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

Sébastien Soubies, Thomas Hoffmann, Guillaume Croville, Thibaut T. Larcher, Mireille Ledevin, Denis Soubieux, Pascale P. Quéré, Jean-Luc Guerin, Daniel Marc, Romain Volmer

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Running title: Role of NS1 C-terminal ESEV domain in avian hosts

Sébastien M. Soubies[1,2], Thomas W. Hoffmann[3,5], Guillaume Croville[1,2], Thibaut Larcher[6], Mireille Ledevin[6], Denis Soubieux[3,5], Pascale Quéré[4,5], Jean-Luc Guérin[1,2], Daniel Marc[3,5] and Romain Volmer[1,2]*

[1]INRA, UMR 1225, Ecole nationale vétérinaire de Toulouse, F-31076 Toulouse, France

[2]Université de Toulouse, ENVT, UMR 1225, F-31076 Toulouse, France

[3]Equipe BioVA, INRA UMR1282, Infectiologie et Santé Publique, ISP, F-37380 Nouzilly, France

[4]Equipe PIA, INRA UMR1282, Infectiologie et Santé Publique, ISP, F-37380 Nouzilly, France

[5]Université François Rabelais de Tours, UMR1282 Infectiologie et Santé Publique, F-37000 Tours, France

[6]INRA UMR 703, APEX, Oniris-La Chantrerie, F-44307 Nantes, France

*Correspondence:
Romain Volmer
rvolmer9@gmail.com

Abstract:

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 $R_{225}VESEV_{230}$ at the C-terminus of NS1. In order to assess how the truncation of NS1 affected virus replication, we generated by reverse genetics a wild-type low pathogenic avian influenza (LPAI) H7N1 virus with a 230 amino acid long NS1 ($H7N1_{230}$) and a mutant virus with a truncated NS1 ($H7N1_{224}$). The six amino acids truncation had no impact on virus replication in duck or chicken cells *in vitro*. The $H7N1_{230}$ virus and the $H7N1_{224}$ virus also replicated to similar levels and induced similar immune responses in ducks or chickens. No significant histological lesion was detected in infected ducks regardless of the virus inoculated. However, in chickens, the $H7N1_{230}$ induced a more severe interstitial pneumonia than the $H7N1_{224}$ virus. These findings indicate that the C-terminal extremity of NS1, including the PDZ-binding motif ESEV, is dispensable for efficient replication of a 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) introducing a stretch of basic amino acids at the HA cleavage site (Pantin-Jackwood & Swayne, 2009). As a consequence, the 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 tract where trypsin-like proteases are expressed. Thus, the cleavability of the 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 in 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 interferon (IFN) competent cells and are strongly attenuated *in vivo* (Egorov *et al.*, 1998; Garcia-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 the evolution from a LPAI H7N1 virus to a HPAI virus. As a consequence, the HPAI viruses lacked the C-terminal ESEV domain in the NS1 protein that had been previously shown 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 LPAI viruses' reservoir species, such as ducks, to poultry is unknown.

In order to address these questions, we generated by reverse genetics H7N1 viruses containing 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 contains a 230 amino acids long NS1 protein with an ESEV C-terminal domain. We called this virus $H7N1_{230}$. In a previously published paper, this virus was referred to as "ESEV" virus (Soubies *et al.*, 2010). In parallel, we mutated the NS segment to obtain a truncated NS1 protein of 224 amino acids. 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 (Figure 1). The recovered mutant virus has a NS1 protein of 224 amino acids and was designated as $H7N1_{224}$. In a previously published paper, this virus was referred to as "NEP S70" virus (Soubies *et al.*, 2010).

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$_{230}$ and H7N1$_{224}$ in duck embryonic fibroblasts (DEF) and chicken embryonic fibroblasts (CEF). In order to assess multicycle growth, we infected DEF and CEF at a multiplicity of infection (MOI) of 0.001 and cultured the cells in the presence of trypsin. H7N1$_{230}$ and H7N1$_{224}$ replicated with a similar kinetics and reached similar titers at 33 hours post-infection (hpi) (Figure 2). At 33hpi, the majority of the H7N1$_{230}$ and H7N1$_{224}$ 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 a MOI=3. We did not detect any difference between the viral titers of H7N1$_{230}$ and H7N1$_{224}$ (Figure 3A&B). We also measured type I IFN production in the supernatant of DEF and CEF infected at a MOI=3. Both viruses induced a detectable type I IFN production (Figure 3C&D). However, in both DEF and CEF, no significant difference could be detected between H7N1$_{230}$ and H7N1$_{224}$. Taken together, these findings indicate that the truncation of NS1 has no impact on virus replication or on type I IFN production in duck and chicken cells.

