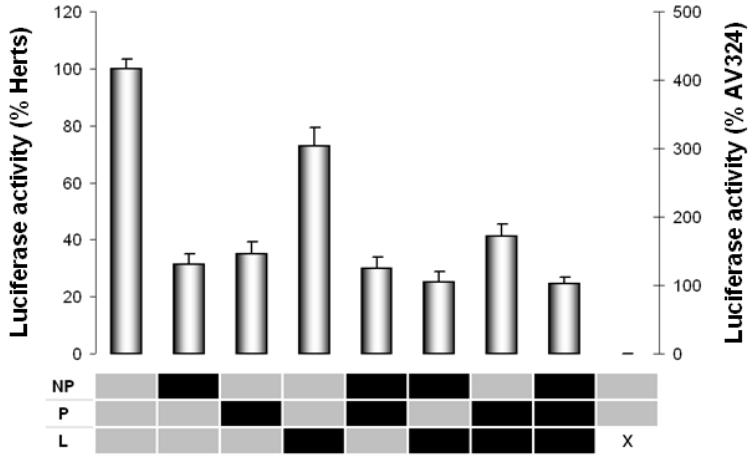

Fig. 4.3. Relative luciferase expression levels after co-transfection of FPV-T7 infected QM5 cells with the viral minigenome plasmid (containing the leader and trailer sequences of Herts flanking the Gluc reporter gene) and plasmids expressing NP, P and L of Herts (grey) or AV324 (black). The background level of luciferase activity was determined by omitting the L plasmid (x). The results show the mean values obtained in six separate experiments. On the left y-axis the luciferase activity relative to Herts are given and on the right y-axis the luciferase activity relative to AV324. Error bars represents SEM.

Viral genome replication

To investigate the onset and kinetics of viral genome replication of the recombinant viruses in cell culture monolayers, a qRRT-PCR that specifically detects negative-strand viral RNA was used. To this end, DF-1 cells were infected at an m.o.i. of 10 and at different time-points up till 10 hpi the amount of genomic RNA was determined. The results showed that the genome of FL-Herts replicated at a higher rate than that of rgAV324 (Fig. 4.4). When the Herts virus was provided with the AV324 replication complex the replication kinetics were similar to those of rgAV324. Unexpectedly, however, the recombinant virus in which the replication genes of AV324 were replaced by those of Herts (rgAV324(NP-P'-L)^{Herts}) showed the lowest replication efficiency of all viruses tested. Because several studies have shown that the M protein may affect transcription and/or replication, we also examined the recombinant viruses in which only the M gene was exchanged. The results showed that the kinetics of FL-Herts(M)^{AV324} were very similar to those of rgAV324(NP-P'-L)^{Herts} (Fig. 4.4), suggesting that the specific combination of the M protein of AV324 and the NP-P'-L complex of Herts results in a decreased replication rate. This effect also seems to affect virulence since the introduction of the AV324 M protein in Herts resulted in a reduction of the ICPI value from 1.54 to 1.18 (Fig. 4.1). The kinetics of genome replication of rgAV324 was not increased by the M protein of Herts and also no significant effect on virulence was noted (cf. rgAV324 vs rgAV324(M)^{Herts}), indicating that the effect is non reciprocal.

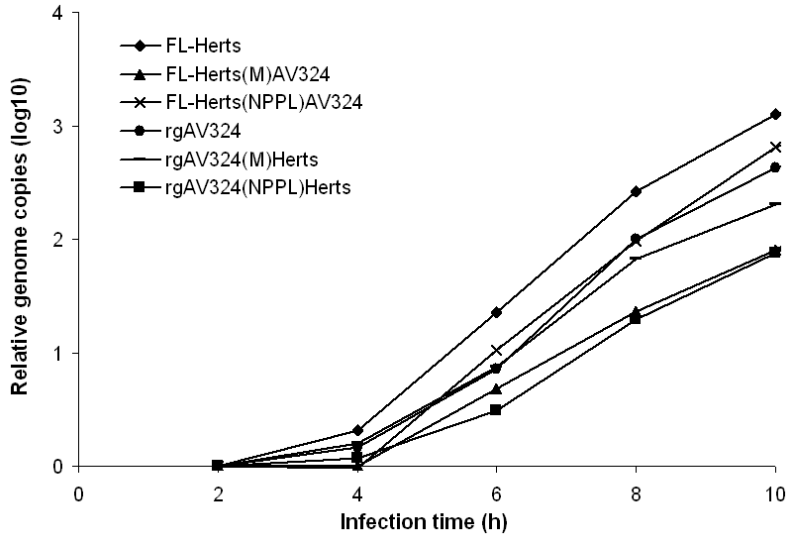

Fig. 4.4. Onset and kinetics of negative-sense genomic RNA replication in the first 10 hours of infection. DF-1 cells were infected at an m.o.i. of 10 and replication was determined by qRRT-PCR. Results are the mean of duplicate infections.

Plaque size

In order to examine the replication of the different viruses in tissue culture cells in an other way, we determined their plaque sizes in monolayers of quail-derived QM5 cells and chicken-derived DF-1 cells at 48 hpi (Fig. 4.5). Relative to FL-Herts, rgAV324 showed a significantly smaller plaque size in QM5 and in DF-1 cells. Furthermore, smaller plaque sizes in both cell types were also observed when the Herts virus was provided with the M protein of AV324 or the replication complex of AV324. When the AV324 virus expressed the M protein or the replication complex of Herts, QM5 cells and DF-1 cells showed contrasting results with regard to plaque size. While the plaque sizes were smaller in QM5 cells, larger plaques were observed in DF-1 cells (Fig. 4.5).

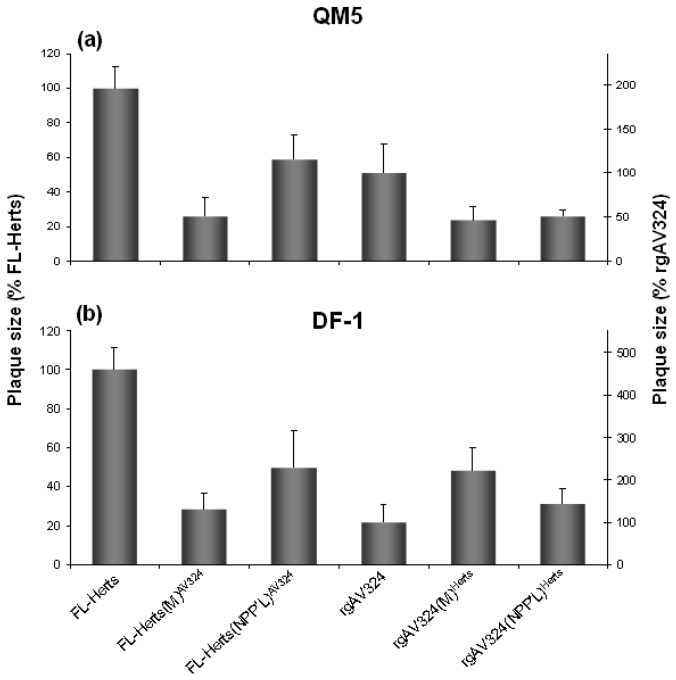

Fig. 4.5. Relative plaque sizes of the recombinant viruses in QM5 (a) and DF-1 (b) cells 48 hours post infection. Plaques were visualized by immunological staining using a mAb against the NDV F protein. The mean plaque size was determined by measuring the area of at least 12 plaques per virus with the Image-Pro Plus software (Media Cybernetics, Inc.). On the left y-axis the plaque size relative to FL-Herts are given and on the right y-axis the plaque size relative to rgAV324. Error bars represent standard deviations.

Discussion

The results of this study show that all three proteins that make up the viral replication complex (NP, P and L) play a significant role in determining the virulence of NDV. By exchanging the replication genes simultaneously, the virulent Herts virus was significantly attenuated, whereas the low virulent AV324 became much more virulent. All individual replication proteins have their own contribution, but act synergistically when exchanged all three together. The matrix (M) protein of AV324 showed a distinct effect on virulence in the Herts background, probably by interacting with the viral replication complex. However, this effect was not reciprocal since the M protein of Herts lacked the ability to increase the virulence of AV324.

The difference in virulence between AV324 and Herts seems to be directly related to the efficiency of *in vivo* viral replication. After exchanging the genes encoding the entire NP-P-L replication complex, the virus titres of the virulent strain Herts in day-old

chickens were decreased in all organs examined, whereas those of the avirulent strain AV324 were increased in three of the four organs tested (Fig. 4.2). Lentogenic NDV strains are generally unable to spread systemically when inoculated intracerebrally. Because these viruses lack the multibasic cleavage motif in their F protein, activation requires trypsin-like proteases which are apparently not present in neuronal tissue. Furthermore, replication of these viruses is often limited to the inoculation site (37, 49, 58). The titre of the AV324 strain in brain tissue gradually decreased over time, but the virus was still able to spread to secondary organs (Fig. 4.2). However, this systemic infection did not sicken its host drastically as evidenced by the virus' ICPI of 0.10 (Fig. 4.1) and the absence of clinical signs (data not shown). Thus, low virulent PPMV-1 isolates may be perfectly adapted to their hosts by being able to spread systemically due to their typical velogenic F cleavage site motif, while replicating at a relatively low level.

The differences in efficiency of the viral replication complexes were confirmed in an *in vitro* replication assay (Fig. 4.3). Optimal replication was observed when all three replication proteins originated from the Herts strain. This matches the results of the ICPI tests (Fig. 4.1). However, none of the possible combinations of the NP, P and L proteins of AV324 and Herts reached the level of replication obtained with Herts replication proteins only. One of the possible explanations may be that the individual Herts replication proteins are inherently more active but that optimal activity is dependent on the presence of the cognate interaction partners. The amino acid sequence of the protein domains responsible for the interaction of the NP and P proteins (24) differ between Herts and AV324. Furthermore, although not well identified for NDV, the interaction domains responsible for the P-L interaction presumably also differ between both viruses, as they have been shown to differ among several other paramyxoviruses (18, 20, 25, 41, 56). Since these complexes are essential for transcription and replication of the viral genome (26) they will probably not function optimally in a heterologous constitution.

The discrepancy between the *in vivo* results (Fig. 4.1 and 4.2) and the *in vitro* results (Fig. 4.4 and 4.5) might be explained by the role of the M protein. While the M protein is considered to be the central organizer of viral morphogenesis (26), in addition, several studies have shown its involvement in regulating viral RNA synthesis (17, 23, 47). With NP as its most likely binding partner (23, 38), the M protein associates with the nucleocapsid (17, 27, 54). Upon viral entry of the target cell the nucleocapsid dissociates from the M protein and is released into the cytoplasm where transcription can occur. The interaction of the M with the nucleocapsid might affect transcription and have a subsequently effect on replication. Furthermore, this interaction may be strain specific and non-reciprocal, and might explain the relative low replication rate (Fig. 4.4) and small plaque size (Fig. 4.5) of rgAV324(NP-P'-L)^{Herts} since similar results were found for FL-Herts(M)^{AV324}. Another possible explanation for our results is that the association of the M protein with host cell factors might differ between both strains. During the infection cycle the M protein of NDV and other paramyxoviruses is trafficking between the cytoplasm and the nucleus (19). Early during infection the M resides primarily in the nucleus, while later during infection M is

localized mainly in the cytoplasm (7, 19). It has been suggested that the function of M being in the nucleus relates to inhibiting host cell functions (1, 16), although this is not yet confirmed for NDV. The nuclear localization signals (7) and the proposed viral late-domain core sequence, ^{24}FPIV^{27}, necessary for budding (50) do not differ between Herts and AV324 M proteins.

The involvement of the NP, P and L proteins in NDV virulence has been examined before (49). In that study, chimeric viruses were generated by exchanging genes between the lentogenic strain LaSota and the mesogenic strain Beaudette C, both classified as members of lineage 2 or genotype II in the avian paramyxovirus type 1 group (2). Surprisingly, a recombinant Beaudette C virus that contained the L gene of LaSota replicated at a higher level and was more virulent than its parental virus. However, no effect was found for the NP and P proteins. These results differ from our findings which show that all three replication proteins are associated with virulence. An explanation for this difference might be that LaSota and Beaudette C belong to the same phylogenetic lineage and genotype, whereas the strains used in this study belong to different lineages and genotypes; Herts is classified as a member of lineage 3b (or genotype IV) whereas AV324 belongs to lineage 4b (or genotype VI) (2). Furthermore, Herts is a chicken derived strain, whereas AV324 is of pigeon origin.

The molecular mechanism for the relation between the level of replication of a virus and its pathogenesis is not fully understood. It is conceivable that higher levels of RNA synthesis lead to higher levels of viral replication and thus to more virus production. This may overwhelm the host immune response hence causing enhanced pathogenesis. A correlation between virulence and the efficiency of viral replication has been observed before. It has, for instance, been reported that reduced levels of RNA synthesis are associated with reduced virulence of NDV (28). For several other paramyxoviruses such as measles virus (5, 55), respiratory syncytial virus and parainfluenza virus (33, 52) it has been described that determinants of virus attenuation are associated with mutations in the P and L genes.

