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# 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

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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 was not 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 index 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 caused by Newcastle disease virus (NDV), or avian paramyxovirus type 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* (Mayo, 2002) and has a non-segmented negative-strand RNA genome consisting of six transcriptional units (Lamb & Parks, 2007). These encode at least six proteins: the nucleocapsid protein (NP), the phosphoprotein (P), the matrix protein (M), the fusion protein (F), the haemagglutinin-neuraminidase (HN) and the polymerase (L) protein. During P gene transcription an additional, non-structural protein (V) is produced by means of mRNA editing (Steward *et al.*, 1993) and functions as an interferon antagonist (Park *et al.*, 2003). 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 (Nagai *et al.*, 1976; Ogasawara *et al.*, 1992). 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 (Lamb & Parks, 2007). 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 (Alexander *et al.*, 1985a). Several Newcastle disease outbreaks in chickens have been attributed to PPMV-1, which makes it a real threat to the poultry industry (Alexander *et al.*, 1985b, 1997, 1998; Anonymous, 1984; Irvine *et al.*, 2009; Liu *et al.*, 2006; Werner *et al.*, 1999). The F proteins of all PPMV-1 strains examined to date contain a poly-basic cleavage site motif, a

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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 (Meulemans *et al.*, 2002). Nevertheless, they do have a virulence potential for chickens, which can emerge upon serial passages in these animals (Alexander & Parsons, 1984; Collins *et al.*, 1994; Kommers *et al.*, 2001, 2003). 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 (Collins *et al.*, 1994, 1996; Kommers *et al.*, 2003). 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 (Dortmans *et al.*, 2009).

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 (Dortmans *et al.*, 2010b). 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.

## 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 World Organization for Animal Health (OIE)-prescribed virulence test for NDV, which is expressed as the 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 ICPI test in day-old chickens (Table 1). 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. 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

**Table 1.** 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 | $P^{N37D}L^{N1564S}L^{V1694E}$ |

mutations in the L protein had been maintained and an additional mutation was observed in the P protein, N37D (Table 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 with 133 available sequences in GenBank, but adaptation did not cause a change into the NDV consensus, which is S37.

### Increase in virulence is associated with the accumulation of 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 (the cDNA clone of PPMV-1 isolate AV324/96), resulting in rgAV324-LL and rgAV324-PLL, respectively. A schematic illustration of the viral genomes is shown in Fig. 2. Because the $L^{V1694E}$ mutation had no effect on the ICPI (Table 1), we did not attempt to construct the single mutant rgAV324-$L^{V1694E}$. Viruses were rescued as described in Methods. 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. 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. 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.

