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Length Variations in the NA Stalk of an H7N1 Influenza Virus Have Opposite Effects on Viral Excretion in Chickens and Ducks

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A deletion of ~20 amino acids in the stalk of neuraminidase is frequently observed upon transmission of influenza A viruses from waterfowl to domestic poultry. A pair of recombinant H7N1 viruses bearing either a short- or long-stalk neuraminidase was genetically engineered. Inoculation of the long-stalk-neuraminidase virus resulted in a higher cloacal excretion in ducks and led conversely to lower-level oropharyngeal excretion in chickens, associated with a higher-level local immune response and better survival. Therefore, a short-stalk neuraminidase is a determinant of viral adaptation and virulence in chickens but is detrimental to virus replication and shedding in ducks.

One of the host range determinants that favor the multiplication of influenza viruses in domestic poultry is the length of the neuraminidase (NA) stalk (13, 16, 25). Viruses of the N1, N2, and N3, and less frequently N6 and N7, neuraminidase types that harbor a 20- to 30-amino-acid deletion in the NA stalk have been isolated from domestic poultry (2, 4, 7, 10, 13, 14, 19). Such a deletion was also observed upon experimental transmission to quail or chickens of viruses belonging to the H2N2 (24, 25), H7N3 (8), H9N2 (9), and H11N9 (11, 12) subtypes. Viruses with a short-stalk NA are rarely found in nongallinaceous hosts (13), and this feature could be detrimental to virus multiplication in ducks (11).

Here, we addressed this issue by comparing, in chickens and in ducks, the levels of multiplication and pathogenicities of two genetically engineered H7N1 viruses differing by the length of the NA stalk.

The low-pathogenicity avian influenza (LPAI) virus A/turkey/Italy/977/1999 (H7N1), isolated in Italy in 1999, is characterized by a short NA stalk (5). A plasmid-based reverse genetics system for this virus has been previously described (26). The NA plasmid was modified by the insertion of a sequence encoding the 22 amino acids ITYENNTWVNQTYVNISNTNFL between residues I⁵³ and T⁵⁴ of the NA stalk, as found in the NA sequence of the A/chicken/Italy/1082/1999 (H7N1) LPAI virus. The H7N1 variants with a short-stalk NA (wt virus) or a long-stalk NA (insNA virus) were rescued by reverse genetics. They both grew efficiently in 10-day-old embryonated eggs, reaching titers up to 2×10^8 to 4×10^8 PFU/ml as determined by plaque assay on QT6 cells. They showed similar growth kinetics on primary chicken fibroblasts as well as duck embryonic fibroblasts (data not shown). Their NAs were both characterized by a Michaelis-Menten constant (K_m) of 32 μ M, as determined using the small fluorogenic substrate MUNANA (21). However, the activity of the short-stalk NA was dramatically reduced compared to that of the long-stalk variant, as measured by the elution time of the corresponding viruses from red blood cells of either chickens or turkeys (>20 h and <3.5 h for the wt and insNA viruses, respectively), in agreement with previously published studies (8, 16).

Four-week-old chickens and 4-week-old Pekin ducks were inoculated in parallel with either the wt or insNA virus (5×10^6 50% egg infective doses [EID₅₀] orally and concomitantly 5×10^5 EID₅₀ intratracheally) or with sterile phosphate-buffered saline (PBS) as a control. At day 2 (d2), d3, d4, and d7 postinoculation (p.i.), oropharyngeal and cloacal swabs as well as tissue samples were taken from euthanized animals for viral RNA quantification, histopathology, and cytokine mRNA quantification.

The virus-inoculated ducks showed no clinical symptoms, which was in agreement with previous observations of an asymptomatic replication of influenza viruses in ducks (26). The lungs were overall histologically normal, except for a mild focal pneumonia that was observed in only 2 out of 20 ducks in each virus-inoculated group. However, in cecal samples, some submucosal infiltration by inflammatory cells, mainly mononuclear cells with several heterophils, was noted (Fig. 1, upper panel). These lesions were mild or marked in 13 out of the 20 insNA virus-inoculated ducks and were detected in only 4 ducks from the wt virus-inoculated group.