Consequences of NS1 truncation on viral replication and pathogenicity in ducks.

We infected Pekin ducks via the oral and intrachoanal routes with 10^7 egg infectious dose 50 (EID_{50}) of H7N1$_{230}$ or H7N1$_{224}$. 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$_{230}$ and H7N1$_{224}$-infected ducks, showing some minimal to mild infiltration of the submucosal chorion mainly composed of heterophilic polynuclear cells from day 2 to 4 pi (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 had previously been shown to be the major site of influenza A virus replication in ducks (Kida *et al.*, 1980). Viral

titers were maximal at day 3 post-infection (pi) for H7N1$_{230}$ and at day 7 pi for H7N1$_{224}$ (Figure 4A). However, no significant difference in the level of virus replication in the colon was detected between H7N1$_{230}$ and H7N1$_{224}$ infected ducks. 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 to non-infected ducks (Figure 4B). However, Mx levels were not significantly different between H7N1$_{230}$ and H7N1$_{224}$ infected ducks.

Consequences of NS1 truncation on viral replication and pathogenicity in chickens.

We infected four-week old White Leghorn chickens with 6.10^4 EID_{50} via the intratracheal route, along with 6.10^5 EID_{50} via the choanal route. Infection with H7N1$_{230}$ and H7N1$_{224}$ caused severe respiratory symptoms and was associated with a significant mortality: out of the 30 inoculated animals in each group, seven died in the H7N1$_{230}$ group and ten died in the H7N1$_{224}$ group during the monitoring week, in addition to the birds that were euthanized for tissue collection. Kaplan-Meier estimate and log-rank test didn't show any difference in survival between H7N1$_{230}$ and H7N1$_{224}$ inoculated chickens (4.4 ± 0.2 days vs 3.5 ± 0.1, $p = 0.195$, Figure 5A). At day 1, 2, 3 and 4 pi, tissue samples were taken from euthanized birds for viral RNA quantification, cytokine mRNA quantification, and histopathology. Due to the high rate of mortality, no bird survived until day 7 pi in the H7N1$_{224}$ infected group, while only two birds survived in the H7N1$_{230}$ infected group.

Virus replication was assessed through RT-qPCR-based quantification of viral RNA. Viral RNA was detected in the lungs from day 1 pi to day 4 pi with a peak day at 3 pi (Figure 5B). Lower levels of viral RNA (1 to 10% of those measured in the lungs) were also detected regularly in the kidney and brain, and less regularly in the caecum, between day 2 and 4 pi (data not shown). The presence of virus in kidney and brain probably reflects a systemic transport of the virus. We observed no significant differences in the viral load between the two viruses. Viral sequence analysis performed on four chickens per group at day 3 or 4 pi revealed that no virus had acquired mutations affecting the coding sequence of NS1 (data not shown). The virus was completely cleared from the two surviving H7N1$_{230}$ infected chickens at

day 7 pi (data not shown).

The levels of various cytokines and Mx mRNAs were measured by qRT-PCR in the lungs of chickens euthanized at day 1, 2, 3, and 4 pi. Mx mRNA levels were significantly upregulated from day 1 to day 4 pi in both $H7N1_{230}$ and $H7N1_{224}$ inoculated chickens compared to non-infected animals ($p<0.01$) (Figure 6A). We did not detect any significant upregulation of IFN-α mRNA in infected chickens (Figure 6B), whereas IFN-β mRNA levels were significantly upregulated in both $H7N1_{230}$ and $H7N1_{224}$ inoculated chickens compared to non-infected animals at day 3 pi ($p<0.05$) (Figure 6C). However, IFN-β mRNA levels were similar between $H7N1_{230}$ and $H7N1_{224}$ inoculated chickens. Thus, our results indicate that the intensity of the type I IFN immune response does not significantly differ between $H7N1_{230}$ and $H7N1_{224}$ infected chickens. We detected an upregulation of IFN-γ (Figure 6D), IL-6 (Figure 6E) and IL-8 (Figure 6F) in infected chickens. At day 3 pi, these mRNAs were significantly increased compared to controls in both $H7N1_{230}$ and $H7N1_{224}$ infected chickens ($p<0.05$). However, IFN-γ, IL-6 and IL-8 mRNA levels did not significantly differ between $H7N1_{230}$ and $H7N1_{224}$ infected chickens.