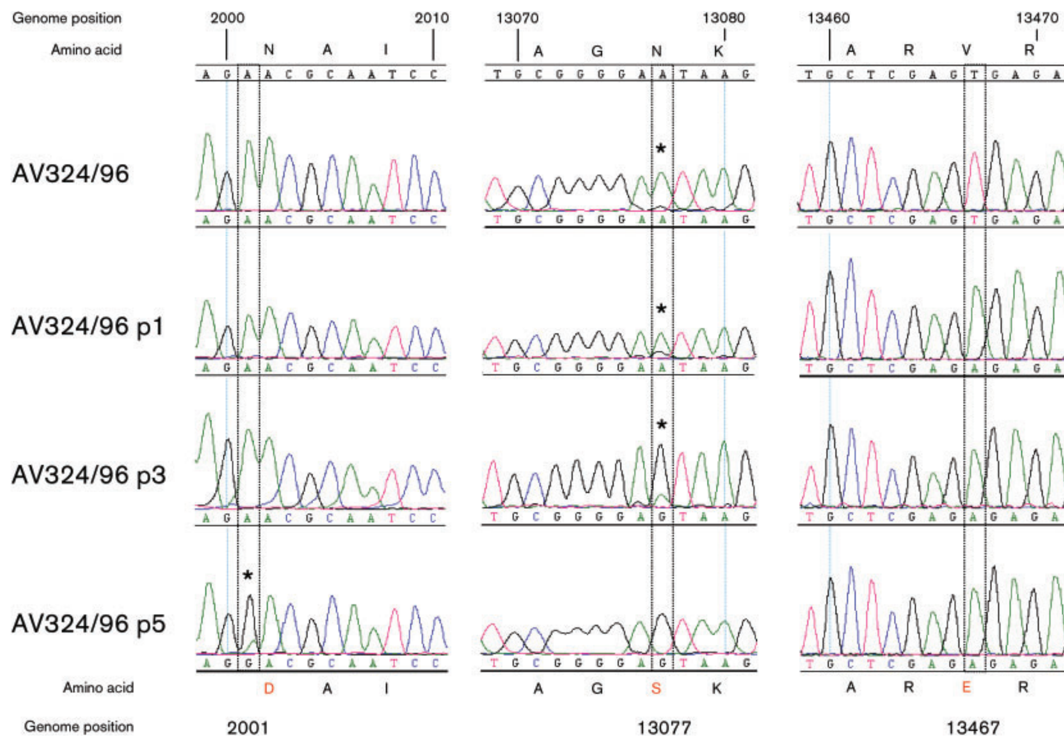


**Fig. 1.** Nucleotide sequences in the regions of the adaptive mutations acquired upon virus passaging and corresponding electropherograms. The genome regions (nucleotide sequence numbers) and the amino acids involved are depicted at the top. The left column represents the region in the P gene, whereas 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.

## 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. 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. 3a). However, in DF-1 cells the presence of the LL and PLL

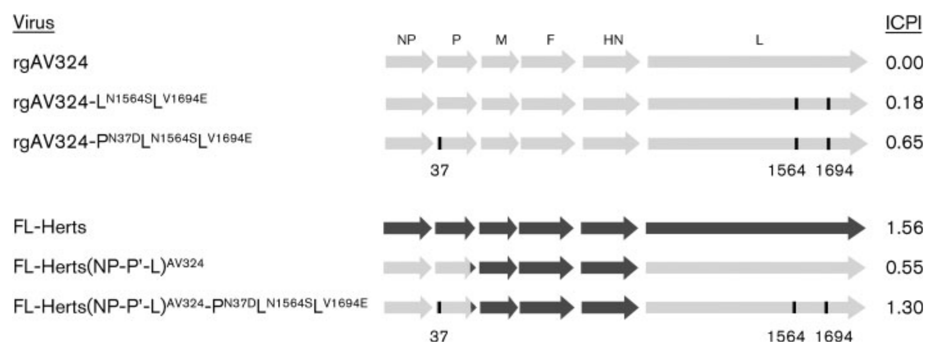


**Fig. 2.** Schematic illustration of the genomic organization of rgAV324 (light grey) and FL-Herts (dark grey). The positions of amino acid changes are indicated and refer to the mutations observed after serial passage (Table 1). The virulence of the parental viruses and their recombinants containing the mutations was determined by the ICPI in day-old chickens (ICPI score: maximum 2.0).

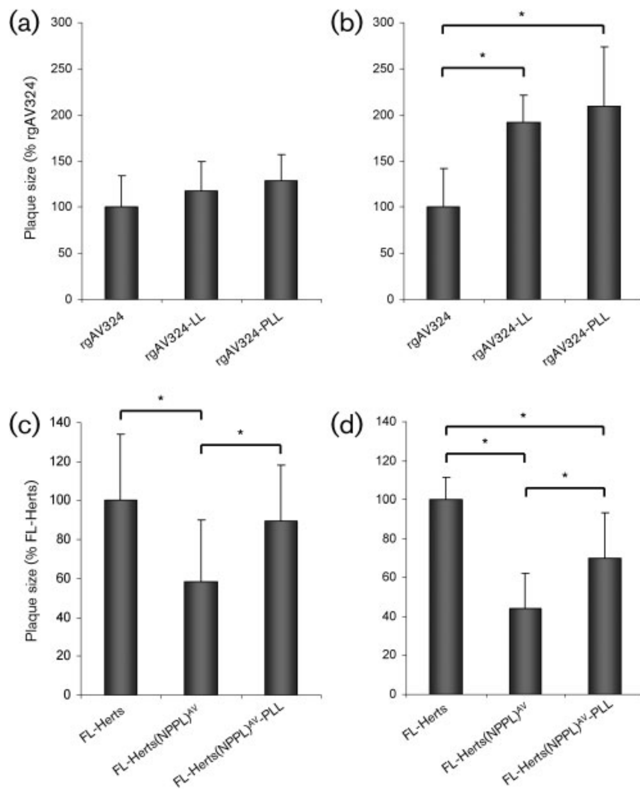


**Fig. 3.** Relative plaque size of the recombinant viruses in QM5 cells (a, c) and DF-1 cells (b, d) 48 h p.i. (a and b) rgAV324 and its derivatives containing the $L^{N1564S}/L^{V1694E}$ mutations (LL) or the $P^{N37D}/L^{N1564S}/L^{V1694E}$ mutations (PLL). (c and d) FL-Herts and the recombinants 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 SD. Differences were significant (*) when $P<0.05$.

mutations resulted in an almost twofold increase in plaque size (Fig. 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 with the virulent Herts virus, the PLL mutations almost completely compensated for this decrease (Fig. 3c, d).

