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Short Communication

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

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Some pigeon paramyxovirus type 1 (PPMV-1) strains exhibit low virulence in chickens, despite their fusion (F) protein's multi-basic cleavage site. To elucidate the molecular basis of the low pathogenicity of these strains, 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 AV324 and a virulent Newcastle disease virus (NDV) strain (Herts) and evaluated the recovered chimeric viruses for their pathogenicity in 1-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.

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*) (Mayo, 2002). 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 1-day-old chickens (Alexander & Gough, 2003).

The amino acid sequence at the fusion (F) protein cleavage site has been shown to be a major determinant of NDV virulence (Nagai *et al.*, 1976; Ogasawara *et al.*, 1992). Cleavage of the precursor glycoprotein F0 into F1 and F2 by host-cell proteases is essential for progeny virus to become infective (Garten *et al.*, 1980; Nagai *et al.*, 1976; Rott & Klenk, 1988). Lentogenic viruses have a monobasic cleavage motif and are processed extracellularly by trypsin-like proteases found in the respiratory and intestinal tracts. Mesogenic and velogenic strains have a dibasic motif in their F protein and can be cleaved intracellularly by ubiquitous furin-like proteases, resulting in a systemic infection (Nagai *et al.*, 1976; Ogasawara *et al.*, 1992).

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 (Meulemans *et al.*, 2002). To study this remarkable behaviour, particularly the role of the F protein, we used PPMV-1 isolate AV324/96p1, originally isolated from a racing-pigeon loft in Ireland by H. de Geus (PV 17/96), that we obtained from the Veterinary Laboratories Agency (Addlestone, Surrey, UK). This isolate is low virulent for chickens (ICPI 0.44), but contains an F protein cleavage-site motif, ^{112}RRKKRF^{117}, typically associated with virulent viruses; it 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 first passaged three times by limiting dilution in specific-pathogen-free eggs and subsequently plaque-purified three times in QM5 cells (Antin & Ordahl, 1991), resulting in a virus (AV324/96p4pp) with an ICPI of 0.00. To prepare a cDNA clone of this virus, genomic RNA was isolated and first-strand cDNA was synthesized by using SuperScript III reverse transcriptase (Invitrogen). Five overlapping subgenomic cDNA fragments were generated, purified and subsequently sequenced (BaseClear, Leiden, The Netherlands). The sequences of the 3′- and 5′-terminal genomic ends were determined as described previously (de Leeuw & Peeters, 1999). Primer sequences used for generation of the overlapping subgenomic cDNA fragments and for genome sequencing are available upon request. The assembled genomic sequence consists of 15 192 nt (GenBank accession no. GQ429292), which puts

The GenBank/EMBL/DDBJ accession number for the sequence of AV324 is GQ429292 and that for Dove/Italy/2736/00 is GQ429293.

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 (Ujvari, 2006) and 248VB (Fuller *et al.*, 2007) (data not shown). Comparison of the AV324 genome sequence 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 (GenBank accession no. AY562989), of which an ICPI of 1.2–1.3 has been documented (Pedersen *et al.*, 2004; Terregino *et al.*, 2003). 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 %) to vaccine strains B1 (GenBank accession no. AF375823), Clone 30 (accession no. Y18898) and LaSota (accession nos AF077761 and AY845400), but not to other known PPMV-1 sequences, made us decide to resequence the complete genome of Dove/Italy/2736/00 (provided by Istituto Zooprofilattico Sperimentale delle Venezie, Italy). The new sequence (GenBank accession no. GQ429293) lacked the vaccine-resembling sequences and had 97 % overall similarity to 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: 10 amino acids differing in NP, nine in P, nine in M, seven in F, seven in HN and 11 in L. Thus, the comparison did not allow us to pinpoint unambiguously the molecular basis of the non-virulent phenotype of AV324/96p4pp.

To establish a reverse-genetics system for AV324/96p4pp, we constructed the full-size cDNA clone and the NP, P and L expression plasmids as described previously (de Leeuw *et al.*, 2005; Peeters *et al.*, 1999). Details of this construction are available upon request. To rescue virus from the cDNA, QM5 cells were infected with recombinant fowlpox virus FPV-T7 (Britton *et al.*, 1996) and subsequently co-transfected with full-length cDNA constructs and helper plasmids expressing P and L as described previously (de Leeuw *et al.*, 2005; Peeters *et al.*, 1999); inclusion of the NP-expressing helper plasmid was not essential. Virus designated rgAV324 could be rescued and was completely non-virulent in chickens, similar to the parental virus AV324/96p4pp, as evidenced by an ICPI of 0.00.