The clinical outcome was very different for virus-inoculated chickens. At 2 days postinoculation (2 d p.i.), all the animals in the wt virus group showed severe signs of respiratory distress, and 5 out of 27 were found dead or had to be euthanized. Seven additional animals were found dead on the following day (3 d p.i.), and by day 4 p.i., a total of 14 out of 27 chickens had died. Chickens in the insNA virus group showed delayed and milder clinical signs, and only 3 animals died between day 3 and day 7 p.i. Figure 2 illustrates that the level of survival of insNA-virus-inoculated

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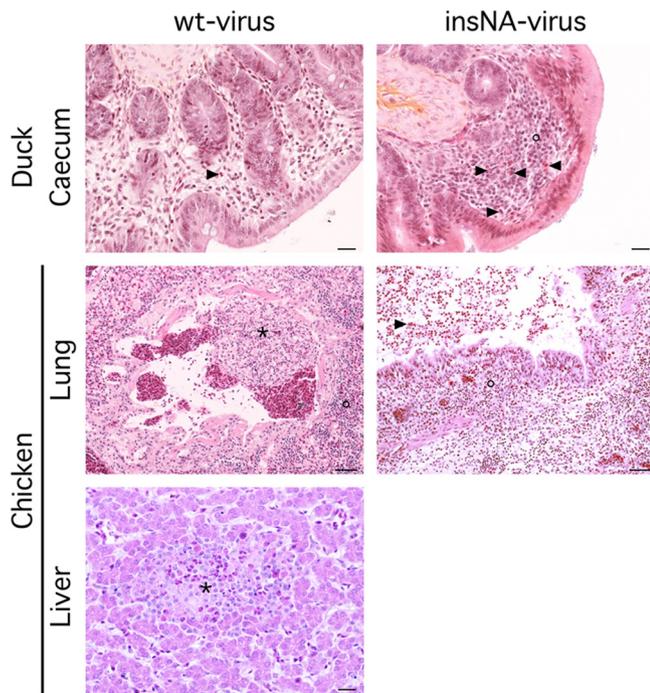


FIG 1 Microscopic examination of tissues sampled from chickens and ducks inoculated with the wt or insNA virus. Mild focal infiltration of mononuclear cells (open circle) and heterophils (filled arrowheads) was more pronounced in the ceca of ducks inoculated with the insNA virus than in those of ducks inoculated with the wt virus at d4 (upper panel). In the lungs of wt virus-inoculated chickens at d2, the lumen of bronchi and parabronchi was occluded by large amounts of necrotic debris of the respiratory epithelium (*) admixed with some heterophils, whereas only an exudate rich in heterophils (filled arrowheads) was present in insNA virus-inoculated animals (middle panel). Mild multifocal hepatic necrosis (*) associated with a heterophilic infiltration was detected in a chicken inoculated with the wt virus at d3 (lower panel). Hemalum-eosin-saffron staining was used. Scale bars, 20 μm (upper and lower panels) and 50 μm (middle panel).

chickens was significantly higher than that of wt virus-inoculated chickens, as estimated with Kaplan-Meier life table analysis (5.7 ± 0.2 days versus 3.2 ± 0.2 days; $P < 0.0001$). At the histological level, wt and insNA virus-inoculated chickens showed lesions of peribronchiolar interstitial pneumonia of similar extension from d2 to d4 p.i. The lung parenchyma was massively infiltrated by lymphocytes and macrophages. At d2 p.i., two of the three wt virus-inoculated chickens that were histologically examined presented a multifocal mild necrosis of the bronchial epithelium. In contrast, necrotic lesions were not recorded in insNA virus-inoculated chickens, which presented only exudative and suppurative bronchial lesions (Fig. 1, middle panel). Both the levels of expression and the localizations of viral antigens, as detected by immunohistochemistry, were similar in the lungs of wt and insNA virus-infected chickens (data not shown). In two animals of the wt virus group, but in none of the insNA virus group, scattered necrotic enterocytes were observed in the cecal mucosa in association with some infiltrating inflammatory cells in the underlying connective tissue of the submucosa. In one animal of the wt virus group, scattered necrotic hepatocytic foci were observed at d3 (Fig. 1, lower panel). Overall, the major histological difference between the two viruses in the respiratory and intestinal tracts of chickens consisted of the necrotic nature of the lesions specifically induced by the wt virus.

