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

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

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## Abstract

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) (Kaleta & Baldauf, 1988). The disease is caused by Newcastle disease virus (NDV) or avian paramyxovirus type 1 (APMV-1), which belongs to the family *Paramyxoviridae* (genus *Avulavirus*) (Mayo, 2002). During the 1980s, a disease caused by an APMV-1 spread worldwide among racing and show pigeons (Alexander *et al.*, 1985). APMV-1 variants like this could be discriminated antigenically by monoclonal antibodies (Russell & Alexander, 1983; Alexander *et al.*, 1984; Alexander *et al.*, 1985; Lana *et al.*, 1988; Collins *et al.*, 1989) and genetically by phylogenetic analysis of the F gene nucleotide sequence (Aldous *et al.*, 2003) 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 (Anonymous, 1984; Alexander *et al.*, 1985; Alexander *et al.*, 1997; Alexander *et al.*, 1998; Werner *et al.*, 1999; Liu *et al.*, 2006; Irvine *et al.*, 2009). 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 (Meulemans *et al.*, 1986; Collins *et al.*, 1996; Ujvari *et al.*, 2003). However, some PPMV-1 strains cause only minimal disease and show a low intracerebral pathogenicity index (ICPI) in chickens (Meulemans *et al.*, 2002). Nevertheless, they are potentially virulent as becomes apparent after serial passages in chickens (Alexander & Parsons, 1984; Collins *et al.*, 1994; Kommers *et al.*, 2001; Kommers *et al.*, 2003; Dortmans *et al.*, 2010a). 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 (Dortmans *et al.*, 2009; Dortmans *et al.*, 2010b). Furthermore, we investigated the molecular basis for the virulence increase of AV324/96 after serial passage in chickens (Dortmans *et al.*, 2010a). 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 and 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 (de Leeuw *et al.*, 2005; Dortmans *et al.*, 2009). 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 (Communities, 1992). 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 haemagglutination 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<sub>50</sub>/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 using QM5 cells (Antin & Ordahl, 1991). After 3 days of incubation at 37°C, cells were fixed and immunological staining was performed to detect the infected cells as previously described (Dortmans *et al.*, 2009). Virus titres were calculated using the Reed and Muench method (Reed & Muench, 1938) and expressed as log<sub>10</sub>TCID<sub>50</sub>/ml.

Serum samples were obtained at day 6 before and day 14 after inoculation. The hemagglutination inhibition (HI) assay was performed by standard methods (Alexander, 2008) 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

**Clinical signs.** To compare the infectivity and virulence in pigeons of strains AV324/96, its recombinant derivative rgAV324, and FL-Herts, animals were inoculated with 10<sup>7</sup> EID<sub>50</sub> 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.

**Virus shedding.** To determine virus shedding, tracheal and cloacal swabs were taken daily. The results are compiled in Table 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. 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 1 and Fig. 1). Thus, although the PPMV-1 virus did not cause any clinical symptoms, infected pigeons shed virus until at least 14 dpi.

**Serology.** 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 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.

## 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 (Erickson *et al.*, 1980; Kapczynski *et al.*, 2006; Wakamatsu *et al.*, 2006; Carrasco *et al.*, 2008).

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 (Duchatel & Vindevogel, 1986; Duchatel *et al.*, 1992; Vindevogel & Duchatel, 1993; Kapczynski *et al.*, 2006; Wakamatsu *et al.*, 2006). 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 (Duchatel & Vindevogel, 1986; Stone, 1989; Duchatel *et al.*, 1992; Vindevogel & Duchatel, 1993; Kapczynski *et al.*, 2006; Wakamatsu *et al.*, 2006; Carrasco *et al.*, 2009), except in one case where cloacal virus shedding was observed from 5-11 dpi (Carrasco *et al.*, 2008), 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.

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. This difference in quasispecies complexity might also 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 (Stone, 1989; Vindevogel & Duchatel, 1993; Maas *et al.*, 1998; Kapczynski *et al.*, 2006; Hu *et al.*, 2009). 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 (Kapczynski & King, 2005; Qin *et al.*, 2008; van Boven *et al.*, 2008; Hu *et al.*, 2009; Miller *et al.*, 2009). PPMV-1 strains belong to a distinct genetic lineage (Aldous *et al.*, 2004) and previous work already suggested that vaccines for use in pigeons should preferably be prepared from pigeon-origin PPMV-1 virus (Alexander *et al.*, 1986).

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 (Vindevogel & Duchatel, 1993; Kapczynski *et al.*, 2006; Wakamatsu *et al.*, 2006; Carrasco *et al.*, 2008; Carrasco *et al.*, 2009). 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% (Marlier & Vindevogel, 2006). However, some experimental and field data show higher mortality rates (Erickson *et al.*, 1980; Duchatel & Vindevogel, 1986). A reason for this might be that the pigeons are suffering from secondary bacterial or viral infections (Marlier & Vindevogel, 2006). 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 (Alexander & Parsons, 1984) and requires only a limited number of adaptive mutations for optimal replication in chickens (Dortmans *et al.*, 2010a). This knowledge, together with the recent NDV outbreaks in pigeons caused by PPMV-1 in Europe ([www.oie.int/wahis/public.php?page=disease\\_immediate\\_summary](http://www.oie.int/wahis/public.php?page=disease_immediate_summary)), underscores the potential danger of pigeon-derived paramyxoviruses for the poultry industry.

