Deletion of the C-terminal ESEV domain of NS1 does not affect the replication of a low-pathogenic avian influenza virus H7N1 in ducks and chickens

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Highly pathogenic avian influenza (HPAI) H7N1 viruses caused a series of epizootics in Italy between 1999 and 2001. The emergence of these HPAI viruses coincided with the deletion of the six amino acids R225VESEV230 at the C terminus of NS1. In order to assess how the truncation of NS1 affected virus replication, we used reverse genetics to generate a wild-type low-pathogenic avian influenza (LPAI) H7N1 virus with a 230 aa NS1 (H7N1230) and a mutant virus with a truncated NS1 (H7N1224). The 6 aa truncation had no impact on virus replication in duck or chicken cells in vitro. The H7N1230 and H7N1224 viruses also replicated to similar levels and induced similar immune responses in ducks or chickens. No significant histological lesions were detected in infected ducks, regardless of the virus inoculated. However, in chickens, the H7N1230 virus induced a more severe interstitial pneumonia than did the H7N1224 virus. These findings indicate that the C-terminal extremity of NS1, including the PDZ-binding motif ESEV, is dispensable for efficient replication of an LPAI virus in ducks and chickens, even though it may increase virulence in chickens, as revealed by the intensity of the histological lesions.

INTRODUCTION
The emergence of highly pathogenic avian influenza (HPAI) viruses is caused by mutations in the haemagglutinin (HA) gene that introduce a stretch of basic amino acids at the HA cleavage site (Pantin-Jackwood & Swayne, 2009). As a consequence, HA can be proteolytically matured by ubiquitously expressed proteases belonging to the furin family. HPAI can thus cause a systemic infection, while low-pathogenic avian influenza (LPAI) viruses are restricted to the respiratory and digestive tracts, where trypsin-like proteases are expressed. Thus, the cleavability of HA is a major virulence determinant for influenza viruses in birds.

The influenza virus non-structural protein NS1 is also a virulence factor in birds and mammals (Hale et al., 2008). NS1 regulates viral gene expression and inhibits the host antiviral response through various mechanisms involving its interaction with several cellular proteins. One of the best-described roles of NS1 is to inhibit the synthesis of type I interferon (IFN) from infected cells. Viruses lacking a functional NS1 protein do not replicate efficiently in type I IFN-competent cells, and are strongly attenuated in vivo (Egorov et al., 1998; García-Sastre et al., 1998; Kochs et al., 2007).

Several epizootics due to HPAI viruses of the H7N1 subtype occurred in Italy between 1999 and 2001 (Capua & Alexander, 2004). Interestingly, all HPAI viruses isolated had a C-terminally truncated NS1 protein, resulting from a point mutation that introduced a premature stop codon at position 225 (Dundon et al., 2006). Of note, the full-length NS1 protein could only be detected in LPAI viruses circulating at that time, suggesting that the C-terminal truncation of NS1 occurred during evolution from an LPAI H7N1 virus to an HPAI virus. As a consequence, the HPAI viruses lacked the C-terminal ESEV domain in the NS1 protein that had been shown previously to modulate virulence in a way that appears to be dependent both on
the host species and on the virus isolate (Jackson et al., 2008; Soubies et al., 2010; Zielecki et al., 2010). The consequences of the truncation on the virulence of this isolate are unknown. In addition, whether the truncation of NS1 contributes to the adaptation of avian influenza viruses from reservoir species of LPAI viruses, such as ducks, to poultry is unknown.

In order to address these questions, we used reverse genetics to generate H7N1 viruses that contained a full-length NS1 protein or a C-terminally truncated NS1 lacking the amino acids RVESEV. We compared the replication of these viruses in duck and chicken embryonic fibroblasts. In addition, we assessed their replication and pathogenicity in ducks and chickens.