## *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 quantify replication activity we used a plasmid harbouring 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. 4). Furthermore, the P mutation N37D had a significant additional effect. Yet, even with the three mutations combined, the AV324 replication proteins did not reach the level obtained with the Herts-virus-derived replication proteins (Fig. 4).

## 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 h 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

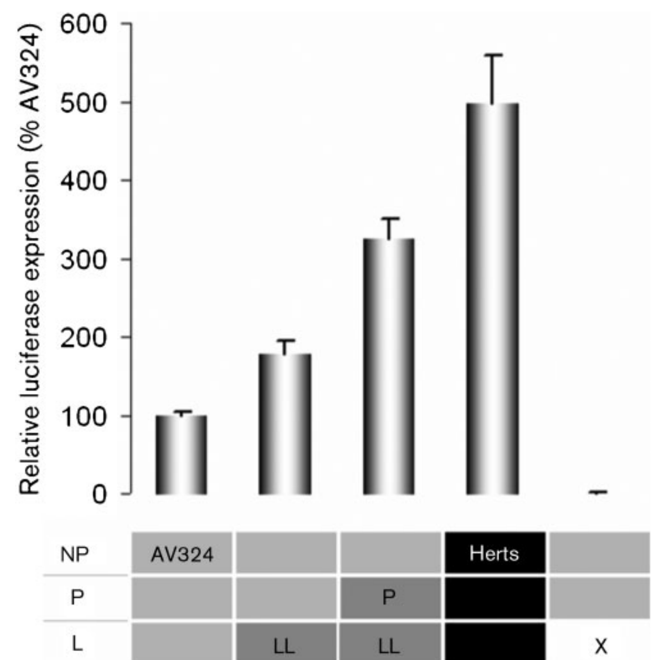


**Fig. 4.** Relative luciferase expression levels after cotransfection of fowlpox recombinant virus fpE-FLT7pol (FPV-T7)-infected QM5 cells with the viral minigenome plasmid (containing the leader and trailer sequences of AV324 flanking the Gluc reporter gene) and plasmids expressing NP, P and L of AV324 (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 represent SEM. All differences were significant ($P<0.05$).

mutant rgAV324-PLL showed an increased replication rate (Fig. 5), which indicates that the three mutations are responsible for the enhanced replication efficiency in QM5 cells.

### *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. 6). The results showed that in brain tissue there is little replication of any of the viruses except for rgAV324-PLL. 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.

## DISCUSSION

Pigeon paramyxoviruses are a hidden threat to the poultry industry. Though generally of low virulence or non-virulent 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. 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. Firstly, the virus containing the three mutations showed enhanced replication in cell culture as evidenced by the increase in plaque size (Fig. 3). Secondly, the viral genome replication rate increased as a result of the mutations (Fig. 5). Thirdly, these mutations led to an increase in the activity of the viral polymerase complex as shown by an *in vitro* replication assay (Fig. 4). Finally, recombinant viruses containing these mutations replicated to higher titres in the organs of infected chickens (Fig. 6).

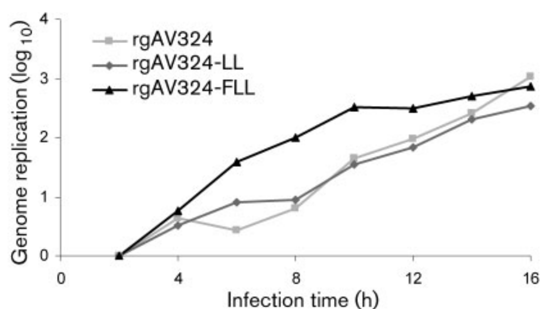


**Fig. 5.** The relative negative-sense genomic RNA replication in the first 16 h of infection. QM5 cells were infected with an m.o.i. of 10 of rgAV324, rgAV324-LL or rgAV324-PLL and replication was determined by qRRT-PCR. Results are the mean of two separate experiments.