Histopathological analysis of the lungs revealed a broncho-interstitial pneumonia in infected chickens from day 2 to 4 pi. (Table 1). The intensity of the lesions was significantly higher in $H7N1_{230}$-infected chickens than in $H7N1_{224}$-infected chickens. The nature of the lesion was roughly similar, associating mononuclear cells around parabronchi and some heterophils in the parabronchial lumen (Figure 7A&B). However, at day 2 and 3 pi, an increased tendency to epithelial necrosis of bronchi and parabronchi was observed in $H7N1_{230}$ infected chickens compared to $H7N1_{224}$ infected chickens. This was associated with the accumulation of cellular debris occluding respiratory duct lumens in $H7N1_{230}$ infected chickens compared to essentially fibrinous exudation in parabronchi of $H7N1_{224}$ infected chickens (Figure 7C&D). Atrial emphysema recognized by severe enlargement of air spaces with surrounding atelectasis was frequently observed secondary to respiratory airway occlusion in $H7N1_{230}$ infected chickens. To specifically detect cells with replicating virus, we performed a NS1 immunolabelling on frozen lung sections (Figure 7E&F). At day 2 pi, NS1-positive cells (Figure 7F) localized at sites with a high cellularity compared to the normal lung parenchyma cellularity observed in controls (Figure 7E). This focal increase in cell density is most likely due to the influx of inflammatory cells in the infected lung areas suggesting that inflammatory cells are recruited to the

site of infection.

Discussion

Our results show that the truncation of the C-terminal RVESEV amino acids of NS1 has no impact on the ability of a LPAI H7N1 virus to replicate in duck cells 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 $H7N1_{230}$ and $H7N1_{224}$ viruses were shown to induce similar levels of type I IFN and of 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 $H7N1_{230}$ than in $H7N1_{224}$ infected chickens. Given the similar viral load and immune response observed with the two viruses, the cause for the severity of the histological lesions observed in $H7N1_{230}$-infected chickens remains unclear. The C-terminal ESEV domain of NS1 was shown to bind to 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 to Scribble and Dlg1 (Golebiewski *et al.*, 2011). The PDZ-protein binding of $H7N1_{230}$ NS1 could damage tight junctions, thereby causing increased lung pathology compared to the $H7N1_{224}$ virus.

Analysis of virus strains collected from different bird species during the Italian avian influenza epizootics from 1999 and 2001 suggested that the C-terminal truncation of NS1 occurred following the transmission from the wild aquatic bird reservoir, such as ducks, to terrestrial poultry, such as chickens (Dundon *et al.*, 2006). Thus, we initially hypothesized that the deletion of the C-terminal RVESEV motif of NS1 could be the result of a selection pressure occurring when the virus enters the chicken species.

Our results do not support this hypothesis. Indeed, the H7N1$_{224}$ virus with a truncated NS1 has no major phenotypic advantage compared to the wild type virus in chickens. During the Italian avian influenza epizootics from 1999 and 2001, only the HPAI viruses were found to harbor the 1-224 truncated variant of NS1 (Dundon *et al.*, 2006), while our studies were performed with LPAI H7N1 viruses. Thus, we cannot rule out that the 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. using the H1N1 PR8 strain (Jackson *et al.*, 2008) 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 significantly increase virulence in ducks and chickens, confirming our previous results in ducks (Soubies *et al.*, 2010) and Zielecki and colleagues' results in chickens (Zielecki *et al.*, 2010). 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 previously shown 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 a NS1 of 230 amino acids, 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 a NS1 of 219 amino acids due to the acquisition of a stop codon at position 220 (Garten *et al.*, 2009). Mutating the stop codon to obtain a 230 amino acid long NS1 had no impact on the replication of the pH1N1 virus in mice or in ferrets (Hale *et al.*, 2010). The 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 contributing to the selection of NS1 proteins of different lengths and with different C-terminal domains remain largely unknown.