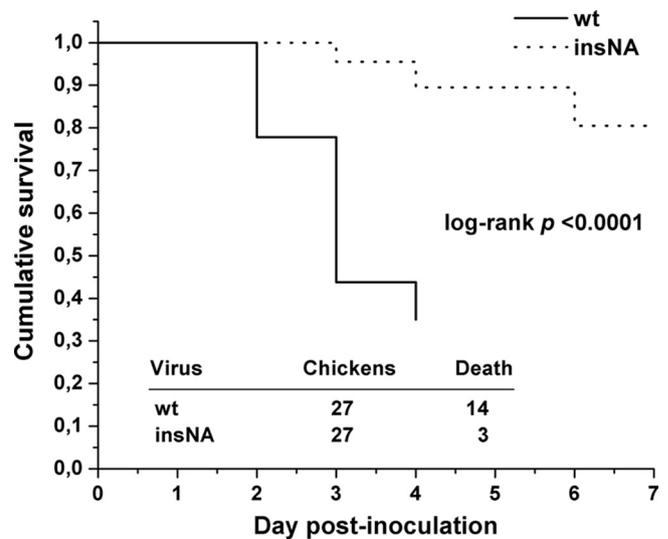


FIG 2 Survival of chickens inoculated with wt or insNA virus. Survival was estimated for 27 wt virus-inoculated chickens and 27 insNA virus-inoculated chickens by Kaplan-Meier life table analysis, and survival data were compared by a log rank test.

Viral loads in the tissues of infected birds were estimated by quantitative real-time PCR (qRT-PCR)-based quantification of M viral RNA (vRNA). In ducks, only 2 lung samples and 1 cecum sample from the wt group (Fig. 3A and B, open circles) and 2 lung samples and 2 cecum samples from the insNA group (Fig. 3A and B, filled circles) were found positive for the presence of vRNA. All kidney samples were negative (Fig. 3C, circles). Most of the oropharyngeal swabs were vRNA positive at d2, d3, and d4 (Fig. 3E) and negative at d7 (data not shown), no significant difference being observed between the two groups of animals. Remarkably, 10 out of the 11 vRNA-positive cloacal swabs (Fig. 3F, circles) corresponded to animals of the insNA virus group. The observed discrepancy between the substantial cloacal excretion and the infrequent detection of vRNA in the cecum samples could result from the rapid desquamation of differentiated intestinal epithelial cells, which were previously found to be the main site of replication of the H7N1 virus (26). Taken together, our observations suggest that the insNA virus replicated more efficiently than the wt virus in the intestinal tract of ducks.

In chickens, all lung and kidney samples were positive for the presence of M vRNA, no difference being observed between the two groups of animals (Fig. 3A and C, triangles). In cecum samples, vRNA levels were slightly higher at day 4 p.i. in the wt virus group than in the insNA virus group ($P = 0.032$; Fig. 3B), and the fraction of vRNA-positive samples (cumulative results for days 2, 3, and 4 p.i.) was relatively higher in the wt virus group (14/15 compared to 10/15; Fig. 3B, triangles). Most of the cloacal swabs that were vRNA positive (5 out of 6; Fig. 3F, triangles) corresponded to chickens inoculated with the wt virus, further indicating a slight replicative advantage of the wt virus in the intestinal tract of chickens. The oropharyngeal swabs showed even more striking differences, vRNA levels being 10- to 100-fold higher in the wt virus group than in the insNA virus group at day 2 p.i. ($P = 0.008$; Fig. 3D). The difference between the two groups was less pronounced at days 3 and 4 p.i. but remained significant when the