In view of its well-established critical role in determining NDV virulence, we focused our study on the F protein. 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 NDV strain FL-Herts (de Leeuw *et al.*, 2005) to exchange the F genes. PCR mutagenesis was used to introduce the unique restriction sites *Asc*I (position 4533) and *Fse*I (position 6353) into the rgAV324 cDNA (Fig. 1). To introduce the *Fse*I site, two PCRs were performed, one with primers p5380 (5′-GACTCAGATCTTGGGTATACAG-3′) and pAV-FseIR (5′-TGGAGGCCGGCCTCTCCGACCG-3′) and the other with primers pAV-FseIF (5′-GAGAGGCCGGCCTCCAA-TCAGG-3′) and p7093 (5′-GTTGATGGAACGCAGA-GTAG-3′), using rgAV324 as a template. The two overlapping PCR fragments were joined in a second PCR using primers p5380 and p7093. The resulting PCR fragment was digested with *Bst*Z17I and *Sbf*I and cloned into a subclone. The *Pml*I–*Sbf*I fragment of this subclone was subsequently cloned into rgAV324, resulting in rgAV324^{F}. To introduce the *Asc*I site into rgAV324^{F}, two PCRs were performed, either with primers p3001 (5′-CTAAGCTTCTGAGTAAGTTG-3′) and pAV-AscIR (5′-TTGGGCGCGCCAGCCGGGATCCAG-3′) or with pAV-AscIF (5′-GGCTGGCGCGCCCAAAGTGCAATA-3′) and p5900R (5′-ATAACTTGAGGATCTAGTATT-3′), using rgAV324^{F} as a template. The two overlapping PCR fragments were joined in a second PCR using primers p3001 and p5900R. The resulting PCR fragment was digested with *Pac*I and *Sbf*I and cloned into rgAV324^{F}, 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 cloned reciprocally into rgAV324^{AF} and FL-Herts^{AF} (J. C. F. M. Dortmans, C. M. Fuller, E. W. Aldous, P. J. M. Rottier & B. P. H. Peeters, unpublished data), resulting in plasmids rgAV324(F)^{Herts} and FL-Herts(F)^{AV324}, respectively (Fig. 1). Both chimeric viruses could be rescued,

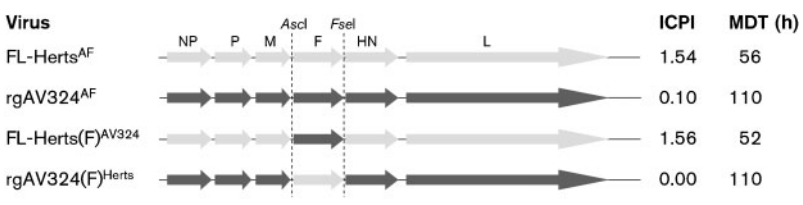

Fig. 1. Schematic illustration of the cloning strategy used to exchange the F gene between FL-Herts^{AF} and rgAV324^{AF}, resulting in FL-Herts(F)^{AV324} and rgAV324(F)^{Herts}, and virus virulence as determined by ICPI and mean death time (MDT). ICPI values range from 0.0 for non-virulent viruses to 2.0 for highly virulent viruses and the MDT is >90 h for lentogenic strains, 60–90 h for mesogenic strains and <60 h for velogenic strains.

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 as described in the European Community Council Directive 92/66/EEC (Council of the European Communities, 1992). FL-Herts^{AF} and FL-Herts(F)^{AV324} had ICPI values 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. 1). In embryonated eggs, strains FL-Herts^{AF} and FL-Herts(F)^{AV324} had MDT values of 56 and 52 h, respectively, compared with 110 h for both rgAV324^{AF} and rgAV324(F)^{Herts} (Fig. 1). 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. Apparently, whilst the multi-basic amino acid cleavage motif is an absolute prerequisite, other decisive factors contribute critically to the virulence phenotype of at least some PPMV-1 isolates. This is consistent with the increasing virulence observed upon passaging PPMV-1 strains through chickens without changes in the primary structure of the F protein (Collins *et al.*, 1996).

In vitro plaque formation, size and morphology have been used to characterize NDV strains (Hanson, 1975). Lentogenic strains need the addition of exogenous trypsin to spread and form syncytia in cell-culture monolayers, whereas mesogenic and velogenic strains do not. To determine whether viruses FL-Herts^{AF}, FL-Herts(F)^{AV324}, rgAV324^{AF} and rgAV324(F)^{Herts} were able to form plaques, QM5 cells were infected 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 30 h post-infection by immunological staining (Wensvoort *et al.*, 1986) using monoclonal antibody (mAb) 8E12A8C3 (CVI of Wageningen UR) against the F protein and a polyclonal rabbit anti-mouse Ig (code P 0260; Dako) conjugate. All viruses produced plaques 30 h post-infection, whereas the lentogenic NDV strain LaSota, derived from the infectious cDNA clone NDFL+ (Peeters *et al.*, 1999), only gave rise to single-cell infections (Fig. 2). 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}. Although plaque formation has often been used to characterize NDV strains (Hanson, 1975), our observations and those of others (Fuller *et al.*, 2007) show that plaque formation and thus F protein cleavage do not always correlate with virulence of NDV.