RESULTS

Rescue of recombinant H7N1 viruses

Plasmid-driven reverse genetics was used to recover a wild-type virus that contained a 230 aa NS1 protein with an ESEV C-terminal domain. We called this virus H7N1\textsubscript{230}. In a previous paper (Soubies et al., 2010), this virus was referred to as ESEV virus. In parallel, we mutated the NS segment to obtain a truncated NS1 protein of 224 aa. Through a single C to U substitution, we introduced a stop codon at position 225, as observed in the HPAI viruses isolated during the 1999–2001 Italian epizootics (Fig. 1). The recovered mutant virus has an NS1 protein of 224 aa and was designated H7N1\textsubscript{224}. In a previous paper (Soubies et al., 2010), this virus was referred to as NEP S70 virus. Importantly, the introduced mutation does not alter the amino acid sequence of the nuclear export protein (NEP). The identity of the amplified viruses was verified by sequencing of amplicons of each viral gene.

Analysis of virus growth and type I IFN production in duck and chicken cells

We compared the growth properties of H7N1\textsubscript{230} and H7N1\textsubscript{224} in duck embryonic fibroblasts (DEF) and chicken embryonic fibroblasts (CEF). In order to assess multicycle growth, we infected DEF and CEF at an m.o.i. of 0.001 and cultured the cells in the presence of trypsin. H7N1\textsubscript{230} and H7N1\textsubscript{224} replicated with similar kinetics and reached similar titres at 33 h post-infection (p.i.) (Fig. 2). At 33 h p.i., the majority of the H7N1\textsubscript{230} and H7N1\textsubscript{224}-infected cells were dead and the multicycle growth analysis was therefore terminated. To assess virus production during single-cycle growth, we infected DEF and CEF at an m.o.i. of 3. We did not detect any differences between the virus titres of H7N1\textsubscript{230} and H7N1\textsubscript{224} (Fig. 3a, b). We also measured type I IFN production in the supernatant of DEF and CEF infected at an m.o.i. of 3. Both viruses induced detectable type I IFN production (Fig. 3c, d). However, in both DEF and CEF, no significant difference could be detected between H7N1\textsubscript{230} and H7N1\textsubscript{224}. Taken together, these findings indicate that the truncation of NS1 has no impact on virus replication or on type I IFN production in duck or chicken cells.

Consequences of NS1 truncation on viral replication and pathogenicity in ducks

We infected Pekin ducks via the oral and intracoanal routes with 10⁷ EID₅₀ (50 % egg infectious dose) of H7N1\textsubscript{230} or H7N1\textsubscript{224}. Infected animals did not show any clinical signs, as classically observed for LPAI infection in ducks (Kida et al., 1980; Pantin-Jackwood & Swayne, 2009; Webster et al., 1978). Histologically, very mild lesions were only observed in trachea and caecum samples of most H7N1\textsubscript{230}- and H7N1\textsubscript{224}-infected ducks, showing minimal to mild infiltration of the submucosal chorion, composed mainly of heterophilic polynuclear cells, from day 2 to day 4 p.i. (data not shown).

We assessed the level of virus replication by measuring the level of viral RNA by quantitative RT-PCR (qRT-PCR) in the colon, which has previously been shown to be the major site of influenza A virus replication in ducks (Kida et al., 1980). Virus titres were maximal at day 3 p.i. for H7N1\textsubscript{230} and at day 7 p.i. for H7N1\textsubscript{224} (Fig. 4a). However, no significant difference was detected between H7N1\textsubscript{230} and H7N1\textsubscript{224}-infected ducks in the level of virus replication in the colon. Finally, we quantified the intensity of the type I IFN induced-immune response by measuring Mx transcripts. Mx is an interferon-stimulated gene and a good indicator of the level of type I IFN produced in situ (Holzinger et al., 2007; Sommereyns et al., 2008). The level of Mx transcripts was increased in both groups of virus-infected ducks compared with non-infected ducks (Fig. 4b). However, Mx levels were not significantly different between H7N1\textsubscript{230} and H7N1\textsubscript{224}-infected ducks.
Consequences of NS1 truncation on viral replication and pathogenicity in chickens

We infected 4-week-old White Leghorn chickens with $6 \times 10^4$ EID$_{50}$ via the intratracheal route and with $6 \times 10^5$ EID$_{50}$ via the choanal route. Infection with H7N1$_{230}$ and H7N1$_{224}$ caused severe respiratory symptoms and was associated with significant mortality: of the 30 inoculated animals in each group, seven died in the H7N1$_{230}$ group.