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 (Dortmans *et al.*, 2010b). When comparing two NDV strains within one genotypic lineage, the L protein alone was characterized as being important (Rout & Samal, 2008). For other paramyxoviruses such as measles virus (Bankamp *et al.*, 2002; Takeda *et al.*, 1998), respiratory syncytial virus and parainfluenza virus (Murphy & Collins, 2002; Skiadopoulos *et al.*, 1998) 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 p5 chicken-selected PPMV-1 are localized within the L protein. This protein is conserved among all members of the order *Mononegavirales* and contains six conserved domains that constitute its enzymic activities (Poch *et al.*, 1990; Sidhu *et al.*, 1993; Wise *et al.*, 2004). 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 viruses in the order *Mononegavirales* and it has been suggested that this particular region evolved separately between individual viruses (Poch *et al.*, 1990; Sidhu *et al.*, 1993). 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 (Hamaguchi *et al.*, 1983; Jahanshiri *et al.*, 2005; Kingston *et al.*, 2004; Lamb & Parks, 2007) 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 (Jahanshiri *et al.*, 2005), 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

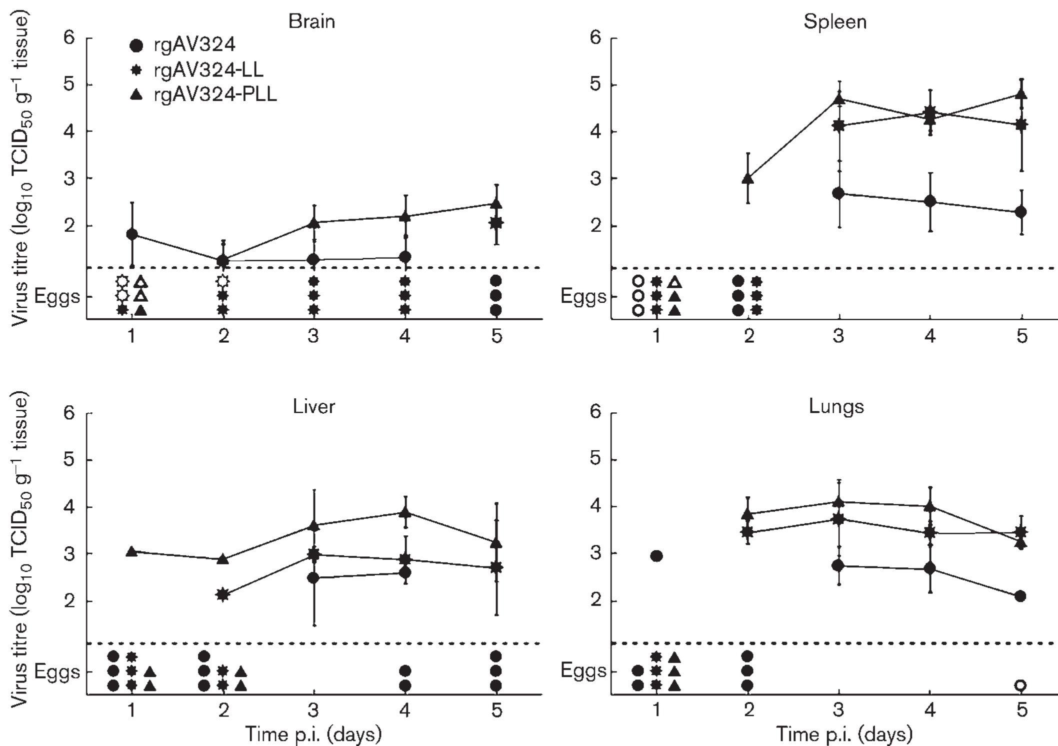


**Fig. 6.** Viral titres of rgAV324 and its derivatives (rgAV324-LL and rgAV324-PLL) containing the LL or PLL mutations acquired during serial passage (Table 1) after intracerebral inoculation of day-old chickens. Each day 3 chickens were sacrificed and brain, spleen, liver and lungs were collected. Virus titres were determined using QM5 cells and are presented as the mean virus titre ($\log_{10}$ $TCID_{50}$ $g^{-1}$ 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 represent SD.