Due to the use of the *Pac*I cloning site, the exchange of the P genes actually resulted in the exchange of chimeric P proteins, in which 7 of the C-terminal 57 amino acids that differ between the P proteins of Herts and AV324 are still similar to those of the backbone strain. Exchanging the chimeric P genes had only a limited effect on virulence (ICPI=1.33 for FL-Herts(P')^{AV324} and ICPI=0.25 for rgAV324(P')^{Herts}). However, we cannot completely exclude the possibility that exchanging the complete P protein may have had a different effect on its function and virulence.

In conclusion, this study shows that, in addition to the F, V, HN and L proteins also the complex of the NP, P and L proteins contribute to virulence of NDV. Additional studies will be required to elucidate whether the proteins have an effect on viral transcription, replication or both. Altogether, these observations illustrate that virulence of NDV is a complex trait determined by multiple genetic factors. Furthermore, the degree to which these factors are involved in NDV virulence seems to be strain and cell-type dependent.

Acknowledgements

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Chapter 5

Passaging of a Newcastle disease virus pigeon variant in chickens results in selection of viruses with mutations in the polymerase complex enhancing virus replication and virulence

J.C.F.M. Dortmans[1,2], P.J.M. Rottier[2], G. Koch[1] and B.P.H. Peeters[1]

[1]Central Veterinary Institute of Wageningen UR, Lelystad, The Netherlands.
[2]Virology Division, Department of Infectious Diseases & Immunology, Faculty of Veterinary Medicine, Utrecht University, The Netherlands.

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Summary

Some Newcastle disease virus (NDV) variants isolated from pigeons (pigeon paramyxovirus type 1; PPMV-1) do not show their full virulence potential for domestic chickens but may become virulent upon spread in these animals. In this study we examined the molecular changes responsible for this gain of virulence by passaging a low-pathogenic PPMV-1 isolate in chickens. Complete genome sequencing of virus obtained after 1, 3 and 5 passages showed the increase in virulence not to be accompanied by changes in the fusion protein - a well known virulence determinant of NDV - but by mutations in the L and P replication proteins. The effect of these mutations on virulence was confirmed by means of reverse genetics using an infectious cDNA clone. Acquisition of three amino acid mutations, two in the L protein and one in the P protein, significantly increased virulence as determined by intracerebral pathogenicity tests in day-old chickens. The mutations enhanced virus replication *in vitro* and *in vivo* and increased the plaque size in infected cell culture monolayers. Furthermore, they increased the activity of the viral replication complex as determined by an *in vitro* minigenome replication assay. Our data demonstrate that PPMV-1 replication in chickens results in mutations in the polymerase complex rather than the viral fusion protein, and that the virulence level of pigeon paramyxoviruses is directly related to the activity of the viral replication complex.

Introduction

Newcastle disease virus (NDV), or avian paramyxovirus type 1 (APMV-1), is an economically important disease of birds. Periodic outbreaks of Newcastle disease severely affect the poultry industry and, therefore, many countries rely on compulsory vaccination. NDV is classified in the genus *Avulavirus* of the family *Paramyxoviridae* (31) and has a non-segmented negative-strand RNA genome consisting of six transcriptional units (29). These encode at least six proteins: the nucleocapsid protein (NP), the phosphoprotein (P), the matrix protein (M), the fusion protein (F), the hemagglutinin-neuraminidase (HN) and the polymerase (L) protein. During P gene transcription an additional, non-structural protein (V) is produced by means of mRNA editing (46) and functions as an interferon antagonist (37). The M, F, and HN proteins are associated with the viral envelope, in which the M protein is involved in budding and morphogenesis, whereas F and HN mediate the entry and release of NDV. The virulence of NDV is mainly determined by the amino acid sequence of the protease cleavage site of the F protein. Virulent NDV strains can be discriminated from low- or non-virulent strains by the presence of multiple basic amino acids at the proteolytic cleavage site of the F protein (35, 36). The NP protein encapsidates the RNA genome to form the nucleocapsid and associates with the P and L proteins. The P protein is essential for viral RNA synthesis and is involved in all of its aspects. The L protein is an RNA-dependent RNA polymerase that associates with the NP and P proteins, together

constituting the viral replication complex (29). This complex is responsible for transcription and replication of the viral genome.

Pigeon paramyxovirus type 1 (PPMV-1) viruses are variant strains of NDV associated with infections of pigeons and have a worldwide distribution (6). Several Newcastle disease outbreaks in chickens have been attributed to PPMV-1, which makes it a real threat to the poultry industry (2, 3, 7, 8, 23, 30, 49). The F proteins of all PPMV-1 strains examined to date contain a poly-basic cleavage site motif, a feature of NDV generally associated with high virulence. However, some PPMV-1 strains cause only minimal disease and have a low intracerebral pathogenicity index (ICPI) in chickens (32). Nevertheless, they do have a virulence potential for chickens, which can emerge upon serial passages in these animals (4, 11, 27, 28). Sequence analysis of such passaged viruses has mainly focused on the F gene, and it was concluded that the increase in virulence was not associated with changes in the F protein sequence (11, 12, 28). This is in agreement with our own observation that replacement of the F gene of a virulent NDV strain by that of a non-virulent PPMV-1 strain resulted in a virulent chimeric virus, indicating that the non-virulent phenotype of the PPMV-1 strain is not caused by the F protein (chapter 3).

By exchanging genes between a low virulent PPMV-1 strain and a highly virulent NDV strain, we recently showed that virulence of NDV (and PPMV-1) is associated with the activity of the viral replication proteins (chapter 4). Consistently, the increase in virulence observed during passaging of PPMV-1 in chickens might also be caused by changes in these proteins. To test this hypothesis, we passaged the low virulent PPMV-1 strain AV324 in chickens. We indeed observed an increase in virulence and here we show this to be due to the accumulation of mutations in the P and L proteins. These mutations resulted in more efficient virus replication both *in vitro* and *in vivo,* indicating that virulence of PPMV-1 for chickens is directly related to the efficiency of virus replication.

Materials & Methods

Cells, viruses and animals

Quail fibrosarcoma cells (QM5) were grown in Fort Dodge QT35 medium (Gibco-BRL/LifeTechnologies) and chicken fibroblast cells (DF-1) were grown in DMEM + glutaMAX (Invitrogen) using cell culture plates (Greiner Bio-One). Both media were supplemented with 5% fetal bovine serum and 1% of an antibiotic stock consisting of penicillin (100 units/ml) and streptomycin (100 μg/ml).

The PPMV-1 isolate AV324/96 was obtained from the Veterinary Laboratories Agency (Addlestone, Surrey, UK) and was passaged once in embryonated eggs to obtain a virus stock. The cDNA clone of AV324/96 (designated rgAV324) and of strain Herts/33 (designated FL-Herts), and their respective helper plasmids expressing NP, P and L have been described previously (16, chapter 3). The fowlpox recombinant virus fpE-FLT7pol (10) (hereafter called FPV-T7), which expresses the bacteriophage T7 RNA polymerase, was used as recently described (chapter 2).

In this study specific-pathogen-free (SPF) chickens were used. Animal experiments were approved by the Ethics Committee for Animal Experiments of the Central Veterinary Institute of Wageningen UR, and comply with the Dutch law on animal experiments.

Pathogenicity tests and chicken passage

The intracerebral pathogenicity test (ICPI) in one-day-old chickens was performed as described in the European Community Council Directive 92/66/EEC (13). Of the chickens that survived the ICPI test, sera were tested in a hemagglutination inhibition assay (13) to verify that the animals had become infected. In order to check the sequences of the different recombinant viruses after the ICPI tests, brains, livers and lungs of dead chickens were collected and virus re-isolation using embryonated SPF eggs was performed as previously described (15).

The PPMV-1 isolate AV324/96 (chapter 3) is low virulent for chickens (ICPI = 0.44), but contains a multibasic F protein cleavage site motif that is typically associated with virulent NDV viruses. Five serial passages were performed by inoculating one-day-old chickens intracerebrally, as described for the ICPI test (13). Brain tissue from dead chickens was collected and virus was re-isolated after inoculation of embryonated SPF eggs. This virus was subsequently used as the inoculum for the next passage.

Sequencing and generation of recombinant viruses

Viral RNA was isolated from virus recovered after passages 1, 3 and 5 using a High Pure Viral RNA kit (Roche Diagnostics). First-strand cDNA synthesis was carried out using a Superscript^{TM} III Reverse Transcriptase kit (Invitrogen) and PCR fragments were purified using a High Pure PCR purification kit (Roche Diagnostics). Nucleotide sequencing (primer sequences are available on request) was carried out using a BigDye Terminator v1.1 cycle sequencing kit (Applied Biosystems) and a 3130 genetic analyzer (Applied Biosystems). The complete genomes were compared with the consensus sequence of AV324 (GenBank accession no. GQ429292).

The mutations found in the passaged viruses were introduced into the full-length cDNA copy rgAV324 (chapter 3) by site directed mutagenesis using PCR, resulting in rgAV324-$L^{N1564S}L^{V1694E}$ (rgAV324-LL) and rgAV324-$P^{N37D}L^{N1564S}L^{V1694E}$ (rgAV324-PLL). The three mutations were also introduced into the FL-Herts(NP-P'-L)^{AV324} cDNA (chapter 4), resulting in FL-Herts(NP-P'-L)^{AV324}-$P^{N37D}L^{N1564S}L^{V1694E}$ (FL-Herts(NPP'L)^{AV}-PLL) and in the corresponding expression plasmids resulting in pCI-P^{N37D} and pCI-$L^{N1564S}L^{V1694E}$. All PCR-derived DNA fragments were verified by sequencing.

Recombinant virus was recovered from cDNA after cotransfection of QM5 cells as previously described (chapter 3). The respective expression plasmids of either rgAV324 or FL-Herts (16) were used along with their respective full-length cDNAs. Three days after transfection, the culture supernatant was harvested and inoculated into 9-11-day-old embryonated SPF eggs to obtain a virus stock.

Determination of plaque size

Monolayers of QM5 cells and DF-1 cells were infected with the parental and mutant viruses and incubated for 2 days under an overlay of Glasgow modification of Eagle medium/Eagle's minimal essential medium (ASG-Lelystad) containing 1% methylcellulose. Plaques were visualized by immunological staining using monoclonal antibody (mAb) Fusie 133 8E12A8C3 (CVI of Wageningen UR) against the NDV F protein and HRPO-conjugated polyclonal rabbit anti-mouse Ig (Dako). The mean plaque size was determined by measuring the surface area using digital images of 10-15 discrete plaques per virus (photographed at a magnification of 6.3) using the Image-Pro Plus software (Media Cybernetics, Inc.). Differences in plaque size were statistically analyzed using the Wilcoxon test. Mean differences were considered significant when the *p* value was less than 0.05.

In vitro replication assay

To test the activity of the viral replication complex, an *in vitro* replication assay was performed as previously described (chapter 4). Briefly, QM5 cells were infected at an m.o.i. of 1 with FPV-T7 for 1 h. Subsequently, the minigenome pAV324-GLuc and helper plasmids expressing NP, P and L originating either from the AV324 strain (chapter 3) or from the Herts strain (16) were cotransfected. After 24 h the expression levels of the secreted luciferase were measured using a luciferase assay kit (Promega) and a GloMax luminometer (Promega). The replication assays were performed in triplicate. The mean of three separate experiments was determined. Differences in luciferase expression were statistically analyzed using the Wilcoxon test and considered significant when the *p* value was less than 0.05.

Analysis of genome replication by quantitation of negative-sense genomic RNA

To investigate the onset of virus replication, the relative amount of negative-sense genomic RNA was determined by quantitative real-time RT-PCR (qRRT-PCR) at different time-points post infection (pi). The qRRT-PCR targets a 129-nucleotide fragment in the L gene, as previously described (chapter 4). QM5 cells were seeded in 12-well plates (Greiner Bio-One) and infected in duplicate with virus at an m.o.i. of 10. At 2, 4, 6, 8, 10, 12, 14 and 16 hours pi plates were frozen at -70ºC. RNA was extracted from the combined infected cells and supernatant, using the MagNA Pure LC Total Nucleic Acid Isolation Kit and the MagNA Pure LC Instrument (Roche Applied Science) according to the manufacturer's instructions. Details of the PCR protocol are available on request.