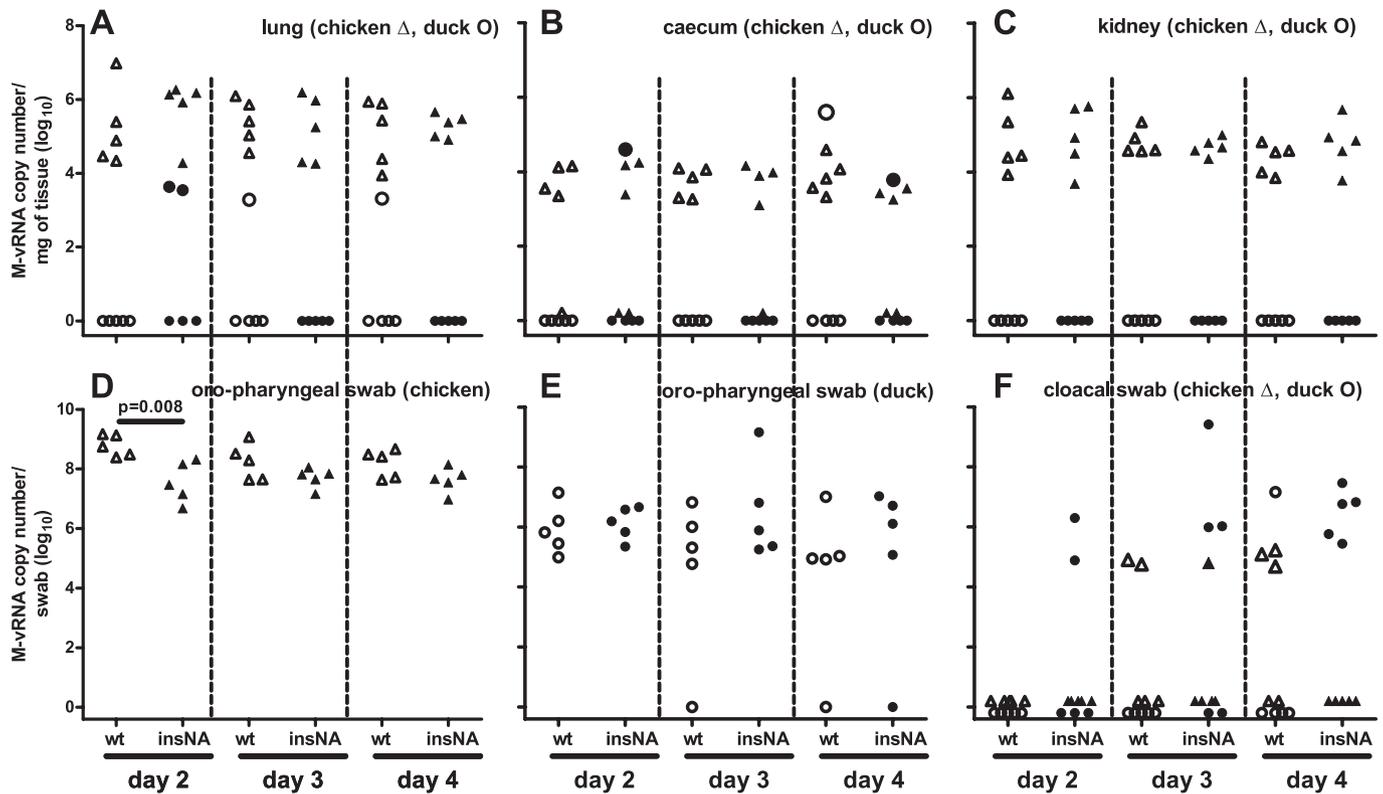


FIG 3 Levels of M vRNA in wt and insNA virus-inoculated birds. Levels of M vRNA were determined by qRT-PCR on viral RNA extracted from lung (A), cecum (B), and kidney (C) homogenates or from oropharyngeal and cloacal swabs (D to F). Data from both chickens and ducks are grouped in panels A to C and F. The M vRNA copy numbers per mg of tissue were calculated as previously described (16) and converted to their \log_{10} values. Differences between the wt and insNA groups were assessed by a Mann-Whitney U test.

accumulated data for days 2, 3, and 4 p.i. were compared ($P = 0.001$).