Methods

Cells and reagents.

Primary duck embryonic fibroblasts (DEF) were obtained from 10-day-old Pekin duck (*Anas platyrhynchos*) embryos. Primary chicken embryonic fibroblasts (CEF) were obtained from 11-day-old chicken embryos. Cells were grown in Dulbecco's modified Eagle's medium (DMEM) supplemented with penicillin (10^4 U/ml), streptomycin (10 mg/ml), and 10% fetal bovine serum at 37°C and 5% CO2.

Virus and reverse genetics.

The low-pathogenicity avian influenza (LPAI) virus A/Turkey/Italy/977/1999 (H7N1) was a kind gift of I. Capua (Istituto Zooprofilattico Sperimentale Delle Venezie, Legnaro, Italy). The recombinant viruses $H7N1_{230}$ 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 viral segment using reverse transcription (RT)-PCR.

Infections and virus titration.

All infections were performed with DMEM supplemented with 0.2% bovine serum albumin (BSA). TPCK trypsin (0.1 µg/ml) was added in the case of multiple-cycle growth analysis. Viral titers were determined by plaque assay with MDCK 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 (Universität Freiburg, Freiburg, Germany) (38) and TK-RLuc, as previously described (42).

In vivo experiments.

All animals used in *in vivo* experiments were treated according to European

297 Economic Community (EEC) recommendations for animal welfare and under the
298 supervision of the local INRA Ethics Committee.
299 Two-week-old Pekin ducks (*Anas platyrhynchos domesticus*) were obtained from a
300 commercial hatchery of controlled sanitary status (Couvoir de la Seigneurtière,
301 Vieillevigne, France). We verified that animals had no anti-H7 antibodies prior to
302 inoculation. Animals were inoculated with 10^7 egg infectious dose 50 (EID_{50}) of virus
303 diluted in PBS to reach a final volume of 500 µL, of which 250 µL was administered
304 via the intrachoanal cleft route and 250 µL was administered via the oral route.
305 Four-week-old specific-pathogen-free histocompatible B13/B13 White Leghorn
306 chickens were housed in biosafety level 3 cabinets under negative pressure with
307 HEPA-filtered air. Briefly, three groups of 30, 30, and 10 birds were inoculated with
308 either virus, or mock-inoculated, respectively. Inoculated birds received 6.10^4 EID_{50}
309 in 0.1 mL via the intratracheal route, along with 6.10^5 EID_{50} in 0.1 mL via the
310 choanal route, while virus was replaced by PBS for control animals. For each tissue,
311 including lung, kidney, caecum, and brain, one sample (50 mg) was dry-frozen for
312 viral RNA quantification, one was fixed in 10% neutral buffered formalin for
313 histopathological evaluation. A sample of lung was also conserved in RNA-later
314 solution (Ambion) for cytokine mRNA quantification.
315
316 **RNA extraction and quantitative PCR.**
317 RNA was extracted by use of the Nucleospin RNA II kit (Macherey Nagel).
318 Quantitative PCR for *in vivo* duck experiments was performed as described
319 previously (Soubies *et al.*, 2010).
320 QiaAmp viral RNA mini kit (Qiagen) was used to prepare vRNAs from 140 µL of
321 chicken tissue homogenates, according to the manufacturer's recommendations. For
322 the quantification of M-vRNAs in chicken by real-time RT-PCR, the Superscript III
323 Platinum SYBR Green one-step quantitative RT-PCR kit (Invitrogen) and Chromo 4
324 instrument (Bio-Rad) were used, according to the manufacturer's recommendations.
325 Total RNA was extracted from chicken lung after dissociation with the Tri Reagent
326 solution, according to the manufacturer's recommendations (Sigma), and treated
327 with RNase-free DNase I (Invitrogen). Reverse transcription was performed on 1 µg
328 of RNA using the Superscript first-strand synthesis system for RT-PCR (Invitrogen)
329 according to the manufacturer's recommendations. Amplification of the cDNA by
330 qPCR (Chromo 4; Bio-Rad) was performed in duplicate, using 2 µL of the 3-fold-