Viral titres in one-day-old chickens

To compare the *in vivo* replication properties of rgAV324, rgAV324-LL and rgAV324-PLL, one-day-old SPF chickens were inoculated intracerebrally with $2x10^3$ $TCID_{50}$ of virus per animal. Three birds were sacrificed daily until 5 days post infection. Brain, spleen, liver and lungs were collected and the virus titres were determined on QM5

cells using 10-fold serial dilutions of cleared tissue homogenates in PBS. Mean virus titres were calculated using the Reed and Muench method (40) and are expressed as $\log_{10}TCID_{50}$ per gram tissue. Undiluted homogenized tissue samples could not be used because these samples were toxic for the cells. Therefore "QM5-negative" samples were additionally tested for the presence of virus by inoculation of undiluted tissue homogenates in embryonated SPF eggs.

For statistical analysis a non-parametric approach based on rank numbers was used, because numbers of animals are modest and some observations fall below the detection limit, as previously described (chapter 4). Tests over times were performed and the chicken groups infected with virus rgAV324-LL or rgAV324-PLL were compared with the rgAV324 infected chickens, employing Wilcoxon's rank sum test (14). Differences were considered to be significant when $p < 0.05$. All calculations were performed with GenStat (38).

Results

Passage of PPMV-1 in chickens results in an increase in virulence

Since it is generally assumed that NDV virulence correlates with the ability of the virus to replicate in the brain of infected animals, we decided to passage the virus by intracerebral inoculation. This route is used in the standard OIE-prescribed virulence test for NDV, which is expressed as the intracerebral pathogenicity index (ICPI). The PPMV-1 isolate AV324/96 was serially passaged intracerebrally in day-old chickens. After each passage the virus showed an increase in virulence as determined by the intracerebral pathogenicity test (ICPI) in one-day-old chickens (Table 5.1).

Table 5.1. Pathogenicity index (ICPI) after passage (p) of AV324/96 in chicken brains. Mutations were found in the L and P genes. Amino acid residues involved and their sequence position are indicated. nd: not determined

virus	ICPI	sequence
AV324/96	0.44	consensus
AV324/96 p1	0.43	L^{V1694E}
AV324/96 p2	0.60	nd
AV324/96 p3	0.80	L^{N1564S} L^{V1694E}
AV324/96 p4	0.83	nd
AV324/96 p5	0.90	$P^{N37D}L^{N1564S}$ L^{V1694E}

Of the viruses obtained after passage (p) 1, 3 and 5 the complete genomic sequence was determined and compared to that of the parent strain AV324/96 (GenBank accession no. GQ429292). The nucleotide mutations and corresponding electropherograms are shown in Fig. 5.1. The resulting amino acid changes in each virus are shown in Table 1. Compared to the parent strain, p1 virus contained a single nucleotide mutation, which resulted in amino acid mutation V1694E in the L protein. Virus of p3 contained an additional mutation in the L protein, N1564S. In virus of p5 these two mutations in the L protein had been maintained and an additional mutation was observed in the P protein, N37D (Table 5.1). When comparing these observations with sequences available in GenBank, it appeared that from a total of 84 available L sequences 81 had V1694 and 83 N1564, as in AV324. Thus, the adaptive mutations in this protein seem to change away from the consensus of known NDV sequences. Furthermore, the N37 in the P protein of AV324 is unique compared to 133 available sequences in GenBank, but adaptation did not cause a change into the NDV consensus, which is S37.

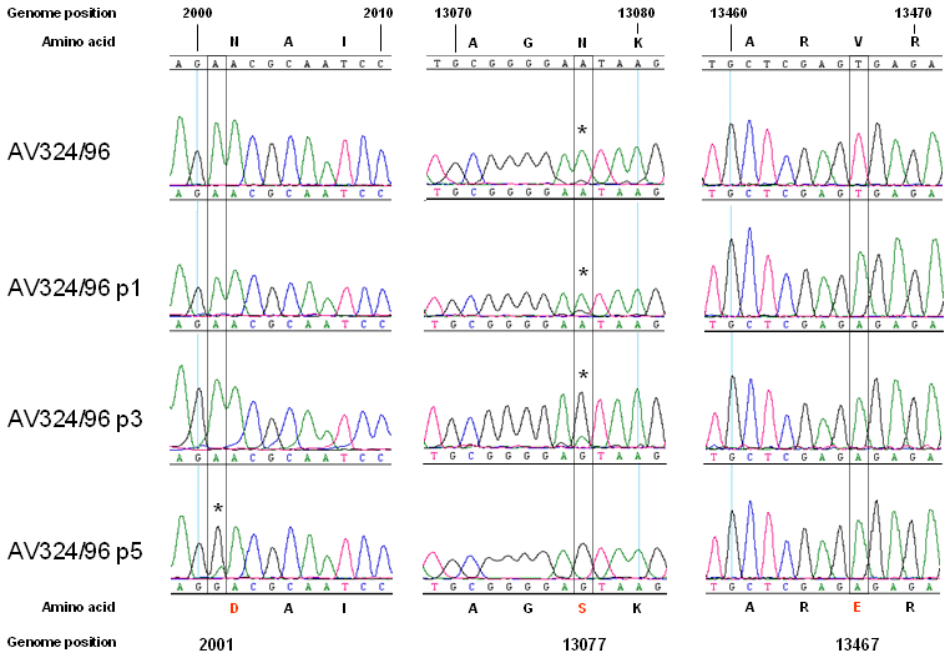

Fig. 5.1. Nucleotide sequences in the regions of the adaptive mutations acquired upon virus passaging. The genome regions (nucleotide sequence numbers) are depicted at the top and the involved amino acids. The left column represents the region in the P gene, whereas the middle and the right column represent the regions in the L gene. The nucleotide positions where mutations occurred are indicated at the bottom of the figure. * Double peak. The amino acid codes are positioned at the second nucleotide of the codon. N: asparagine, A: alanine, I: isoleucine, G: glycine, K: lysine, R: arginine, V: valine, D: aspartic acid, S: serine, E: glutamic acid.

Increase in virulence is associated with mutations in the L and P proteins

To show unambiguously that the differences in virulence between viruses from p1, p3 and p5 were due to the observed mutations in the L and P proteins, we used the reverse genetics system that we previously developed for strain AV324. The double (LL) and triple (PLL) mutations were engineered into rgAV324, resulting in rgAV324-LL and rgAV324-PLL, respectively. A schematic illustration of the viral genomes is shown in Fig. 5.2. Because the L^{V1694E} mutation had no effect on the ICPI (Table 5.1), we did not attempt to construct the single mutant rgAV324-L^{V1694E}. Viruses were rescued as described in the Materials and Methods section. The ICPI of the recombinant viruses was determined and compared with that of the parental virus rgAV324 (ICPI = 0.00). The two mutations in rgAV324-LL resulted in a slight increase in virulence (ICPI = 0.18), while the triple mutant rgAV324-PLL exhibited a relatively large increase in virulence (ICPI = 0.65; Fig. 5.2). To further study the significance of these mutations, the effects of the PLL substitutions were also determined in a different background, i.e. the chimeric virus FL-Herts(NP-P'-L)^{AV324}, which comprises the NP, P and L genes of rgAV324 in the background of the virulent NDV strain FL-Herts. While this chimeric virus had a moderate virulence with an ICPI of 0.55, the combined PLL mutations enhanced its virulence strongly (ICPI = 1.30; Fig. 5.2). Taken together, these results show that the triple (PLL) mutations observed after serial passage in chickens are indeed responsible for the increase in virulence.

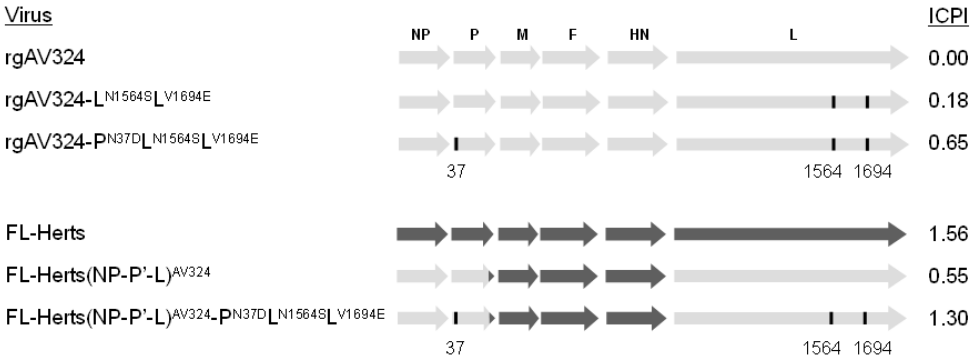

Fig. 5.2. Schematic illustration of the genomic organization of rgAV324 (light grey) and FL-Herts (dark grey). The positions of amino acid changes are indicated and refer to the mutations observed after serial passage (Table 5.1). The virulence of the parental viruses and their recombinants containing the mutations was determined by the intracerebral pathogenicity index in day-old chickens (ICPI score: max 2.0).

Plaque size of recombinant viruses

The observation that the adaptive mutations occurred in viral proteins that are involved in transcription and replication suggested that they affected virus replication. In order to examine replication of the different viruses, their plaque size in tissue culture monolayers of quail-derived QM5 cells and chicken-derived DF-1 cells was determined (Fig. 5.3). Introduction of the LL and PLL mutations in the rgAV324 backbone did not seem to have a significant effect on plaque size in QM5 cells (Fig. 5.3a). However, in DF-1 cells the presence of the LL and PLL mutations resulted in an almost two-fold increase in plaque size (Fig. 5.3b). While the chimeric Herts virus containing the NP, P and L genes of AV324 showed a halving of the plaque size in both cell types compared to the virulent Herts virus, the PLL mutations almost completely compensated for this decrease (Fig. 5.3c and d).

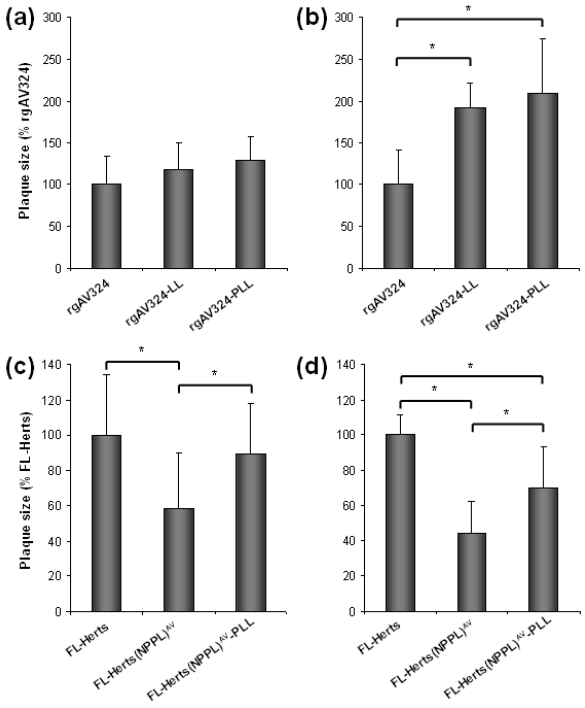

Fig. 5.3. Relative plaque size of the recombinant viruses in QM5 cells (a and c) and DF-1 cells (b and d) 48 hours post infection. (a and b) rgAV324 and its derivatives containing the L^{N1564S}/L^{V1694E} mutations (LL) or the $P^{N37D}/L^{N1564S}/L^{V1694E}$ mutations (PLL). (c and d) FL-Herts and the recombinants containing the NP, P and L proteins of AV324 with or without the PLL mutations. Plaques were visualized by immunological staining using a mAb against the NDV F protein. The mean plaque size was determined by measuring the area of at least 11 plaques per virus with the Image-Pro Plus software (Media Cybernetics, Inc.). Error bars represent standard deviations. Differences were significant (*) when $p < 0.05$.

In vitro replication assay

To show that the increase in plaque size was directly related to the efficiency of viral genome replication, we performed an *in vitro* replication assay using cotransfection of a minigenome plasmid and plasmids expressing the NP, P and L proteins. To quantitate replication activity we used a plasmid harboring a NDV-derived minigenome encoding Gaussia luciferase (GLuc). Transcription of pAV324-GLuc using T7 RNA polymerase generates negative-sense minigenome RNA. Expression of the GLuc gene is dependent on the conversion of this RNA into positive-sense RNA, a process that is entirely dependent on the combined action of the viral replication complex consisting of the NP, P and L proteins. The replication assay revealed that the LL mutations caused a significant increase in the activity of the replication complex (Fig. 5.4). Furthermore, the P mutation N37D had a significant additional effect. Yet, even with the 3 mutations combined, the AV324 replication proteins did not reach the level obtained with the Herts virus derived replication proteins (Fig. 5.4).