The levels of cytokine and Mx1 mRNAs were measured by qRT-PCR in the lungs of chickens euthanized at days 2, 3, and 4 p.i. All virus-inoculated chickens showed significantly increased levels of Mx1 mRNA from day 2 to day 4 p.i. (Fig. 4C), although no virus-induced alpha interferon (IFN- α) response was detected (Fig. 4A). An appreciable IFN- β response was found only in 5 out of 15 chickens inoculated with the insNA virus (Fig. 4B). Strikingly, at 2 d p.i. the levels of IFN- γ , interleukin 6 (IL-6), and CXCL12 (analogous to mammalian IL-8) mRNAs were 20- to 50-fold higher in the group of chickens inoculated with the less pathogenic insNA virus (Fig. 4D, E, and F, respectively). IL-15 mRNA levels in the insNA virus group were also slightly but significantly higher than those in the wt virus group at day 2 p.i. (Fig. 4G), whereas no differences were observed for IL-1 β and transforming growth factor β 4 (TGF- β 4) (Fig. 4H and I, respectively). These observations suggest that infection with the long-stalk insNA virus induced a stronger local immune response which limited virus-induced damages. These results are in contrast with our previous observations that shortening of the NA stalk of an H1N1 virus resulted in higher levels of proinflammatory cytokines at 3 d p.i. and 4 d p.i. (16). A likely hypothesis is that the short-stalk H7N1 virus, with higher replication efficiency than its H1N1 counterpart, can outcompete the antiviral response in infected chickens (20). The fact that the H7N1 virus has an allele B NS1 while the H1N1 virus has an allele A NS1 could also contribute to

differences in the modulation of the cytokine response (17). We observed no difference in the abilities of the insNA and wt viruses to induce cytokine transcription upon infection of the chicken lung epithelial cell line CLEC213 (6) (data not shown). Therefore, the differences observed *in vivo* could involve other cell types from the inflammatory response, including cells of myeloid origin and lymphocytes, activated or recruited locally in response to infection (22, 28).

Overall, our comparative study clearly indicates that ducks are better than chickens at controlling virus replication, which could partly result from the presence of a functional RIG-I in ducks but not in chickens (3). It points to species-specific effects of length variations in the NA stalk of an H7N1 virus. The short-stalk-NA virus was much more virulent than the long-stalk-NA virus in chickens, inducing necrosis of epithelial cells, lower-level local immune responses, and higher mortality. This increased virulence could reflect differences in the tissue or cell tropism, which was previously shown to be influenced by the NA stalk length (11, 25) and could be related to an altered balance between hemagglutinin and neuraminidase activities (1, 15, 27). The NA stalk length may also differentially modulate the activation of cellular components of the inflammatory and T-cell responses (18, 23). Furthermore, the short-stalk virus replicated at significantly higher levels in the upper airways of chickens, which could favor aerial transmission (25), and was also excreted by the fecal route at higher levels than the insNA virus. In ducks, in contrast, the long-stalk virus showed higher levels of replication and fecal shedding than the short-stalk