diluted cDNA sample, 7.5 μL of 2X iQ Supermix SYBR green (Bio-Rad), 4 μL of 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, GAPDH, G10, and ubiquitin was assessed. The sequence of primers can be provided upon request. For each target sequence, a standard curve was obtained by subjecting 2 to 2.10^6 copies of a corresponding DNA plasmid to qPCR in parallel. Only PCR products showing a unique temperature of fusion after raising the temperature to 95°C at a transition rate of 0.5°C/s were retained for further quantitative automated analysis with the Opticon Monitor 3 software (Bio-Rad). The copy numbers of cytokine cDNAs determined experimentally were normalized to 10^7 copies of the geometric mean of 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 thick sections, and dropped off on positive-charge slides. Sections were then stained using a routine Hematoxylin-Eosin-Saffron staining (HES). Histopathological analysis was done by a ECVP-certified veterinary pathologist who was blinded to the experimental conditions. Two sections of each sample were analysed. Semi-quantitative evaluations were performed for extension and intensity of lung lesions. Scores were as follows: 0, no lesion ; 1, focal lesion (less than 500μm diameter); 2, focal extensive or multifocal coalescent lesions; 3, generalized lesion. NS1 immunolabelling was performed using IG12 anti-NS1 mouse monoclonal antibody on frozen lung samples as described previously (Munier *et al.*, 2010).

Data analysis.

Data are presented as means ± standard errors of the 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, log-rank test was used to compare survival estimated by Kaplan-Meier analysis between $H7N1_{230}$ and $H7N1_{224}$-inoculated chickens.

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460

461 **Figure legends**

462

463 **Figure 1**

464 **NS segments of H7N1$_{230}$ and H7N1$_{224}$.** The NS segment of influenza A viruses
465 encodes two proteins, named NS1 and NEP. The nucleotide mutated in H7N1$_{224}$ is
466 underlined.

467

468 **Figure 2**

469 **Multicycle growth analysis in duck and chicken cells.** (A) Primary duck
470 fibroblasts (DEF) and (B) primary chicken fibroblasts (CEF) were infected at an MOI
471 of 0.001 in the presence of TPCK trypsin to allow multiple-cycle virus growth.
472 Supernatants were collected at the indicated times post-infection (pi), and viral titers
473 were determined with MDCK cells. The results are representative of at least three
474 independent experiments.

475

476 **Figure 3**

477 **Single-cycle growth analysis and type I IFN production in duck and chicken**
478 **cells.** (A) Primary duck fibroblasts (DEF) and (B) primary chicken fibroblasts (CEF)
479 were infected with the H7N1$_{230}$ and H7N1$_{224}$ viruses at an MOI of 3. Supernatants
480 were collected at the indicated times pi, and viral titers were determined with MDCK
481 cells. (C and D) Mx-Firefly luciferase activity (Mx-FFLuc) normalized to TK Renilla
482 luciferase activity (TK-Rluc) was measured in duck cells stimulated with supernatant
483 collected 20 h pi from infected DEF (C) and in chicken cells stimulated with
484 supernatant collected 20 h pi from infected CEF (D) or increasing concentrations of
485 recombinant duck IFN-α and chicken IFN-α, respectively (triangles, individual level;
486 bars, mean level).

487

488 **Figure 4**

489 **Replication and pathogenesis in ducks.** (A) Viral RNA level in the ileal and colonic
490 mucosae at days 1, 2, 3 and 6 pi. Viral RNA levels from scraped mucosae were
491 determined by RT-qPCR and normalized to glyceraldehyde 3-phosphate
492 dehydrogenase (GAPDH) levels (triangles, individual level; bars, mean level). (B)
493 RT-qPCR analysis of Mx expression in the ileal and colonic mucosae at days 1, 2, 3