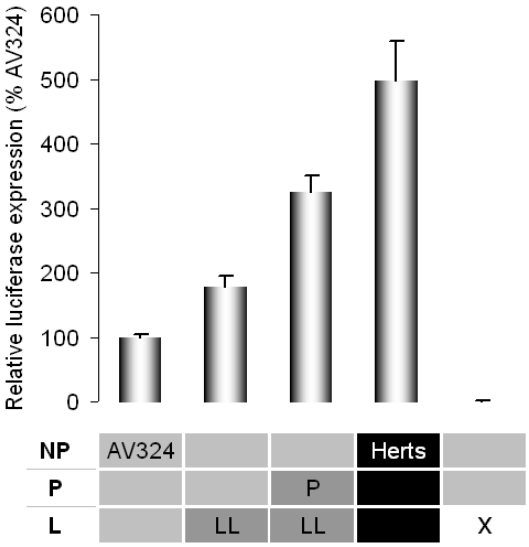

Fig. 5.4. Relative luciferase expression levels after cotransfection of FPV-T7 infected QM5 cells with the viral minigenome plasmid (containing the leader and trailer sequences of AV324 flanking the Gluc reporter gene) and plasmids expressing NP, P and L of AV324 (light grey) or Herts (black), or plasmids containing the L^{N1564S}/L^{V1694E} mutations (LL) or the $P^{N37D}/L^{N1564S}/L^{V1694E}$ mutations (PLL) (grey). The background level of luciferase activity was determined by omitting the L plasmid (x). The data are representative of at least three separate experiments. Error bars represents SEM. All differences were significant ($p < 0.05$).

Viral genome replication

To investigate the kinetics of viral genome replication of the recombinant viruses in cell culture monolayers, the relative amount of negative-sense genomic RNA was determined in the first 16 hours of infection. A specific quantitative real time PCR (qRRT-PCR) was used. The results showed that the replication kinetics of rgAV324-LL were similar to those of rgAV324. However, the triple mutant rgAV324-PLL showed an increased replication rate (Fig. 5.5), which indicates that the three mutations are responsible for the enhanced replication efficiency in QM5 cells.

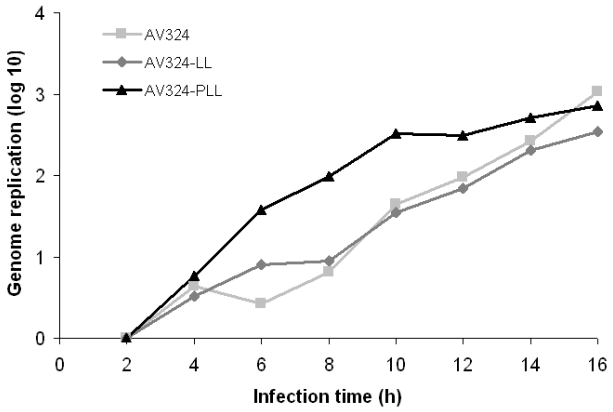

Fig. 5.5. The relative negative-sense genomic RNA replication in the first 16 hours of infection. QM5 cells were infected with an m.o.i. of 10 and replication was determined by quantitative real-time RT-PCR. Results are the mean of two separate experiments.

In vivo replication of recombinant viruses

The *in vivo* replication levels of the AV324 based mutant viruses was determined in day-old chickens. To this end, virus was recovered from several tissues at different days after intracerebral inoculation (Fig. 5.6). The results showed that there is little replication of any of the viruses except for rgAV324-PLL in brain tissue. However, in the other three organs tested, liver, lungs and spleen, recombinants rgAV324-LL and rgAV324-PLL replicated to significantly higher levels than the parental virus rgAV324 ($p < 0.05$). These observations indicate that the adaptive mutations are responsible for more efficient replication in chickens.

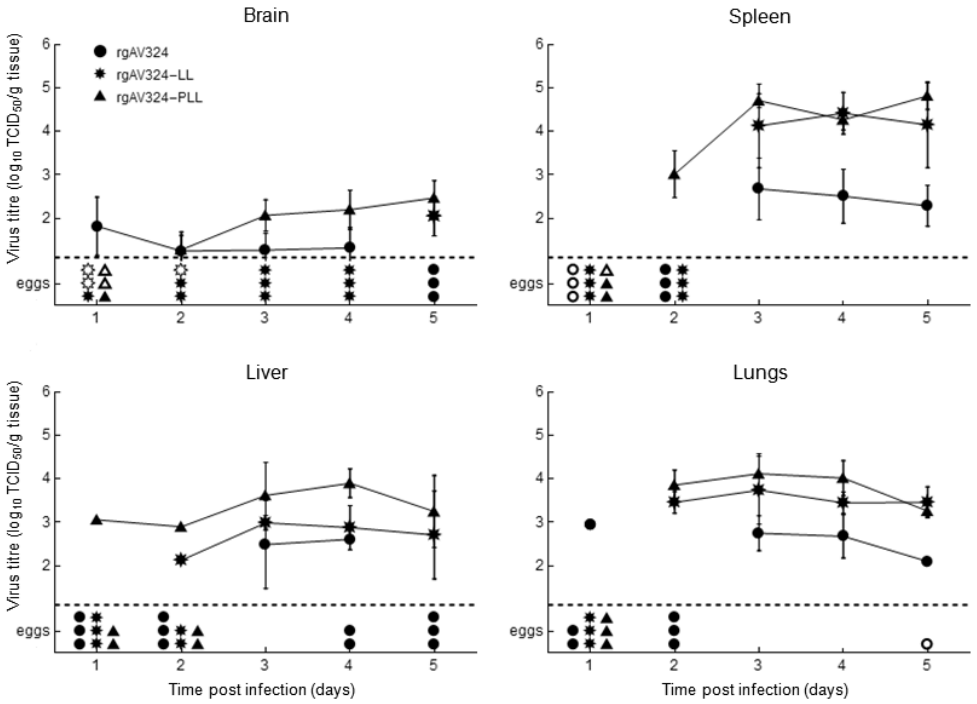

Fig. 5.6. Viral titres of rgAV324 and its derivatives containing the LL or PLL mutations acquired during serial passage (Table 5.1) after intracerebral inoculation of one-day-old chickens. Each day three chickens were sacrificed and brain, spleen, liver and lungs were collected. Virus titres were determined using QM5 cells and are presented as the mean virus titre (log_{10} TCID50/g tissue). The dotted line indicates the detection limit of the virus detection assay in QM5 cells. "QM5-negative" samples were tested for the presence of virus by inoculation in embryonated eggs. Open symbols represent that inoculated eggs had remained virus negative, whereas closed symbols indicate that inoculated eggs had become virus positive. Error bars show standard deviations.

Discussion

Pigeon paramyxoviruses are a hidden threat to the poultry industry. Though generally low- or nonvirulent for chickens, these viruses may gain virulence during spread through the flock, probably not in the least because they already carry the F protein cleavage motif associated with high virulence. Here we have serially passaged the low virulent PPMV-1 isolate AV324 in chickens. After five passages the virus exhibited significantly enhanced virulence. We identified the accompanying mutations, which appeared to map to the replication proteins P and L. Using reverse genetics and two different genetic backgrounds, we demonstrated these mutations to be responsible for the acquired phenotype by showing that they have a direct effect on virulence as

determined by ICPI (Fig. 5.1). Furthermore, we showed that virulence of PPMV-1 for chickens is directly associated with the efficiency of viral genome replication. This conclusion is based on four independent observations. First, the virus containing the three mutations showed enhanced replication in cell culture as evidenced by the increase in plaque size (Fig. 5.3). Second, the viral genome replication rate increased as a result of the mutations (Fig. 5.5). Third, these mutations led to an increase in the activity of the viral polymerase complex as shown by an *in vitro* replication assay (Fig. 5.4). Finally, recombinant viruses containing these mutations replicated to higher titres in the organs of infected chickens (Fig. 5.6).

These conclusions are consistent with earlier observations indicating a relationship between virulence and replication efficiency. By exchanging genes between a low virulent PPMV-1 and a highly virulent NDV we were able to map these features to differences in all three replication proteins (chapter 4). When comparing two NDV strains within one genotypic lineage, the L protein alone was characterized as being important (42). For other paramyxoviruses such as measles virus (9, 47), respiratory syncytial virus and parainfluenza virus (34, 44) it has also been described that determinants of virus attenuation are associated with mutations in the P and L genes. Thus, changing the efficiency of viral genome replication seems to be a general mechanism to modulate paramyxovirus virulence.

Two of the three mutations that we observed in our passage 5 chicken-selected PPMV-1 are localized within the L protein. This protein is conserved among all members of the *Mononegavirales* and contains six conserved domains that constitute its enzymatic activities (39, 43, 50). The two L mutations are positioned in the variable region between domains V and VI. This specific region is more variable than other non-conserved regions in the L protein of the *Mononegavirales* and it has been suggested that this particular region evolved separately between individual viruses (39, 43). This region may interact with unique host cell factors and variations in this region may be associated with the adaptation of PPMV-1 to chickens. The vast majority of the known NDV L protein sequences contain V1694 and N1564. Surprisingly, adaptation to chickens causes amino acid changes away from the consensus sequence of all NDV sequences known to date. The two L substitutions may be mutations that change the conformation of the protein, thereby improving its intrinsic activity or its interaction with the P protein and/or specific host factors. The NP-P complex and the P-L complex are essential for transcription and replication of the viral genome (19, 24, 26, 29) and our results imply that the N37D mutation in the P protein is important for these processes. Although the C-terminal half of the P protein is involved in the NP-P interaction (24), the position of the L-binding site on P has not yet been identified for NDV. Because N37 of the P protein of AV324 is unique among all NDV strains, it is probably no coincidence that this very position changed during passaging. However, it did not revert to the consensus amino acid of most NDV strains although this would have been possible by a single nucleotide change in the amino acid codon (AAC > GAC).

Molecular changes are often associated with a switch in host species (in this case adaptation of pigeon-origin virus to chickens). In many cases these changes are found in replication proteins, which may be cell-type specific. This might be the reason for the

different plaque sizes obtained with different cells (Fig. 5.3). Other studies have already shown that plaque size is highly dependent on the use of certain viral mutants, strains and cell types (21, 22, 33, 48). For avian influenza virus it has been described that adaptation to a mammalian host is often associated with changes in the viral polymerase complex (18). Furthermore, particular mutations in PB2 and PA of influenza virus may influence pathogenesis in mice or humans (17, 20, 41, 45). Adaptation of Borna disease virus to mice resulted in three amino acid changes affecting the polymerase L and the polymerase cofactor P. When all three mutations were combined, a strong increase in virulence was observed (1). These results show a striking similarity with those of the present study.

The selective cycle of collection, amplification into eggs and passage into chicken brain is not representative of a natural route of infection and the adaptive mutations that we found may thus not be representative of mutations occurring under field conditions. Furthermore, ICPI levels in one-day old chickens may not always correspond to the severity of clinical disease in adult chickens infected via the natural route. Although we cannot completely rule out these considerations, it should be noted that the mutated viruses differ in replication efficiency not only in brain tissue, but also in other tissues such as lungs, spleen and liver. Therefore, we expect that these specific mutations will also result in an increase in pathogenicity after natural infection. However, this has to be verified by appropriate animal experiments. Furthermore, it should be noted that the ICPI is a generally accepted method not only to determine the virulence of a virus, but also to define a ND outbreak and, as a consequence, the control measures to be taken.

It could also be argued that the mutations that we found were not solely the result of adaptation to chickens but also to selection in the embryonated eggs used to recover the virus as the inoculum for the next passage. It should however be considered that this is not a bottle neck procedure such as e.g. limiting dilution. Moreover, it is a method generally used to generate a virus stock. We therefore do not suppose that this has had a major contribution in selecting the mutations we found. The fact that the mutations in the consensus sequence arise and accumulate in a given order may suggest that they are stable in the virus population. For instance, while the P^{N37D} mutation had a significant effect on the ICPI value in the recombinant virus containing the double L mutant (Fig. 5.2), it did not exert an effect in the recombinant without the L mutations (data not shown). As implied above, this again suggests an interaction between the domains of the L and P proteins containing these particular amino acids.

It is possible that more mutations would come up upon further virus passage. Apparently, there is a selection for PPMV-1 mutants with enhanced replication efficiencies in chickens, which is probably not yet optimal considering that the replication complex of the Herts virus is still more active (Fig. 5.4). Studies have shown that passaging of PPMV-1 isolates in chickens does not always lead to a virulence increase for chickens. Not the particular passage procedure utilized but the particular virus strain appears to determine the outcome (4, 5, 25, 27, 28).

The ICPI levels of the passaged viruses differed from those observed for their corresponding recombinant viruses. While, for example, passage 5 virus had an ICPI of 0.90, that of its recombinant counterpart rgAV324-PLL was only 0.65. This discrepancy

is most likely caused by the presence of different subpopulations (quasispecies) in the original virus population. This can, for instance, be seen by the presence of “double” peaks in Fig. 5.1. These subpopulations together determine the phenotype of that particular virus in the assay. Virus generated from cDNA copies is far less heterogeneous and will therefore not represent the original virus population completely. In this respect it should be noted that the described mutations may be the result of selection of a virus subpopulation already present in the original virus AV324/96. For example, more than one nucleotide seems to occur at position 13077 in the sequence reading of the original stock (Fig. 5.1). Yet, by using viruses generated by reverse genetics we demonstrated that the specific mutations in the P and L proteins that were selected during the passage procedure were indeed responsible for the phenotypic differences that we observed.