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**Table 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

virus	pigeon	days post infection														
		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	-/-	+/-	+/-	+/-	+/-	-/-	-/-	-/-	-/-	-/-	-/-	-/-	-/-	-/-	-/-

**Table 2.** Antibody titers determined with antigens rgAV324, FL-Herts and Ulster at 14 days post infection on a log<sub>2</sub> scale. Positive when HI ≥ 3. Nd, not determined

virus	pigeon	HI antigen		
		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

**Fig. 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.*

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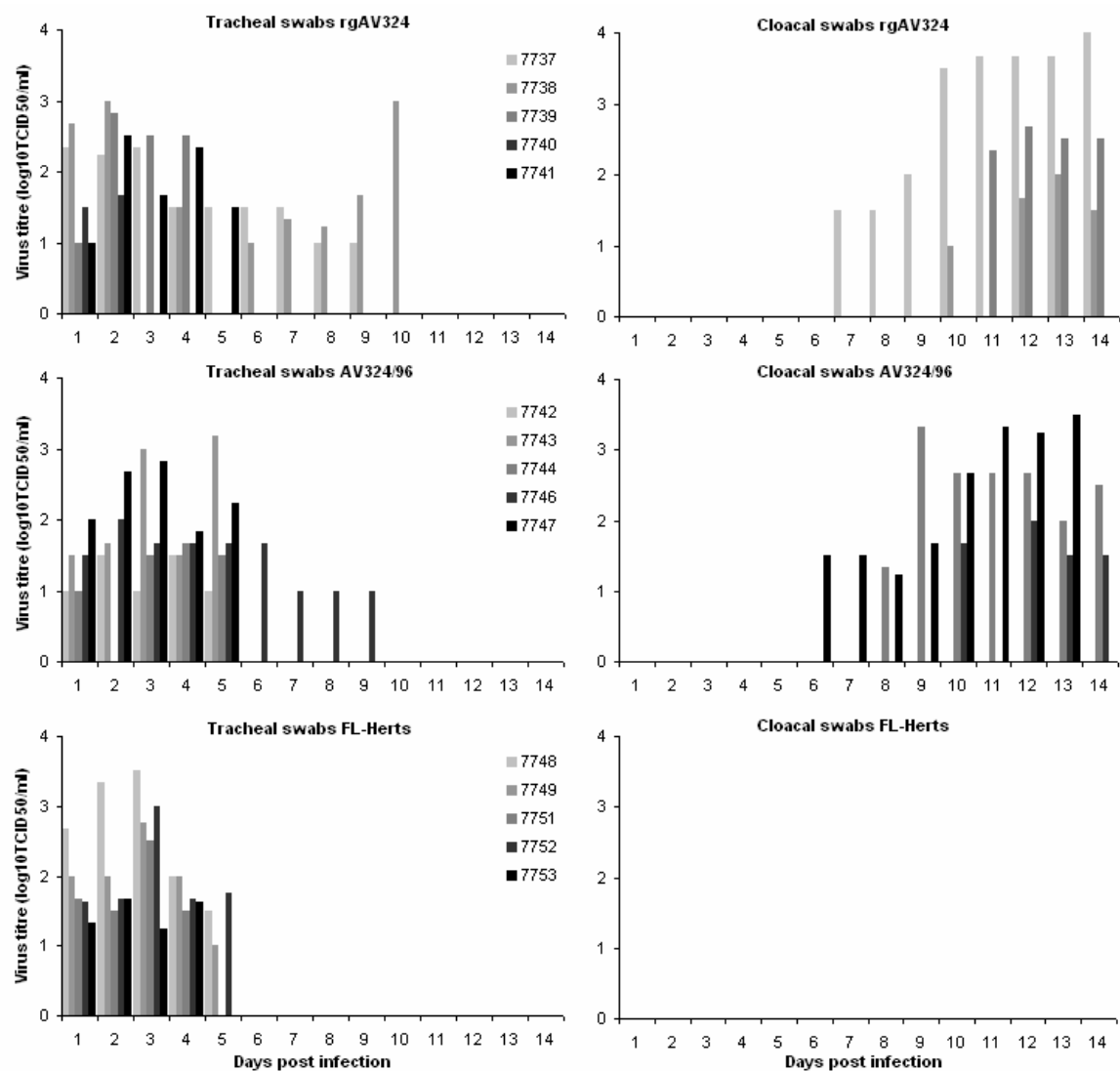


Fig. 1. Virus titres determined in tracheal (left column) and cloacal (right column) swabs of individual pigeons. Titres (log<sub>10</sub>TCID<sub>50</sub>/ml) were determined by serial end-point dilution in 96-well plates using QM5 cells.