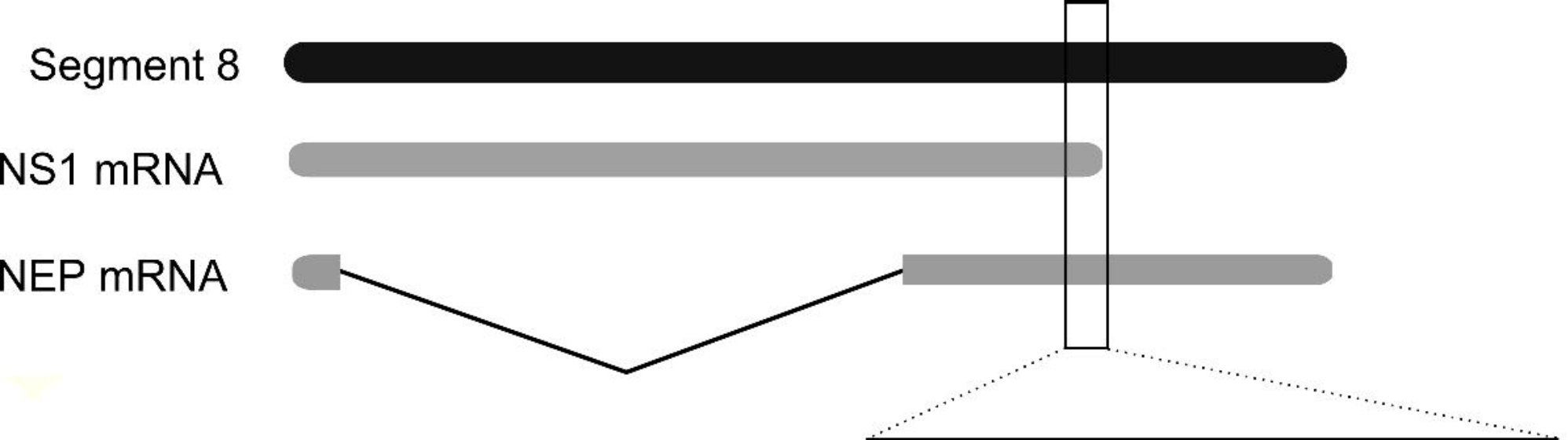
494 and 6 pi. Mx levels are normalized to GAPDH levels (triangles, individual level; bars,
495 mean level).
496
497 **Figure 5**
498 **Survival and viral load in chickens.** (A) Survival was estimated for 30 wt-
499 inoculated chickens (line) and 30 truncNS1-inoculated chickens (dots) by Kaplan
500 Meier life-table analysis, and was compared between the 2 groups by log-rank test.
501 (B) Viral RNA level in the lung at days 1, 2, 3 and 4 pi. Levels of viral RNA were
502 determined by qRT-PCR on RNA extracted from the lung and expressed in number
503 of copies/mg of lung (triangles, individual level; bars, mean level).
504
505 **Figure 6**
506 **Level of cytokine mRNAs in the lungs of chickens.** The levels of the indicated
507 cytokine mRNAs at the indicated time points were determined using quantitative RT-
508 PCR. The results are expressed as mRNA copy numbers normalized with 10^7 copies
509 of the geometric mean of 3 housekeeping genes cDNA copy numbers (GAPDH, G10
510 and ubiquitin). Triangles: individual level; bars, mean level.
511
512 **Figure 7**
513 **Histopathological observations of chicken lungs.** 4 days after inoculation of
514 $H7N1_{230}$ virus (A) or $H7N1_{224}$ (B), focal extensive broncho-interstitial pneumonia (*) is
515 noted with large amounts of material in airways (arrowheads). Note the presence of
516 normal parenchyma (°) surrounding the relatively well-delineated lesion in the
517 $H7N1_{224}$ inoculated animal. Higher magnification of A and B are presented in C and
518 D respectively. Following $H7N1_{230}$ inoculation (C), airways are obliterated by necrotic
519 debris and heterophils (arrow), disrupting parabronchus wall (dotted lines), whereas
520 $H7N1_{224}$ inoculation (D) elicits essentially fibrinous exudation (arrowhead) in airways
521 with a few heterophils and no necrosis. Haemalun-Eosin-Saffron staining. Upper
522 panel : bar = 250 μm ; Lower panel : bars = 100 μm. (E to F) Immunochemistry
523 analysis of lung sections at day 2 pi, using an anti-NS1 monoclonal antibody (green).
524 TOPRO3 nuclear staining (blue). (E) Mock-inoculated chicken. (F) $H7N1_{230}$ virus-
525 inoculated chicken. The viral NS1 antigen is detected in the centre of areas of higher
526 cellular density.
527