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Chapter 6

A comparative infection study of pigeon and avian paramyxovirus type 1 viruses in pigeons: evaluation of clinical signs, virus shedding and seroconversion

J.C.F.M. Dortmans[1,2], G. Koch[1], P.J.M. Rottier[2] and B.P.H. Peeters[1]

[1]Central Veterinary Institute of Wageningen UR, Lelystad, The Netherlands.
[2]Virology Division, Department of Infectious Diseases & Immunology, Faculty of Veterinary Medicine, Utrecht University, The Netherlands.

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Summary

The pathogenesis of pigeon paramyxovirus type 1 (PPMV-1) isolate AV324/96 and of its recombinant derivative, rgAV324, was studied in pigeons. For comparison the virulent chicken virus FL-Herts, which is a recombinant derivative of strain Herts/33, was also included. After inoculation by the combined intraocular, intranasal and intratracheal route, clinical signs, virus shedding and serological responses were examined. Clinical symptoms were only observed in the FL-Herts infected group. All virus-inoculated pigeons had positive tracheal swabs until 5 days post infection. However, only the AV324/96 and rgAV324 infected birds, and not the FL-Herts infected animals, shed virus in the cloaca. The AV324/96 infected pigeons showed higher mean antibody titres than the rgAV324 infected animals, whereas the antibody titres of the FL-Herts infected group were rather low. The results show that the pigeon strain AV324 is not virulent for pigeons, but underlines the potential risk of poultry becoming infected by PPMV-1 shed by non-symptomatic pigeons.

Introduction

Newcastle disease (ND) is a highly contagious viral disease of chickens but can also affect a wide variety of other avian species including domestic pigeons and doves (Columbiformes) (25). The disease is caused by Newcastle disease virus (NDV) or avian paramyxovirus type 1 (APMV-1), which belongs to the family *Paramyxoviridae* (genus *Avulavirus*) (34). During the 1980s, a disease caused by an APMV-1 spread worldwide among racing and show pigeons (9). APMV-1 variants like this could be discriminated antigenically by monoclonal antibodies (8, 10, 15, 30, 40) and genetically by phylogenetic analysis of the F gene nucleotide sequence (2) and have become known as pigeon paramyxovirus type 1 (PPMV-1).

In the past, a number of ND outbreaks in poultry have been attributed to PPMV-1, which makes these pigeon-derived viruses a real and continuous threat to the poultry industry (4, 5, 10, 11, 24, 31, 47). The F proteins of PPMV-1 strains contain a poly-basic cleavage site motif, a feature generally associated with virulent NDV strains. It has therefore been suggested that PPMV-1 strains have evolved from virulent NDV strains (17, 35, 42). However, some PPMV-1 strains cause only minimal disease and show a low intracerebral pathogenicity index (ICPI) in chickens (36). Nevertheless, they are potentially virulent as becomes apparent after serial passages in chickens (6, 16, 28, 29, chapter 5). Thus, upon natural transmission from pigeons to chickens, PPMV-1 strains may evolve into virulent viruses and lead to major outbreaks.

In our recent studies, we investigated the molecular characteristics of PPMV-1 isolate AV324/96, originally isolated from a racing-pigeon. Despite having an F protein cleavage site motif typical of virulent viruses, this isolate is low virulent for chickens (ICPI=0.44, as determined by the Veterinary Laboratories Agency, Addlestone, Surrey, United Kingdom). Using a reverse-genetics system we investigated the contribution of

the F, NP, P, M and L proteins to virulence for chickens by exchanging genes between this AV324/96 strain and the highly virulent NDV Herts strain (chapter 3 and 4). Furthermore, we investigated the molecular basis for the virulence increase of AV324/96 after serial passage in chickens (chapter 5). In view of the demonstrated potential of infected pigeons transmitting NDV to poultry, more knowledge about these PPMV-1 viruses is needed quite urgently. One important aspect is the pathogenesis of PPMV-1 in its original host, the pigeon. Therefore we investigated the susceptibility of pigeons to the PPMV-1 isolate AV324/96 and to a recombinant derivative prepared from it (rgAV324). For comparison we also used the recombinant APMV-1 strain FL-Herts which is highly virulent for chickens.

Materials & Methods

Viruses

The PPMV-1 isolate AV324/96 was obtained from Dr. M.S. Collins (Virology Department, Veterinary Laboratories Agency, Addlestone, Surrey, United Kingdom) and was originally isolated by H. de Geus (PV 17/96) from a racing pigeon loft in Ireland. The generation of full-length infectious cDNA clones of AV324/96 and Herts/33 and rescue of infectious virus from these clones, designated rgAV324 and FL-Herts, respectively, has been described previously (19, chapter 3). Working stocks of the wild-type AV324/96 and the recombinant viruses rgAV324 and FL-Herts were prepared in embryonated specific-pathogen-free (SPF) eggs. The virulence level of each virus was assessed by intracerebral inoculation of day-old chickens as described in the European Community Council Directive 92/66/EEC (18). The ICPI values were 0.2 for AV324/96, 0.0 for rgAV324 and 1.6 for FL-Herts.

Animals

A total of 19 clinically healthy pigeons (*Colomba livia*), born in captivity, were used in this study, which was approved by the Ethics Committee for Animal Experiments of the Central Veterinary Institute of Wageningen UR and was in compliance with the Dutch law on animal experiments. One week before experimental infection, the pigeons, then 12-18 weeks of age and all lacking NDV-specific hemagglutination inhibition (HI) antibodies, were moved to a high containment unit of the Central Veterinary Institute of Wageningen UR.

Experimental design

Three groups of 5 pigeons each were inoculated with virus by the combined intraocular, intranasal and intratracheal route, using a total dose of 10^7 EID_{50}/pigeon. The negative control group consisted of 4 pigeons which received PBS. The experiment was terminated at 14 days post infection (dpi).

The animals were observed for clinical signs, and tracheal and cloacal swabs were taken daily. The swabs were placed in 2.95% tryptose phosphate buffer with

appropriate antibiotics and infectious virus was quantified by serial end-point dilution in 96-well plates (Greiner Bio-One) using QM5 cells (12). After 3 days of incubation at 37ºC, cells were fixed and immunological staining was performed to detect the infected cells as previously described (chapter 3). Virus titres were calculated using the Reed and Muench method (39) and expressed as $\log_{10}TCID_{50}$/ml.

Serum samples were obtained at day 6 before and day 14 after inoculation. The hemagglutination inhibition (HI) assay was performed by standard methods (3) using 8 hemagglutinating units of antigen. The antigens used in this study were rgAV324, FL-Herts and strain Ulster. The latter is used as an antigen in standard NDV diagnostics.

Results

To compare the infectivity and virulence in pigeons of strains AV324/96, its recombinant derivative rgAV324, and FL-Herts, animals were inoculated with 10^7 EID_{50} of virus and observed daily for clinical signs. Hardly any symptoms were observed in the AV324/96 and rgAV324 infected group. In the FL-Herts infected group, two pigeons, 7752 and 7753, showed signs of paralysis from day 4 and 7 onwards, respectively, but pigeon 7753 recovered whereas pigeon 7752 died at 6 dpi.

To determine virus shedding, tracheal and cloacal swabs were taken daily. The results are compiled in Table 6.1. While the mock-inoculated birds remained virus negative throughout the experiment, in the three virus-inoculated groups almost all pigeons had positive tracheal swabs from 1 to 5 dpi. Pigeon 7738 incurred a dislocated beak at 3 dpi; hence, a throat swab had to be taken instead of a tracheal swab, which may explain the negative results at 3 and 5 dpi for this particular animal. Two pigeons in the rgAV324 infected group (7737 and 7738) and one in the group infected with AV324/96 (7746) were positive until 9-10 dpi. No differences in the amounts of virus shedding were observed among the three virus groups (Fig. 6.1). This was confirmed by estimating and summing up the area under the curve of each individual animal per group ($p > 0.05$; data not shown). Surprisingly, although all pigeons in the FL-Herts infected group became virus positive in the trachea, none of these birds showed detectable virus in the cloacal swabs. In contrast, three pigeons from both the rgAV324 and the AV324/96 infected group became virus positive in the cloaca (Table 6.1 and Fig. 6.1). Thus, although the PPMV-1 virus did not cause any clinical symptoms, infected pigeons shed virus until at least 14 dpi.

To evaluate the antibody responses of the animals to the infections, serum samples were obtained at day 6 before and day 14 after inoculation. Serum samples taken before inoculation were used to confirm the lack of NDV-specific HI antibodies of the pigeons. At 14 dpi, using the homologous antigens, all pigeons of the rgAV324 and AV324/96 infected group responded positively in the HI test, whereas one pigeon of the FL-Herts infected group did not respond (Table 6.2). AV324/96 infected pigeons showed a broader range of reactivity compared to the animals in the rgAV324 infected group. Notably, FL-Herts infected pigeons did not show any cross reactivity with the rgAV324 antigen.

Table 6.1. Virus shedding in tracheal (light grey) and cloacal (dark grey) swabs (tracheal swab/cloacal swab) determined by inoculating monolayers of QM5 cells. *, throat swab instead of tracheal swab. nd, not determined. †, pigeon died

		days post infection														
virus	pigeon	0	1	2	3	4	5	6	7	8	9	10	11	12	13	14
PBS	7732	-/-	-/-	-/-	-/-	-/-	-/-	-/-	-/-	-/-	-/-	-/-	-/-	-/-	-/-	nd
	7733	-/-	-/-	-/-	-/-	-/-	-/-	-/-	-/-	-/-	-/-	-/-	-/-	-/-	-/-	nd
	7734	-/-	-/-	-/-	-/-	-/-	-/-	-/-	-/-	-/-	-/-	-/-	-/-	-/-	-/-	nd
	7735	-/-	-/-	-/-	-/-	-/-	-/-	-/-	-/-	-/-	-/-	-/-	-/-	-/-	-/-	nd
rgAV324	7737	-/-	+/-	+/-	+/-	+/-	+/-	+/-	+/+	+/+	+/+	-/+	-/+	-/+	-/+	-/+
	7738	-/-	+/-	+/-	-/-*	+/-*	-/-*	+/-*	+/-*	+/-	+/-	+/+	-/-*	-/+	-/+	-/+
	7739	-/-	+/-	+/-	+/-	+/-	+/-	-/-	-/-	-/-	-/-	-/-	-/+	-/+	-/+	-/+
	7740	-/-	+/-	+/-	-/-	-/-	-/-	-/-	-/-	-/-	-/-	-/-	-/-	-/-	-/-	-/-
	7741	-/-	+/-	+/-	+/-	+/-	+/-	-/-	-/-	-/-	-/-	-/-	-/-	-/-	-/-	-/-
AV324/96	7742	-/-	+/-	+/-	+/-	+/-	+/-	-/-	-/-	-/-	-/-	-/-	-/-	-/-	-/-	-/-
	7743	-/-	+/-	+/-	+/-	+/-	+/-	-/-	-/-	-/-	-/-	-/-	-/-	-/-	-/-	-/-
	7744	-/-	+/-	-/-	+/-	+/-	+/-	-/-	-/-	-/+	-/+	-/+	-/+	-/+	-/+	-/+
	7746	-/-	+/-	+/-	+/-	+/-	+/-	+/-	+/-	+/-	+/-	-/+	-/-	-/+	-/+	-/+
	7747	-/-	+/-	+/-	+/-	+/-	+/-	-/+	-/+	-/+	-/+	-/+	-/+	-/+	-/+	-/-
FL-Herts	7748	-/-	+/-	+/-	+/-	+/-	+/-	-/-	-/-	-/-	-/-	-/-	-/-	-/-	-/-	-/-
	7749	-/-	+/-	+/-	+/-	+/-	+/-	-/-	-/-	-/-	-/-	-/-	-/-	-/-	-/-	-/-
	7751	-/-	+/-	+/-	+/-	+/-	-/-	-/-	-/-	-/-	-/-	-/-	-/-	-/-	-/-	-/-
	7752	-/-	+/-	+/-	+/-	+/-	+/-	†								
	7753	-/-	+/-	+/-	+/-	+/-	-/-	-/-	-/-	-/-	-/-	-/-	-/-	-/-	-/-	-/-

Discussion

From the results of this study we conclude that pigeon strain AV324 is not virulent for pigeons, while FL-Herts is only modestly virulent for pigeons. Although we did not infect chickens via the natural mucosal route, the ICPI values suggest that the difference in virulence of APMV-1 and PPMV-1 is much smaller in pigeons than in chickens. This indicates that chickens and pigeons are not equally susceptible to infection by the same strain of virus, and confirms the findings of others (13, 22, 27, 46).