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**Fig. 2.** Multicycle growth analysis in duck and chicken cells. Primary DEF (a) and primary CEF (b) were infected at an m.o.i. of 0.001 in the presence of TPCK–trypsin to allow multiple-cycle virus growth. Supernatants were collected at the indicated times p.i., and virus titres were determined with Madin–Darby canine kidney (MDCK) cells. The results are representative of at least three independent experiments.

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**Fig. 3.** Single-cycle growth analysis and type I IFN production in duck and chicken cells. (a, b) Primary DEF (a) and primary CEF (b) were infected with the H7N1$_{230}$ and H7N1$_{224}$ viruses at an m.o.i. of 3. Supernatants were collected at the indicated times p.i., and virus titres were determined with MDCK cells. (c, d) Mx–firefly luciferase (Mx-FFLuc) activity normalized to TK Renilla luciferase activity (TK-RLuc) was measured in duck cells stimulated with supernatant collected 20 h p.i. from infected DEF (c) and in chicken cells stimulated with supernatant collected 20 h p.i. from infected CEF (d) or increasing concentrations of recombinant duck IFN-α and chicken IFN-α, respectively (circles, individual values; bars, means). n.i., Not infected.
and ten died in the H7N1224 group during the monitoring week, in addition to the birds that were euthanized for tissue collection. Kaplan–Meier estimates and log-rank tests did not show any differences in survival between H7N1230- and H7N1224-inoculated chickens (4.4 ± 0.2 vs 3.5 ± 0.1 days, P = 0.195; Fig. 5a). On days 1, 2, 3 and 4 p.i., tissue samples were taken from euthanized birds for viral RNA quantification, cytokine mRNA quantification and histopathology. Because of the high rate of mortality, no bird survived until day 7 p.i. in the H7N1224-infected group, while only two birds survived in the H7N1230-infected group.

Virus replication was assessed through qRT-PCR-based quantification of viral RNA. Viral RNA was detected in the lungs from day 1 to day 4 p.i., with a peak at day 3 p.i. (Fig. 5b). Lower levels of viral RNA (1–10% of those measured in the lungs) were also detected regularly in the kidney and brain, and less regularly in the caecum, between days 2 and 4 p.i. (data not shown). The presence of virus in kidney and brain probably reflects systemic transport of the virus. We observed no significant differences in virus load between the two viruses. Virus sequence analysis performed on four chickens per group at day 3 or 4 p.i. revealed that no virus had acquired mutations that affected the coding sequence of NS1 (not shown). The virus was completely cleared from the two surviving H7N1230-infected chickens at day 7 p.i. (data not shown).

The levels of various cytokine mRNAs and Mx mRNA were measured by qRT-PCR in the lungs of chickens euthanized at days 1, 2, 3 and 4 p.i. Mx mRNA levels were significantly upregulated from day 1 to day 4 p.i. in both H7N1230- and H7N1224-inoculated chickens compared with non-infected animals (P < 0.01) (Fig. 6a). We did not detect any significant upregulation of IFN-α mRNA in infected chickens (Fig. 6b), whereas IFN-β mRNA levels were significantly upregulated in both H7N1230- and H7N1224-inoculated chickens compared with non-infected animals at day 3 p.i. (P < 0.05) (Fig. 6c). However, IFN-β mRNA levels were similar between H7N1230- and H7N1224-inoculated chickens. Thus, our results indicate that the intensity of the type I IFN immune response does not differ significantly between H7N1230- and H7N1224-infected