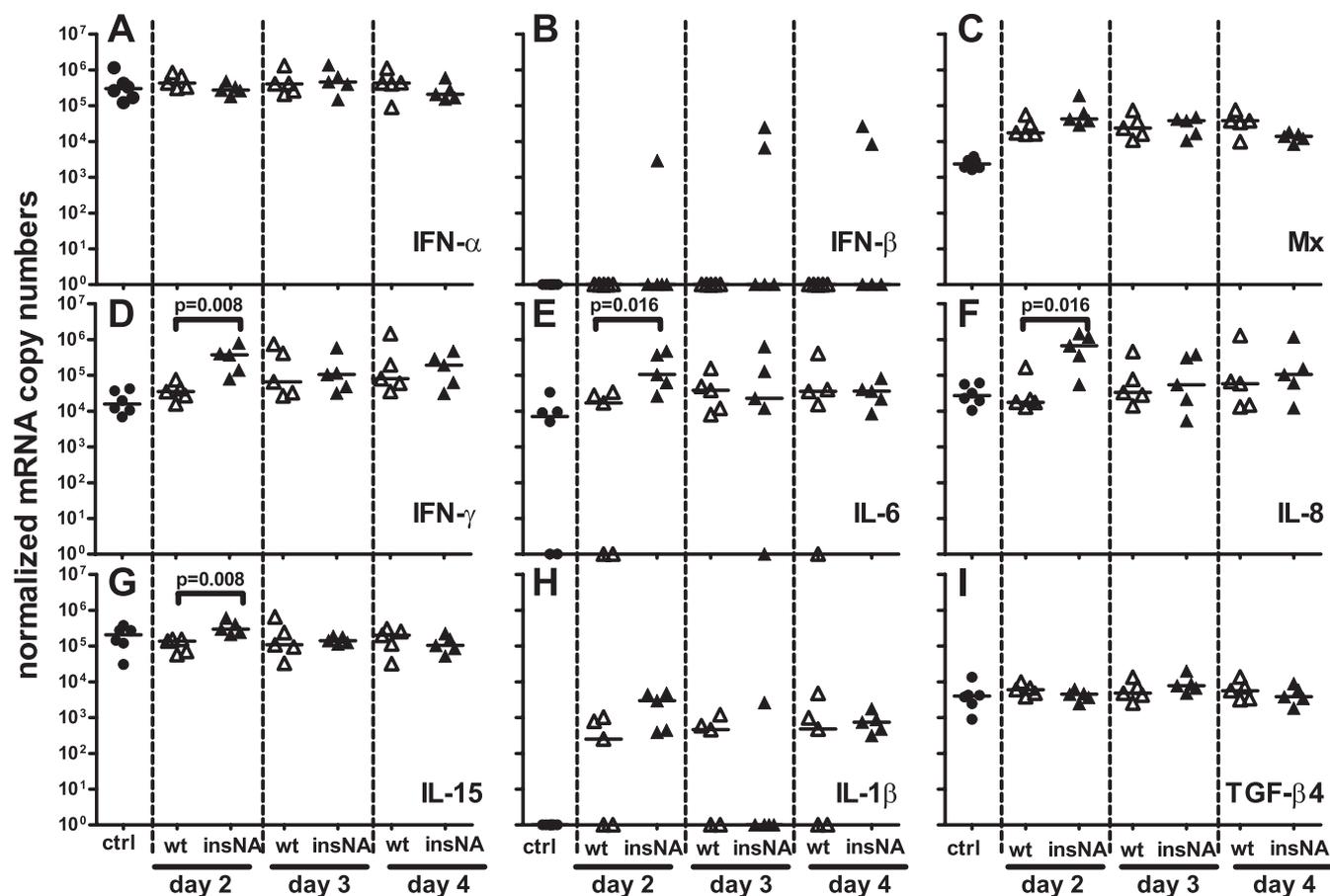


FIG 4 Levels of cytokine mRNAs in the lungs of wt and insNA virus-inoculated chickens. Total RNA was extracted from the lungs of 5 wt virus-inoculated chickens, 5 insNA virus-inoculated chickens, and 2 mock-inoculated chickens (ctrl) at days 2, 3, and 4 postinoculation (p.i.). The levels of the indicated cytokine mRNAs at the indicated time points were determined using quantitative RT-PCR as previously described (16). The results are expressed as mRNA copy numbers (y axis, left scale) normalized with respect to 10^7 copies of the geometric mean of 3 reference gene cDNA copy numbers (those corresponding to glyceraldehyde-3-phosphate dehydrogenase [GAPDH], G10, and ubiquitin), as measured in the same sample. Values from the six mock-inoculated chickens were grouped. The median value for each experimental group is indicated by a horizontal bar. Differences between the wt and insNA groups were assessed by a Mann-Whitney U test with Bonferroni's correction (corrected cutoff *P* for significance, 0.017).

virus. Altogether, our data provide further evidence that a deletion in the NA stalk represents both a host range determinant and a pathogenicity determinant in chickens and show for the first time that this feature is detrimental for a sustained replication and transmission in ducks, which likely contributes to the limited persistence of short-stalk-NA viruses (13).

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There is no conflict of interest for any of the authors.

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