Several earlier studies have shown that pigeons infected with either APMV-1 or PPMV-1 shed virus via the oral, tracheal or laryngeal route from 2 dpi onwards (20, 21, 27, 44, 46). Cloacal shedding was also observed in most of these studies, but the onset of this shedding was generally much earlier, starting at 2-3 dpi (14, 20, 21, 27, 41, 44, 46), except in one case where cloacal virus shedding was observed from 5-11 dpi (13), comparable to our results (6-11 dpi). While, in these studies, virus shedding was determined either by inoculating embryonated eggs or chicken embryo fibroblast cultures or by RT-PCR, we used QM5 cells for our infection assay, which may be less

sensitive. Alternatively, the AV324/96 strain might replicate at a lower rate than the other investigated viruses, hence resulting in a later onset of viral shedding via the cloacal route.

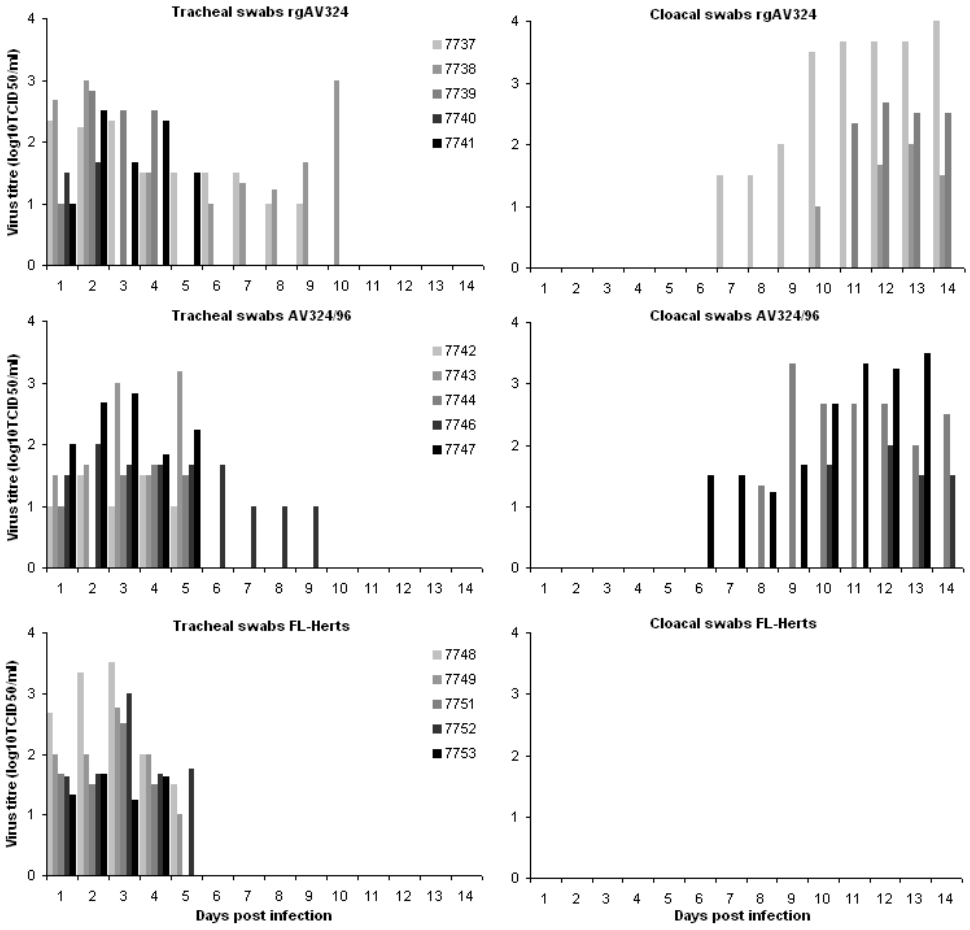

Fig. 6.1. Virus titres determined in tracheal (left column) and cloacal (right column) swabs of individual pigeons. Titres ($log_{10}TCID_{50}$/ml) were determined by serial end-point dilution in 96-well plates using QM5 cells.

The lower antibody response to rgAV324 infection compared to infection with its parental virus AV324/96 might be explained by the broader quasispecies distribution of the latter. Furthermore, this difference in quasispecies complexity might explain the difference in ICPI value we observed between recombinant and parental viruses in chickens. These results also show that using standard diagnostics with Ulster antigen, low antibody responses against PPMV-1 in pigeons could be missed.

Differences in HI antibody titres depending on the virus strain used as the antigen in the HI assay have been noted before (23, 27, 32, 41, 44). Such differences might well relate to observations suggesting that current ND vaccines may provide less protection due to antigenic divergence between the vaccine strain and the circulating field strains (23, 26, 37, 38, 43). PPMV-1 strains belong to a distinct genetic lineage (1) and previous work already suggested that vaccines for use in pigeons should preferably be prepared from pigeon-origin PPMV-1 virus (7).

Table 6.2. Antibody titres determined with antigens rgAV324, FL-Herts and Ulster at 14 days post infection on a $\log_2$ scale. Positive when HI ≥ 3. nd, not determined

		HI antigen		
virus	**pigeon**	**AV324**	**Herts**	**Ulster**
PBS	**7732**	0	0	0
	7733	0	0	0
	7734	0	0	0
	7735	0	0	0
rgAV324	**7737**	3	0	0
	7738	3	0	0
	7739	6	0	0
	7740	4	0	0
	7741	6	0	0
AV324/96	**7742**	5	0	0
	7743	7	0	3
	7744	8	3	4
	7746	7	2	0
	7747	7	5	4
FL-Herts	**7748**	0	3	3
	7749	0	3	0
	7751	0	0	0
	7752	nd	nd	nd
	7753	0	4	5

To assess the virulence of the different viruses used in this study, the ICPIs were determined in day-old chickens. The ICPI value for AV324/96 was 0.2, which is lower than the value for the original isolate which was 0.44. This difference might be explained by the fact that the ICPI assays were performed at different institutes using slightly different virus preparations. Although the ICPI is a standardized method, minor variations such as the genetic background of the birds used, difference in reading clinical signs and or small differences in the exact time of onset of clinical signs may result in small differences in the ICPI value for viruses of low virulence. Furthermore, the virus inocula used at both locations might have differed slightly with respect to the composition of subpopulations.

Although PPMV-1 or velogenic NDV infections in pigeons can cause morbidity and mortality, we found strain AV324 not to be virulent at all. Yet, despite the lack of clinical symptoms, the animals do excrete virus at levels that are comparable to those obtained with virulent viruses for both chickens and pigeons (13, 14, 27, 44, 46). Although mortality of chickens infected with velogenic NDV can reach 100%, mortality of pigeons is usually less than 40% and for PPMV-1 it is even less than 10% (33). However, some experimental and field data show higher mortality rates (21, 22). A reason for this might be that the pigeons are suffering from secondary bacterial or viral infections (33). In pigeon experiments, SPF animals are rarely used, in contrast to chicken experiments.

PPMV-1 is capable of being transmitted from infected pigeons to chickens placed in contact (6) and requires only a limited number of adaptive mutations for optimal replication in chickens (chapter 5). This knowledge, together with the recent NDV outbreaks in pigeons caused by PPMV-1 in Europe (45), underscores the potential danger of pigeon-derived paramyxoviruses for the poultry industry.

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Chapter 7

General discussion

Pathogenicity and virulence

Pathogenicity is the ability of a virus to cause disease in the host it infects and virulence is the degree of pathology caused by the virus. The virulence of a virus may vary from no apparent clinical symptoms to 100% mortality. Whether a host will suffer from infection depends upon various factors such as its state of health, age, environmental stress and the virus strain. The virulence of a virus is determined by multiple genetic factors, which may involve its cellular tropism, its ability to escape from host immunity and/or its replication abilities. The study of virulence and the identification of viral determinants of disease severity is important because an understanding of the underlying mechanisms may enable a more effective therapeutic approach to viral diseases.

Tropism

Cellular tropism in a susceptible host is often determined by virus-receptor interactions. Infections are initiated when virus particles bind to receptor molecules on the surface of target cells. However, the entry of enveloped viruses into host cells often requires the activation of viral envelope glycoproteins through cleavage by either intracellular or extracellular proteases. Previous studies have shown that viral glycoprotein activation is often mediated by proteases recognizing either monobasic or multibasic cleavage sites (47). In retroviruses, orthomyxoviruses and paramyxoviruses, cleavage of viral glycoproteins has been demonstrated to regulate virus entry and fusion (53, 60, 65). For effective virus spread in the infected host, proteolytic activation is indispensable and appears to be determined mainly by the amino acid sequence at the cleavage site. Avirulent viruses are cleaved extracellularly by secreted proteases which limits their spread in hosts to tissues where the appropriate proteases are present, while virulent viruses are cleaved intracellularly by ubiquitously occurring proteases and therefore have the capacity to infect virtually all cell types and cause systemic infections. This feature largely determines the outcome of the infection of virulent and avirulent strains as has been shown for influenza viruses, Sendai virus and Newcastle disease virus (31, 48, 100). However, although the amino acid sequence at the F protein cleavage site has been shown to be a major determinant of NDV virulence (65, 67), other proteins like HN (25, 42, 81, 106), V (41, 61, 69) and L (83) also appear to be involved. The results described in this thesis now show that also the viral replication complex is involved.

Studies in the last decade have already shown that the consequences of an infection are not solely determined by the presence of a multibasic cleavage site motif in the viral glycoprotein. For avian influenza A virus, for example, it has been described that, although a multibasic cleavage site is required, additional factors mainly determine the outcome of the disease (37). Also for certain strains of NDV it has been observed that a multibasic amino acid sequence at the F cleavage site is not necessarily correlated with a high virulence in chickens (62). This observation is confirmed by our studies presented in chapter 3 in which we used an infectious cDNA clone to prove that

the low virulence is an inherent property of the particular virus and is not due to a mixture of low- and high-virulent viruses. Furthermore, as shown in that same chapter, replacement of the F gene of a virulent NDV strain by that of a non-virulent PPMV-1 strain and *vice versa* did not affect the virulence of the recipient virus, indicating that the non-virulent phenotype of the PPMV-1 strain must be determined by other factors. The F proteins of all PPMV-1 strains known to date contain a multibasic cleavage site motif and are therefore probably functionally not different from those of virulent NDV strains.

Immune escape

Many viruses have evolved mechanisms to evade or antagonize the innate immune response of its host (54, 77, 86). For instance, the NS1 protein of influenza A virus contributes to viral pathogenesis, primarily by enabling the virus to disarm the type I interferon (IFN) defense system (32, 107). This phenomenon has also been well documented for paramyxoviruses, which have evolved mechanisms to escape or prevent both IFN production and IFN responsive signal transduction. In many cases, the paramyxovirus IFN evasion activities are mediated by the virus-encoded V protein. The paramyxovirus V protein is derived from the polycistronic P gene (94, 103) and is characterized by a cysteine-rich C-terminal domain that is highly conserved among several paramyxovirus species. The V protein has been demonstrated experimentally and crystallographically to bind two zinc atoms per protein chain (56, 74, 93). The contribution of the V protein to NDV virulence by acting as an interferon antagonist has been described (41, 61, 69, 70). In chapter 4 we show that exchanging the P gene, and thus also the V gene, between the virulent Herts strain and the non-virulent AV324 strain had no major effect on virulence as judged by its ICPI. Although the amino acid homology between the V proteins of Herts and AV324 is low (80%), our results suggest that there is no significant difference in interferon antagonistic function between the V proteins of both strains in young chickens.

Replication

A correlation between virulence and the efficiency of viral replication has been observed before. Although not fully understood, it is conceivable that higher levels of RNA synthesis lead to higher levels of viral replication and consequently to more virus production. This may overwhelm the host immune response hence causing enhanced pathogenesis. It has been reported for NDV that reduced levels of RNA synthesis are associated with reduced virulence (58). For several other paramyxoviruses such as measles virus (13, 99), respiratory syncytial virus and parainfluenza virus (64, 89), it has been described that determinants of virus attenuation are associated with mutations in the replication complex. For influenza virus the viral polymerase proteins PB2 and PA have been shown to contribute to virulence (80, 85, 91, 92). Also, the replicase gene of the avian coronavirus infectious bronchitis virus and mutations in the helicase of West Nile virus have been shown to be involved in pathogenicity (12, 15). In addition, transcriptional and translation control signals may also modulate virulence by controlling protein expression as has, for instance, been described for vesicular

stomatitis virus, measles, canine distemper virus and NDV (10, 14, 46, 71, 95, 110, 111).