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. 3). Other studies have already shown that plaque size is highly dependent on the use of certain viral mutants, strains and cell types (Hohdatsu *et al.*, 1990; Horimoto & Kawaoka, 1997; Moore *et al.*, 2002; Tearle *et al.*, 2003). For avian influenza virus it has been described that adaptation to a mammalian host is often associated with changes in the viral polymerase complex (Gabriel *et al.*, 2005). Furthermore, particular mutations in PB2 and PA of influenza virus may influence pathogenesis in mice or humans (de Wit *et al.*, 2010; Hatta *et al.*, 2001; Rolling *et al.*, 2009; Song *et al.*, 2009). 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 (Ackermann *et al.*, 2007). 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 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 Newcastle disease 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 bottleneck procedure such as 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. 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. 4). Studies have shown that passaging of PPMV-1 isolates in chickens does not always lead to a virulence increase for chickens. It appears that it is the particular virus strain that determines the outcome, not the particular passage procedure utilized (Alexander & Parsons, 1984, 1986; King, 1996; Kommers *et al.*, 2001, 2003).

The ICPI levels of the passaged viruses differed from those observed for their corresponding recombinant viruses. While, for example, p5 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. 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. 1). Yet, by using viruses generated by reverse genetics we demonstrate 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.

## 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). Both media were supplemented with 5 % FBS and 1 % of an antibiotic stock consisting of penicillin (100 U $ml^{-1}$) and streptomycin (100 μg $ml^{-1}$).

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 (de Leeuw *et al.*, 2005; Dortmans *et al.*, 2009). The fowlpox recombinant virus fpE-FLT7pol (Britton *et al.*, 1996) (designated FPV-T7), which expresses the bacteriophage T7 RNA polymerase, was used as recently described (Dortmans *et al.*, 2010a).

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 ICPI test in 1-day-old (referred to as 'day-old') chickens was performed as described in The Council of the European Communities (1992) directive 92/66/EEC. Of the chickens that survived the ICPI test, sera were tested in a haemagglutination inhibition assay (The Council of the European Communities, 1992) 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 reisolation using embryonated SPF eggs was performed as previously described (de Leeuw *et al.*, 2003).

The PPMV-1 isolate AV324/96 (Dortmans *et al.*, 2009) is of low virulence for chickens (ICPI=0.44), but contains a multi-basic F protein cleavage site motif that is typically associated with virulent NDV viruses. Five serial passages were performed by inoculating day-old chickens intracerebrally, as described for the ICPI test (The Council of the European Communities, 1992). Brain tissue from dead chickens was collected and virus was reisolated 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 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 analyser (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 (Dortmans *et al.*, 2009) 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 (Dortmans *et al.*, 2010b), 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 (Dortmans *et al.*, 2009). The respective expression plasmids of either rgAV324 or FL-Herts (de Leeuw *et al.*, 2005) 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 mAb Fusie 133 8E12A8C3 (CVI of Wageningen UR) against the NDV F protein and HRP-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). Differences in plaque size were statistically analysed 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 (Dortmans *et al.*, 2010b). 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 (Dortmans *et al.*, 2009) or from the Herts strain (de Leeuw *et al.*, 2005) 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 analysed using the Wilcoxon test and considered significant when the *P* value was less than 0.05.

**Analysis of genome replication by quantification of negative-sense genomic RNA.** To investigate the onset of virus replication, the relative amount of negative-sense genomic RNA was determined by qRRT-PCR at different time points post-infection (p.i.). The qRRT-PCR targets a 129 nt fragment in the L gene, as previously described (Dortmans *et al.*, 2010b). QM5 cells were seeded in 12-well plates (Greiner) and infected in duplicate with virus at an m.o.i. of 10. At 2, 4, 6, 8, 10, 12, 14 and 16 h p.i. plates were frozen at $-70$ °C. 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 day-old chickens.** To compare the *in vivo* replication properties of rgAV324, rgAV324-LL and rgAV324-PLL, day-old SPF chickens were inoculated intracerebrally with $2 \times 10^3$ $TCID_{50}$ of virus per animal. Three birds were sacrificed daily until 5 days p.i. 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 & Muench (1938) method and are expressed as $\log_{10}$ $TCID_{50}$ $g^{-1}$ 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 (Dortmans *et al.*, 2010b). Tests over time were performed and the chicken groups infected with virus rgAV324-LL or rgAV324-PLL were compared with the rgAV324-infected chickens, employing Wilcoxon rank sum test (Conover, 1980). Differences were considered to be significant when $P<0.05$. All calculations were performed with GenStat (Payne *et al.*, 2009).

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