![Fig. 4. Replication and pathogenesis in ducks. (a) Viral RNA level in the ileal and colonic mucosae at days 1, 2, 3 and 7 p.i. Viral RNA levels from scraped mucosae were determined by qRT-PCR and normalized to glyceraldehyde-3-phosphate dehydrogenase (GAPDH) levels (triangles, individual values; bars, means). (b) qRT-PCR analysis of Mx expression in the ileal and colonic mucosae at days 1, 2, 3 and 7 p.i. Mx levels are normalized to GAPDH levels (triangles, individual values; bars, means). n.i., Not infected.](http://vir.sgmjournals.org)
chickens. We detected upregulation of IFN-γ, interleukin-6 (IL-6) and IL-8 in infected chickens (Fig. 6d–f). At day 3 p.i., these mRNAs were increased significantly compared with controls in both H7N1230- and H7N1224-infected chickens (P<0.05). However, again, IFN-γ, IL-6 and IL-8 mRNA levels did not differ significantly between H7N1230- and H7N1224-infected chickens.

Histopathological analysis of the lungs revealed a broncho-interstitial pneumonia in infected chickens from days 2 to 4 p.i. (Table 1). The intensity of the lesions was significantly greater in H7N1230-infected chickens than in H7N1224-infected chickens. The nature of the lesion was roughly similar, associating mononuclear cells around parabronchi and some heterophils in the parabronchial lumen (Fig. 7a, b). However, on days 2 and 3 p.i., an increased tendency to epithelial necrosis of bronchi and parabronchi was observed in H7N1230-infected chickens compared with H7N1224-infected chickens. This was associated with the accumulation of cellular debris that occluded respiratory duct lumens in H7N1230-infected chickens compared with essentially fibrinous exudation in parabronchi of H7N1224-infected chickens (Fig. 7c, d). Atrial emphysema, recognized by severe enlargement of air spaces with surrounding atelectasis, was frequently observed secondarily to respiratory airway occlusion in H7N1230-infected chickens. To detect cells specifically with replicating virus, we performed immunolabelling of NS1 on frozen lung sections (Fig. 7e, f). At day 2 p.i., NS1-positive cells (Fig. 7f) localized at sites with a high cellularity, compared with the normal lung parenchyma cellularity observed in controls (Fig. 7e). This focal increase in cell density is probably due to the influx of inflammatory cells in infected lung areas, suggesting that inflammatory cells are recruited to the site of infection.

**DISCUSSION**

Our results show that truncation of NS1 by deletion of the C-terminal amino acids RVESEV has no impact on the ability of an LPAI H7N1 virus to replicate in duck or chicken cells *in vitro*, as well as *in vivo*. We further provide evidence that the truncation of NS1 has no impact on the ability of NS1 to antagonize the host immune response. Indeed, the H7N1230 and H7N1224 viruses were shown to induce similar levels of type I IFN and pro-inflammatory cytokines and IL-8. Infected ducks had no detectable symptoms and no histological lesions, in accordance with the well-reported low morbidity observed in LPAI virus-infected ducks (Kida et al., 1980; Webster et al., 1978). By contrast, respiratory symptoms are more commonly observed in chickens infected with LPAI viruses, including LPAI H7 viruses (Spackman et al., 2010). In this study, infected chickens suffered from severe respiratory signs and a high rate of mortality, in accordance with the results from a previous study using the same wild-type virus and a similar inoculation protocol (Hoffmann et al., 2012). Histopathological analysis of the lungs from infected chickens revealed an interstitial pneumonia that was significantly more severe in H7N1230-infected chickens than in H7N1224-infected chickens. Given the similar virus load and immune response observed with the two viruses, the cause of the severity of the histological lesions observed in H7N1230-infected chickens remains unclear. The C-terminal ESEV domain of NS1 was shown to bind PDZ domains containing cellular proteins (Obenauer et al., 2006; Tonikian et al., 2008). NS1 proteins with a PDZ protein-binding domain have been shown to disrupt cellular tight junctions by binding Scribble and Dlg1 (Golebiowski et al., 2011). The PDZ protein binding of H7N1230 NS1 could damage tight junctions, thereby...
causing increased lung pathology compared with the H7N1224 virus.