In chapter 4 we describe the finding that all three proteins that make up the viral replication complex (NP, P and L) play a significant role in determining the virulence of NDV. By simultaneously exchanging these replication genes between Herts and AV324, the virulent Herts virus became significantly attenuated, whereas the low virulent AV324 became much more virulent. This was also confirmed by the *in vitro* results, which showed that the replication proteins of Herts are more active than those of AV324. However, the role of the individual replication proteins remains less conclusive. One of the possible explanations may be that the individual Herts replication proteins are inherently more active but that optimal activity is dependent on the presence of the cognate interaction partners. This feature has also been previously observed for the recovered influenza 1918 virus, where the gene segments show a synergistic effect (45). Another possible explanation might be that although the proteins of the two investigated viruses are compatible with each other, they originate from two distinct phylogenetic lineages or genotypes (2) and the exchange of entire genes might hence cause changes in specific interactions among the replication proteins that subsequently have an effect on interactions with yet unknown cellular factors.

The viral matrix (M) protein has been identified as another regulator involved in viral replication. While the M protein is considered to be the central organizer of viral morphogenesis and budding (51), it has also been found to interact with the replication complex and therefore affects viral transcription and/or replication, as has been shown for rhabdoviruses and some paramyxoviruses (29, 34, 44, 55, 68, 96). Furthermore, there are some studies showing that the M protein is also involved in inhibiting host cell functions (1, 33). It is known that the M protein traffics between the cytoplasm and the nucleus during the infection cycle (20, 36). However, whether this trafficking is related to the effect of M on host cell functions or viral transcription and replication still has to be elucidated.

Host adaptation

Several factors can determine the species specificity of a virus. Viruses of animal origin that have successfully entered the human species are, for example, human immunodeficiency virus (HIV), severe acute respiratory syndrome coronavirus (SARS) and influenza A virus. The viral genome probably needs multiple genetic changes to adapt successfully to a new host. These changes might include differential receptor usage, evasion of innate and adaptive host immune defenses and/or interactions with intracellular host proteins. Viruses, particularly RNA viruses, are change-adept and have several mechanisms for altering their genomes. The basic mechanisms are mutation, recombination and shuffling of gene segments by reassortment. If these alterations provide an advantage, these viruses become selected under certain conditions.

Mutation is an important mechanism of change for all RNA viruses because viral RNA-dependent RNA polymerases are error-prone and have no built-in mechanism for correcting mistakes (52). Polymerase errors, plus the lack of proofreading, results on average in about one mistake every 10,000 nucleotides. Furthermore, progeny viruses are produced in large numbers by infected cells. Most of these mutations will be deleterious, but occasionally a mutation will be advantageous. It has recently been shown in animal models of polio and foot-and-mouth disease that this ability to vary is important for virulence (87, 104). Other examples are viruses like measles, mumps and influenza, that need only a few mutations to attenuate or to increase their virulence after several passages (26, 59, 76). An additional mechanism for virus evolution is recombination, where two similar viruses that infect the same cell can recombine different portions of their genomes to produce new viruses. This is of current importance for poliovirus where the live virus vaccine includes three strains of polio that recombine with themselves and other enteroviruses to produce strains that may have increased virulence (19, 109). The most important virus that evolves by reassortment is influenza virus and this process has resulted in the antigenic shifts that occurred prior to the influenza pandemics of 1957, 1968 and, most recently, 2009 (30).

Strain AV324 belongs to the group of PPMV-1 viruses that cause minimal disease, but have a virulence potential in chickens that can emerge upon serial passages in these animals (8, 21, 49, 50). In the past, sequence analysis of such passaged viruses has mainly focused on the F gene and it was concluded that the F protein sequence had not changed and could thus not explain the increase in virulence (21, 22, 50). In chapter 5 we describe the accumulation of mutations in the P and L proteins of viruses which correlates with an increase in virulence upon passaging in chickens. These mutations resulted in more efficient replication both *in vitro* and *in vivo,* indicating that virulence of PPMV-1 for chickens is directly related to the efficiency of virus replication.

After five passages in chickens the AV324 virus had acquired three nucleotide mutations. It is considered likely that more mutations would arise upon further virus passage. Apparently, there is a selection for PPMV-1 mutants with enhanced replication efficiencies in chickens. This goes along with an increase in virulence which is probably not yet optimal, since the replication complex of the Herts virus is still more active. Because the NP protein also contributes to virulence as described in chapter 4, selection of adaptive mutations in the NP gene might eventually occur during further passage in this protein as well. Moreover, since the natural host for PPMV-1 is the pigeon, selection of chicken adaptive mutations in all proteins might ultimately arise. However, it is questionable whether the virulence for chickens of this particular PPMV-1 strain would ever reach the level of a high virulent APMV-1 strain. Further studies are required to address this issue.

The mechanism by which these adaptive mutations affect replication and/or transcription remains unclear. For these processes one of the most important interactions is that between the P and L proteins since these proteins form the viral polymerase complex. The finding that the P protein changed only after the acquisition of the two mutations in the L protein is suggestive of an interaction between the C-terminal region of the L protein and the N-terminal region of the P protein. This has also

been reported for vesicular stomatitis virus (VSV), the prototype rhabdovirus (17, 18, 28, 98). However, it is not the case for the paramyxoviruses Sendai, SV5 or measles virus. For these viruses, the domain required for L binding was mapped to the C-terminal region of the P protein while the N-terminal half of L protein is necessary for P binding (24, 39, 72, 90). Further investigations will be needed to map the interacting domains of the P and L proteins of NDV and to unravel the exact mechanisms of viral transcription and replication.

It would be interesting to repeat the passaging experiment in order to find out whether exactly the same adaptive mutations would arise. Furthermore, the order in which these mutations arise might also be important. While the N37D mutation in the P protein significantly increased the ICPI of the double L mutant rgAV324-$L^{N1564S}L^{V1694E}$, the same mutation did not result in an increase of the ICPI value of the parent virus rgAV324 (the ICPI value for rgAV324-P^{N37D} is 0.0; data not shown in chapter 5). It is also possible that the specific positions of certain amino acid mutations are less important as long as they occur in a specific interacting domain. Thus, the same changes in interaction between such domains may be determined by different combinations of amino acids. Notably, both adaptive mutations in the L protein changed away from the consensus sequence of known NDV sequences. This suggests that these mutations are not chicken specific, but rather compensate for suboptimal interaction(s) between the P and L proteins or for their interaction with yet unknown host factors. Further research will be necessary to elucidate the exact nature of these molecular interactions.

The fact that PPMV-1 strains that cause minimal disease in chickens can easily increase in virulence during passaging makes these viruses a serious threat to the poultry industry. Furthermore, although pigeons infected with AV324 do not develop disease, they do shed virus which can potentially be transmitted to susceptible birds, as we showed in chapter 6. It has been described before that PPMV-1 is capable of being transmitted from infected pigeons to chickens placed in contact (8), and several ND outbreaks in poultry have thus been attributed to PPMV-1 (6, 7, 9, 11, 43, 57, 108). The last few years in Europe there is even a trend of increasing NDV outbreaks in pigeons caused by PPMV-1 (16, 105). Therefore, such developments involving pigeon-derived paramyxoviruses should be closely monitored.

Determination of virulence

Low virulent NDV strains need the addition of exogenous trypsin to spread from cell to cell and to form syncytia in cell culture monolayers, whereas virulent strains do not (35, 82). Interestingly, an old study already suggested that the NDV plaque size correlates with virulence (79). In this thesis, we investigated the plaque size of the recombinant viruses in cell culture monolayers (chapters 3, 4 and 5). We showed that plaque size does not correlate with NDV virulence, confirming a recent study in which it was found that deletion mutant viruses which had plaque sizes comparable to the parental virus, still showed differences in attenuation in chickens (111). Other studies have shown that

the plaque size is highly dependent on the use of certain viral mutants, strains and cell types (38, 40, 63, 101). Hence, plaque size cannot be considered a reliable marker for viral virulence, at least not for NDV, leaving the study of this crucial biological feature unfortunately to the realm of animal experimentation.

Besides differences in plaque size we also observed a difference in plaque morphology in cell culture monolayers (chapter 3 and unpublished results). Recombinant viruses containing the AV324 F protein showed a more extensive cytopathic effect (CPE) than viruses containing the F protein from Herts. Several viruses are able to induce apoptosis in infected cells (27, 66, 73, 84, 88, 97) and it has been suggested that virus-induced apoptosis contributes to CPE. Recently, it was shown that also NDV-induced CPE *in vitro* is caused by apoptosis (78). It would be interesting to investigate which features of the F protein are involved in determining this difference in CPE.

Accepted *in vivo* tests for the assessment of virulence are the mean death time (MDT) in embryonated eggs, the intravenous pathogenicity index (IVPI) in six-week-old chickens and the intracerebral pathogenicity index (ICPI) in one-day old chickens. Although in most cases the MDT and the IVPI may give a useful indication of virulence, they are considered to be imprecise, particularly when used to assess viruses isolated from hosts other than chickens (3, 5, 75). Consequently, these assays are not considered reliable tests for the characterization of NDV isolates associated with outbreaks, and they are hence not included in Directive 92/66/EEC (23, 102). Therefore, we used the ICPI as a measure of virulence because of its established accuracy and sensitivity (3). The results obtained with this standardized method can easily be confirmed by repetition and therefore this method is generally accepted and used to characterize NDV strains (4). However, it should be noted that minor variations in the number of birds and time of onset of sickness may result in small differences in the ICPI value for viruses of low virulence. In chapter 4, for example, the ICPI values observed for rgAV324^{AFS}, rgAV324(NP)^{Herts} and rgAV324(P')^{Herts} (0.10, 0.04 and 0.25, respectively) should probably not be considered significantly different. Several repetitions of the test could make the ICPI values statistically more valid. However, in view of the relatively large numbers of animals required this is not desirable for ethical reasons. The use of the ICPI for virulence determination may be criticized because intracerebral inoculation is not the natural route of infection. It may, for instance, be possible that phenotypic differences observed after intracerebral infection are not observed when another route would be used. Therefore, it would be interesting to investigate the virulence of the recombinant viruses described in this thesis using a natural route of infection.

Final remarks

More and more studies illustrate that virus virulence is a complex trait determined by multiple genetic factors. The results presented in this thesis have contributed to our knowledge of NDV virulence. However, as mentioned in the previous paragraphs, there

are still many questions remaining about the molecular mechanisms used by these viruses to regulate transcription and translation, how these processes affect virulence, and how these viruses interfere with the host defense machinery. The development of a reverse genetics system for NDV has been a big step forward in order to be able to study these viruses. Genetic modification of the negative-strand RNA virus genome has made it possible to investigate the functions of the viral genes and proteins and their role in pathogenesis. However, the way in which these viruses were tested or applied in target animals has remained predominantly artificial. This applies in particular to the virulence test (ICPI) and the passaging experiment described in chapter 5 of this thesis. As a consequence, the results obtained with the animal experiments may be questioned regarding their relevance in a natural setting. Yet, while additional studies might try to overcome these considerations, the observations described in this thesis will serve as an important guide for our understanding of the biology of NDV under field circumstances.

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Nederlandse samenvatting

Dankwoord

Curriculum vitae

List of publications

Nederlandse samenvatting

Pseudovogelpest ofwel Newcastle disease (ND) is een van de belangrijkste infectieziekten bij vogels en kan grote economische schade veroorzaken in de pluimveesector. De ziekteverwekker is het Newcastle disease virus (NDV) dat voor het eerst werd beschreven in de jaren twintig van de vorige eeuw. De ziekte heeft zich wereldwijd verspreid en is inmiddels in veel landen endemisch. De ziekte van Newcastle behoort tot de aangifteplichtige ziekten. In 2010 werden uitbraken gemeld in België, Belize, Duitsland, Frankrijk, Honduras, Israël, Japan, Mongolië, Peru en Spanje. De verspreiding van het virus vindt vooral plaats via direct contact tussen gezonde en besmette vogels. Het virus wordt overgedragen via uitwerpselen en afscheidingen uit de snavel en de ogen. De algemene symptomen bij pluimvee zijn een daling van de eiproductie, verminderde eetlust, lusteloosheid, abnormale dorst en groenachtig diarree. Verder kunnen er problemen ontstaan met de luchtwegen, coördinatie stoornissen, zwelling van de weefsels rond de ogen en verlamming van de ledematen. Dieren kunnen heel plotseling sterven, sommige zonder duidelijke klinische symptomen.

NDV behoort tot de familie van de paramyxovirussen en heeft een RNA genoom dat de genetische code bevat voor de productie van verschillende virale eiwitten. Het fusie (F) en het haemagglutinine-neuraminidase (HN) eiwit zijn envelop eiwitten die samen een complex vormen en betrokken zijn bij herkenning van, binding aan en het binnendringen van de gastheercel. Een andere structurele component van het virusdeeltje is het matrix (M) eiwit. Het nucleoprotein (NP), het phosphoprotein (P) en het polymerase (L) eiwit zijn betrokken bij transcriptie en replicatie van het virale genoom.