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. Cells were grown in six-well plates and inoculated in triplicate with the appropriate dose of virus. During the subsequent incubation, samples were taken from the culture supernatant at 8, 16, 24, 32, 40 and 48 h post-infection. Virus titres of the samples were determined by serial end-point dilution in 96-well plates using QM5 cells and an immunoperoxidase monolayer assay as described above. Virus titres ($TCID_{50}$ ml^{-1}) were calculated by using the method of Reed & Muench (1938). 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). Exchange of the F genes affected the productive capacities of both viruses. 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 than FL-Herts^{AF} and rgAV324(F)^{Herts}, which may explain why FL-Herts(F)^{AV324} grew to lower titres than FL-Herts^{AF}. This feature does not, however, seem to correlate with

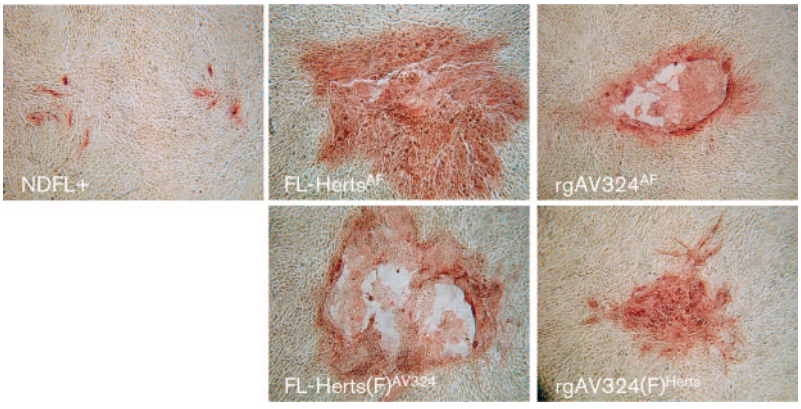

Fig. 2. Plaque formation in QM5 cells in the absence of exogenous trypsin in medium containing 1 % methylcellulose at 30 h post-infection. Plaques were visualized by immunological staining using a mAb against the NDV F protein.

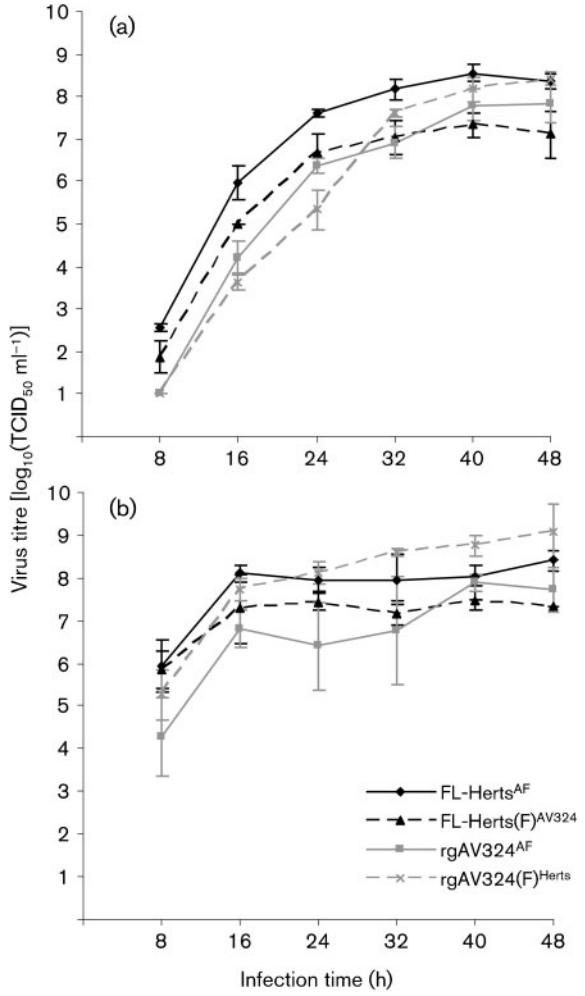

Fig. 3. Multi-cycle (a) and single-step (b) growth kinetics of the F-chimeric viruses along with their respective parental viruses in QM5 cells. Six-well plates of QM5 cell monolayers were infected with virus at an m.o.i. of 0.001 (a) or 10 (b) $TCID_{50}$ per cell for 1 h. The cells were washed with PBS and then incubated in QT35 medium at 37 °C 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 as $TCID_{50}$. Error bars show SD.

differences in virulence in embryonated eggs or 1-day-old chickens.

The current definition of the World Organization for Animal Health (OIE) of a Newcastle disease outbreak is the isolation of an NDV strain that either has an ICPI >0.7 or carries a typical velogenic amino acid motif at the F protein cleavage site. Infections of poultry by strains with characteristics similar to those of 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 of which viral or cellular factors are responsible for the differences in pathogenicity of AV324 in pigeons and chickens. By using the available infectious AV324 and NDV cDNA clones, we will initially focus on virus 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.

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