Analysis of virus strains collected from different bird species during the Italian avian influenza epizootics of 1999–2001 suggested that the C-terminal truncation of NS1 occurred following transmission from a wild aquatic bird reservoir, such as ducks, to terrestrial poultry, such as chickens (Dundon et al., 2006). Thus, we initially hypothesized that deletion of the C-terminal RVESEV motif of NS1 could be the result of selection pressure that occurs when the virus enters the chicken species. Our results do not support this hypothesis. Indeed, the H7N1224 virus with a truncated NS1 has no major phenotypic advantage in chickens compared with the wild-type virus.

Fig. 6. Levels of cytokine mRNAs in the lungs of chickens. (a) Mx; (b) IFN-α; (c) IFN-β; (d) IFN-γ; (e) IL-6; (f) IL-8. The levels of the indicated cytokine mRNAs at the indicated time points were determined using qRT-PCR. Results are expressed as mRNA copy numbers normalized to $10^7$ copies of the geometric mean of the copy numbers of three housekeeping gene cDNAs (GAPDH, G10 and ubiquitin). Triangles, individual values; bars, means. n.i., Not infected.

Table 1. Histological lesions in chickens inoculated with wild-type or mutant H7N1 virus

Lesion intensity was evaluated semiquantitatively (0, no lesion; 1, mild; 2, marked; 3, severe) and a mean score was calculated for each batch of animals. Frequency of lesions/number of chickens is reported in parentheses. Histological scores from day 2 + 4 (grouping day 2 and day 4 animals) were significantly higher in H7N1230-infected chickens than in H7N1224-infected chickens ($P<0.05$).

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<th>Time (days p.i.)</th>
<th>Bronchial lesion intensity</th>
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<tr>
<td></td>
<td>NS1$_{230}$</td>
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<tr>
<td>2</td>
<td>2.7 (3/3)</td>
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<tr>
<td>4</td>
<td>2.3 (3/3)</td>
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<tr>
<td>2 + 4</td>
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truncated variant of NS1 (Dundon et al., 2006), while our studies were performed with LPAI H7N1 viruses. Thus, we cannot rule out the possibility that truncation of NS1 could have other consequences on the replication and pathogenesis of HPAI H7N1 virus.

The C-terminal ESEV domain of NS1 was previously described as a virulence motif in mice by Jackson et al. (2008) using the H1N1 PR8 strain and by our group using the H7N1 strain used in this work (Soubies et al., 2010). The results of the current study indicate that the C-terminal ESEV domain of NS1 does not increase virulence in ducks and chickens significantly, confirming our previous results in ducks (Soubies et al., 2010) and the results of Zielecki et al. (2010) in chickens. Thus, although it is highly conserved in avian influenza viruses, the ESEV domain of NS1 does not seem to increase virus replication and virulence in bird species. We have shown previously that a virus with a C-terminal ESEV domain replicates to lower levels than a virus with a C-terminal RSKV domain in ducks, but that it is excreted for a longer period (Soubies et al., 2010). As a consequence, viruses with an ESEV domain could contaminate more individuals and therefore be selected during virus evolution in birds.