Virulentie is de mate van ziekteverwekkend vermogen van een virus. Deze virulentie wordt bepaald door meerdere genetische factoren die een rol kunnen spelen bij bijvoorbeeld het bepalen van het gastheer- of weefsel-tropisme, het vermogen om te ontsnappen aan het immuunsysteem van de gastheer, en/of de snelheid van virusvermenigvuldiging (replicatie efficiëntie). De virulentie van verschillende NDV stammen kan sterk variëren. Er bestaan laag-virulente (lentogene) stammen die vrijwel geen klinische symptomen veroorzaken, mild-virulente (mesogene) stammen die milde symptomen veroorzaken en virulente (velogene) stammen die ernstige ziekte en sterfte veroorzaken.

Er bestaat een duidelijke correlatie tussen de mate van virulentie en de aminozuursequentie rondom de proteolytische splitsingsplaats van het F eiwit van NDV. Om een nieuwe infectie van het nakomeling-virus mogelijk te maken is splitsing van het F eiwit in F1 en F2 door gastheerproteasen essentieel. Mesogene en velogene stammen hebben een multi-basisch aminozuurmotief dat herkend en gesplitst wordt door intracellulaire furine-achtige proteasen. Deze proteasen komen in vrijwel alle weefsels en organen voor en het virus kan zich daarom door vrijwel het gehele lichaam verspreiden. Het aminozuurmotief in lentogene virussen is niet multi-basisch en wordt alleen herkend en gesplitst door extracellulaire trypsine-achtige proteasen. Deze

proteasen komen vrijwel uitsluitend voor in het ademhalingsstelsel en het maag-darmkanaal en een infectie met deze virussen blijft daarom grotendeels tot deze locaties beperkt.

De mate van virulentie van een NDV stam wordt bepaald door middel van een gestandaardiseerde methode genaamd de intracerebrale pathogeniteits index (ICPI). In deze test worden tien eendagskuikens in de hersenen geïnoculeerd. Vervolgens worden de dieren elke 24 uur klinisch geobserveerd en gescoord met 0 als ze normaal zijn, 1 als ze ziek zijn, en 2 als dood zijn. De ICPI is de gemiddelde score per vogel per waarneming in een 8-daagse periode en kan variëren van 0.0 tot 2.0. Velogene stammen hebben een waarde van boven de 1.5 en de waarde voor lentogene stammen ligt onder de 0.7. Hoewel de aminozuurvolgorde van de splitsingsplaats van het F eiwit de primaire virulentiefactor is, spelen andere virale eigenschappen ook een belangrijke rol in het bepalen van de mate van NDV virulentie. Deze andere virale determinanten zijn nog onvoldoende in kaart gebracht.

In de jaren tachtig van de vorige eeuw veroorzaakte ND veel sterfte in duiven en de uit zieke of dode dieren geïsoleerde virussen bleken duidelijk van de klassieke NDV stammen te verschillen. De ziekte verspreidde zich wereldwijd als gevolg van contact tussen duiven tijdens wedstrijden en tentoonstellingen en door de internationale handel in deze vogels. De virusstammen lieten een compleet ander bindingspatroon zien met antilichamen gericht tegen het F en HN eiwit. Tegenwoordig kunnen deze virussen worden onderscheiden door fylogenetische analyse van de nucleotidenvolgorde van het F gen. Deze NDV varianten worden dan ook aangeduid als pigeon paramyxovirus type 1 (PPMV-1) virussen. Een aantal ND uitbraken bij pluimvee zijn toegeschreven aan PPMV-1, waardoor deze duivenvirussen een reële en continue bedreiging vormen voor de pluimveesector.

Het F eiwit van alle tot nu toe bekende PPMV-1 stammen bevat een multi-basisch aminozuurmotief, een kenmerk dat geassocieerd wordt met hoge virulentie. Echter, sommige PPMV-1 stammen hebben een lage ICPI waarde in kippen en veroorzaken slechts minimale klinische symptomen. Wel zijn deze stammen potentieel virulent hetgeen blijkt uit een toename in virulentie na herhaalde passages in kippen. Omdat sequentieanalyse van dergelijke gepasseerde virussen zich vooral geconcentreerd heeft op het F gen en daarin geen mutaties werden aangetroffen, is het mechanisme van de virulentietoename tot nu toe onopgehelderd gebleven. Vast staat echter dat na transmissie van duiven naar kippen, PPMV-1 stammen kunnen evolueren naar meer virulente virussen en kunnen leiden tot ND uitbraken.

Het doel van het onderzoek beschreven in dit proefschrift is het bepalen welke andere virale factoren, naast de splitsingsplaats van het F eiwit, bijdragen aan de virulentie van NDV en waarom sommige PPMV-1 stammen niet virulent zijn voor kippen, ondanks de aanwezigheid van een multi-basisch aminozuurmotief in het F eiwit. Daarbij rijst tevens de vraag wat het moleculaire adaptatie mechanisme van PPMV-1 is als het wordt gepasseerd in kippen en vervolgens een verhoogde virulentie verkrijgt.

Om antwoorden te vinden op deze vragen is het belangrijk om de studies uit te voeren met een virus met dezelfde goed-gedefinieerde genetisch achtergrond. Dit bleek in hoofdstuk 2, waarin werd geprobeerd om de moleculaire basis op te helderen van het verschil in virulentie van twee genetisch nauw verwante PPMV-1 varianten met sterk contrasterende virulentiepatronen. Met behulp van het *reverse genetics* systeem kan het virale genoom worden gemodificeerd en wordt het mogelijk om de rol van virale genen en eiwitten te onderzoeken. Echter, omdat er op dat moment geen reverse genetics systeem voor het PPMV-1 virus beschikbaar was, moest gebruik worden gemaakt van een ander, wel beschikbaar recombinant kippenvirus. Dit virulente virus is afgeleid van het NDV Herts virus dat tot een ander fylogenetisch genotype behoort dan de onderzochte PPMV-1 isolaten. Helaas bleek het onmogelijk om met behulp van deze aanpak de fenotypische verschillen tussen beide PPMV-1 varianten te verklaren.

Het maken van een infectieuze cDNA kloon van een PPMV-1 virus was dus essentieel. Zoals beschreven in hoofdstuk 3 had het recombinant PPMV-1 virus AV324 een pathogeniteitsindex (ICPI) van 0.0, wat betekent dat het virus niet virulent is voor kippen. Bovendien bleek uit onderzoek met virussen, waarbij het F gen van het virulente Herts virus en van het laag virulente virus AV324 werden uitgewisseld, dat het recombinant virus geen verschil in virulentie vertoonde ten opzichte van het betreffende ouder-virus. Dit betekent dat het F eiwit van PPMV-1 virussen qua functionaliteit niet verschilt van dat van virulente NDV stammen. Geconcludeerd kon worden dat de multi-basische splitsingsplaats van het F eiwit niet noodzakelijkerwijs gecorreleerd is met een hoge virulentie in kippen.

In hoofdstuk 4 wordt beschreven dat alle drie de eiwitten die het virale replicatie complex vormen (NP, P en L) een belangrijke rol spelen bij het bepalen van de virulentie van NDV. Door gelijktijdig deze replicatiegenen uit te wisselen tussen het virulente Herts virus en het avirulente AV324 virus, nam de virulentie van het Herts virus aanzienlijk af, terwijl de virulentie van AV324 sterk toenam. Uit *in vitro* testen bleek dat de replicatie-eiwitten van Herts veel actiever zijn dan die van AV324. De hoeveelheid virus die gevonden werd in geïnfecteerde cellen en in geïnfecteerde kippen was significant hoger voor het Herts virus ten opzichte van het AV324 virus. Het matrix (M) eiwit bleek ook een invloed te hebben op het virale replicatiemechanisme, maar had minder invloed op de virulentie van het virus.

Mutaties vormen een belangrijk mechanisme voor de verandering van het fenotype van alle RNA-virussen. Omdat virale RNA-afhankelijke RNA polymerasen relatief veel fouten maken en ze geen ingebouwd mechanisme hebben voor het corrigeren van deze fouten, leidt dit gemiddeld tot ongeveer één fout op elke 10.000 nucleotiden. Veel van de door geïnfecteerde cellen geproduceerde nakomeling-virussen bevatten dan ook mutaties. De meeste van deze mutaties zijn schadelijk en daardoor zullen deze virussen niet levensvatbaar zijn. Maar af en toe kan een mutatie ook heel nuttig zijn. In dat geval zal het virus met de betreffende mutatie de meerderheid in de viruspopulatie vormen. Stam AV324 behoort tot de groep van PPMV-1 virussen die minimale ziekte veroorzaken, maar die meer virulent kunnen worden in kippen gedurende seriële passages in deze dieren. Vijf passages in kippen zorgden ervoor dat het virus meer virulent werd en sequentieanalyse liet zien dat er drie mutaties geselecteerd waren;

twee in het L eiwit en één in het P eiwit. In zowel celkweek als in geïnfecteerde kippen leidden deze mutaties tot een verhoogde replicatiesnelheid van het virus. Dit impliceert dat virulentie van PPMV-1 voor kippen direct gerelateerd is aan de efficiëntie van virusreplicatie.

Het feit dat PPMV-1 stammen die minimale ziekte veroorzaken in kippen gemakkelijk een verhoogde virulentie kunnen verkrijgen tijdens herhaalde passage in kippen maakt deze virussen een serieuze bedreiging voor de pluimveesector. Er is beschreven dat PPMV-1-geïnfecteerde duiven in contact geplaatste kippen kunnen besmetten, en verschillende ND uitbraken bij pluimvee worden toegeschreven aan PPMV-1. De laatste jaren is er in Europa zelfs een trend van toenemende ND uitbraken in duiven veroorzaakt door PPMV-1. Hoewel AV324-geïnfecteerde-duiven niet ziek worden van de infectie, zijn ze wel in staat om grote hoeveelheden virus uit te scheiden hetgeen potentieel gevaarlijk kan zijn voor vatbare dieren (hoofdstuk 6).

Concluderend kan worden gezegd dat het onderzoek zoals beschreven in dit proefschrift een belangrijke bijdrage heeft geleverd aan de kennis van virulentie van NDV. Het replicatiecomplex speelt niet alleen een belangrijke rol in het bepalen van de mate van virulentie van NDV, maar is ook betrokken bij gastheer adaptatie. In het licht van de huidige aanhoudende PPMV-1 epidemie is het niet ondenkbeeldig dat deze virussen uitbraken kunnen veroorzaken in pluimvee en het is daarom raadzaam om de aanwezigheid van deze duivenvirussen goed te monitoren.

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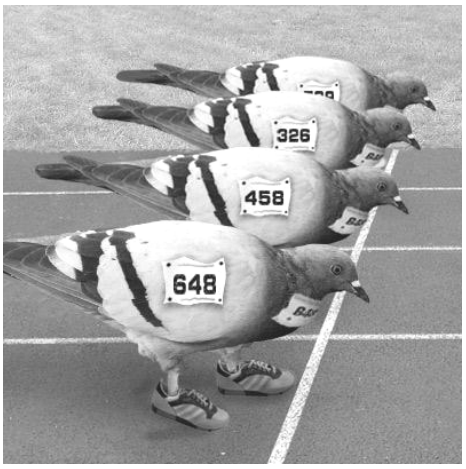

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Curriculum vitae

Johannes Cornelius Franciscus Maria (Jos) Dortmans werd op 29 augustus 1977 geboren te Veghel. In 1994 behaalde hij het HAVO diploma en twee jaar later het VWO diploma aan het Comenius College in Uden. In 1996 begon hij de studie Bioprocestechnologie aan de Wageningen Universiteit. Tijdens deze studie heeft hij bij de vakgroep Virologie gewerkt aan baculovirussen. Bij de vakgroep Celbiologie en Immunologie heeft hij onderzoek gedaan naar NK-receptoren van de karper. De stage vond plaats bij de vakgroep Entomologie van het onderzoeksinstituut CSIRO in Canberra, Australië. Daar werkte hij aan een biopesticide gebasseerd op een tetravirus. Na het behalen van het diploma in 2003 trad hij in dienst als onderzoeksassistent bij de Animal Sciences Group van Wageningen UR. Hier deed hij onderzoek naar klassieke varkenspest en het effect van intradermale vaccinatie. In januari 2006 is begonnen met het promotie onderzoek bij de Faculteit Diergeneeskunde van de Universiteit Utrecht en werd hij gestationeerd bij het Centraal Veterinair Instituut van Wageningen UR (CVI). Dat heeft geleid tot dit proefschrift. Vanaf 1 juli 2010 is hij werkzaam bij het CVI als onderzoeker op het project: “Nieuwe generatie Newcastle disease vaccins”.

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