Although the vast majority of influenza virus strains have an NS1 of 230 aa, a number of strains with a C-terminal truncation in the NS1 protein have been identified (Dundon & Capua, 2009). One remarkable example is the 2009 pandemic influenza A H1N1 virus (pH1N1), which has an NS1 of 219 aa as a result of the acquisition of a stop codon at position 220 (Garten et al., 2009). Mutating the stop codon to obtain a 230 aa NS1 had no impact on the replication of the pH1N1 virus in mice or ferrets (Hale et al., 2010). pH1N1 thus seems to tolerate truncations in the C-terminal domain of NS1, as does the LPAI H7N1 described in the current study. At present, the mechanisms that contribute to the selection of NS1 proteins of different lengths and with different C-terminal domains remain largely unknown.

**METHODS**

**Cells and reagents.** Primary DEF were obtained from 10-day-old Pekin duck (Anas platyrhynchos) embryos. Primary CEF were obtained from 11-day-old chicken embryos. Cells were grown in Dulbecco’s modified Eagle’s medium (DMEM) supplemented with penicillin (10^6 U ml^-1), streptomycin (10 mg ml^-1) and 10% FBS at 37 °C and 5% CO₂.

**Virus and reverse genetics.** The LPAI virus A/Turkey/Italy/977/1999 (H7N1) was a kind gift of I. Capua (Istituto Zooprofilattico Sperimentale Delle Venezie, Legnaro, Italy). The recombinant virus H7N1_230 was generated as described previously (Soubies et al., 2010). In order to generate the mutant virus H7N1_224, site-directed mutagenesis was performed on the NS segment by using the QuikChange II kit (Stratagene) according to the manufacturer’s protocol. We verified the identity of amplified viruses by sequencing of amplicons of each virus segment using RT-PCR.

**Infections and virus titration.** All infections were performed with DMEM supplemented with 0.2% BSA. TPCK–trypsin (0.1 μg ml^-1) was added in the case of multiple-cycle growth analysis. Virus titres were determined by plaque assay with cells.

**Chicken and duck type I IFN titration.** Titration of chicken and duck type I IFN was performed by transfecting CEF and DEF, respectively, with a plasmid containing the sequence of firefly luciferase under the control of a chicken Mx promoter (Mx-FFLuc), kindly provided by P. Staeheli (Universita¨ t Freiburg, Freiburg, Germany) (Schumacher et al., 1994) and TK-RLuc, as described previously (Soubies et al., 2010).

**In vivo experiments.** All animals used in in vivo experiments were treated according to European Economic Community (EEC)
recommendations for animal welfare and under the supervision of the local INRA Ethics Committee.

Two-week-old Pekin ducks (A. platyrhynchos domesticus) were obtained from a commercial hatchery of controlled sanitary status (Couvoir de la Seigneurtie, Vieillevigne, France). We verified that animals had no anti-H7 antibodies prior to inoculation. Animals were inoculated with 10^5 EID₅₀ virus diluted in PBS to reach a final volume of 500 µl, of which 250 µl was administered via the intrachoanal cleft route and 250 µl was administered via the oral route.

Four-week-old specific-pathogen-free histocompatible B13/B13 White Leghorn chickens were housed in biosafety level 3 cabinets under negative pressure with HEPA-filtered air. Briefly, three groups of 30, 30 and 10 birds were respectively inoculated with either virus, or mock-inoculated. Inoculated birds received 6 × 10⁵ EID₅₀ in 0.1 ml via the intratracheal route and 6 × 10⁵ EID₅₀ in 0.1 ml via the choanal route, while virus was replaced by PBS for control animals. For each tissue, including lung, kidney, caecum and brain, one sample (50 mg) was freeze-dried for viral RNA quantification and one was fixed in 10% neutral-buffered formalin for histopathological evaluation. A sample of lung was also conserved in RNA Later solution (Ambion) under negative pressure with HEPA-filtered air. Briefly, three groups of 6, 6 and 2 chickens were inoculated by the intratracheal route and 250 µl, of which 7.5 µl 2 × Q Supermix SYBR green (Bio-Rad), 4 µl ultrapure water (Invitrogen) and Chromo 4 instrument (Bio-Rad) were used, according to the manufacturers’ recommendations.

Total RNA was extracted from chicken lung after dissociation with the Tri Reagent solution, according to the manufacturer’s recommendations (Sigma), and treated with RNase-free DNase I (Invitrogen). Reverse transcription was performed on 1 µg RNA using the SuperScript first-strand synthesis system for RT-PCR, the Superscript III Platinum SYBR Green one-step quantitative RT-PCR kit (Invitrogen) and Chromo 4 instrument (Bio-Rad) were used, according to the manufacturers’ recommendations. The Quantitative PCR for in vivo duck experiments was performed as described previously (Soubies et al., 2010).

RNA extraction and qRT-PCR. RNA was extracted by use of the Nucleospin RNA II kit (Macherey Nagel). Quantitative PCR for in vivo duck experiments was performed as described previously (Soubies et al., 2010).

The QiaAmp viral RNA mini kit (Qiagen) was used to prepare viral RNAs from 140 µl chicken tissue homogenate, according to the manufacturer’s recommendations. For the quantification of M-vRNAs in chicken by real-time RT-PCR, the Superscript III Platinum SYBR Green one-step quantitative RT-PCR kit (Invitrogen) and Chromo 4 instrument (Bio-Rad) were used, according to the manufacturers’ recommendations.

Total RNA was extracted from chicken lung after dissociation with the TRI Reagent solution, according to the manufacturer’s recommendations (Sigma), and treated with RNase-free DNase I (Invitrogen). Reverse transcription was performed on 1 µg RNA using the SuperScript first-strand synthesis system for RT-PCR (Invitrogen) according to the manufacturer’s recommendations. Amplification of the cDNA by qPCR (Chromo 4; Bio-Rad) was performed in duplicate, using 2 µl threefold-diluted cDNA sample, 7.5 µl 2 × IQ Supermix SYBR green (Bio-Rad), 4 µl ultrapure water (Invitrogen) and 0.75 µl of each specific primer (10 µM). mRNA expression of chicken IFN-α, IFN-β, IFN-γ, Mx, interleukin-6 (IL-6), IL-8, IL-15, glyceraldehyde-3-phosphate dehydrogenase (GAPDH), G10 and ubiquitin was assessed. The sequences of primers can be obtained by subjecting 2 to 2 x 10^6 copies of a corresponding DNA plasmid to qPCR in parallel. Only PCR products showing a unique fusion temperature after raising the temperature to 95 °C at a transition rate of 0.5 °C s⁻¹ were retained for further quantitative automated analysis using the Opticon Monitor 3 software (Bio-Rad). The copy numbers of cytokine cDNAs determined experimentally were normalized to 10^6 copies of the geometric mean of the GAPDH, G10 and ubiquitin cDNA copy numbers, as measured in the same sample.

Histopathology and immunohistochemistry. Lung, caecum, colon and liver were collected and fixed in 4% neutral-buffered formalin, embedded in paraffin wax, sliced into 5 µm sections and dropped onto positively charged slides. Sections were then stained by routine haematoxylin–eosin–safron (HES) staining. Histopathological analysis was done by an ECVP-certified veterinary pathologist who was blinded to the experimental conditions. Two sections of each sample were analysed. Semiquantitative evaluations were performed for extension and intensity of lung lesions. Scores were as follows: 0, no lesion; 1, focal lesion (<500 µm diameter); 2, focal extensive or multifocal coalescent lesions; 3, generalized lesion. NS1 immunolabelling was performed using IG12 anti-NS1 mouse mAb on frozen lung samples as described previously (Munier et al., 2010).

Data analysis. Data are presented as means ± SEM for virus growth and as means and individual values for all other analyses. Statistical significance was assessed by using an unpaired, two-tailed Mann–Whitney test. In addition, the log-rank test was used to compare survival estimated by Kaplan–Meier analysis between H7N1230- and H7N1234-inoculated chickens.

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REFERENCES


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