Table 1

Histological lesions in chickens inoculated with wild type or mutant H7N1 virus. Each lesion intensity was semi quantitatively evaluated (0=no lesion, 1=mild, 2=marked, 3=severe) and a mean score was calculated for each batch of animals. Frequency of lesions is reported into brackets. Histological scores from day 2 + 4 (grouping day 2 and day 4 animals) were significantly higher in $H7N1_{230}$ virus chickens than in $H7N1_{224}$ infected chickens ($p<0.05$).

	bronchial lesions	
dpi	**$NS1_{230}$**	**$NS1_{224}$**
2	2.7 (n=3/3)	0.7 (n=1/3)
4	2.3 (n=3/3)	0.7 (n=1/3)
2 + 4	**2.5 (n=6/6)**	**0.7 (n=2/6)**

dpi = days post inoculation. n = total number of chickens.



"H7N1-NS1$_{230}$ "

mRNA sequence GACGAGUUGAGUCAGAAGUUU

NS1 protein R_{225} V_{226} E_{227} S_{228} E_{229} V_{230}

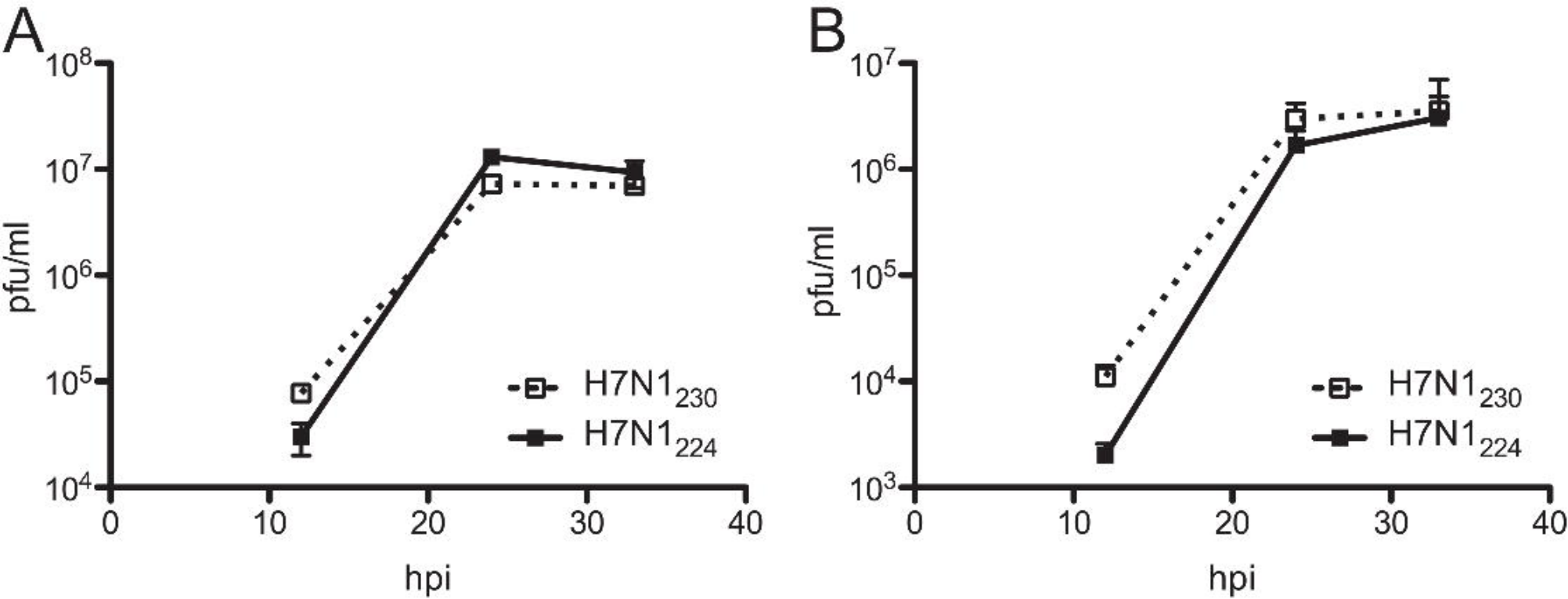
NEP protein D_{67} E_{68} L_{69} S_{70} Q_{71} K_{72} F_{73}

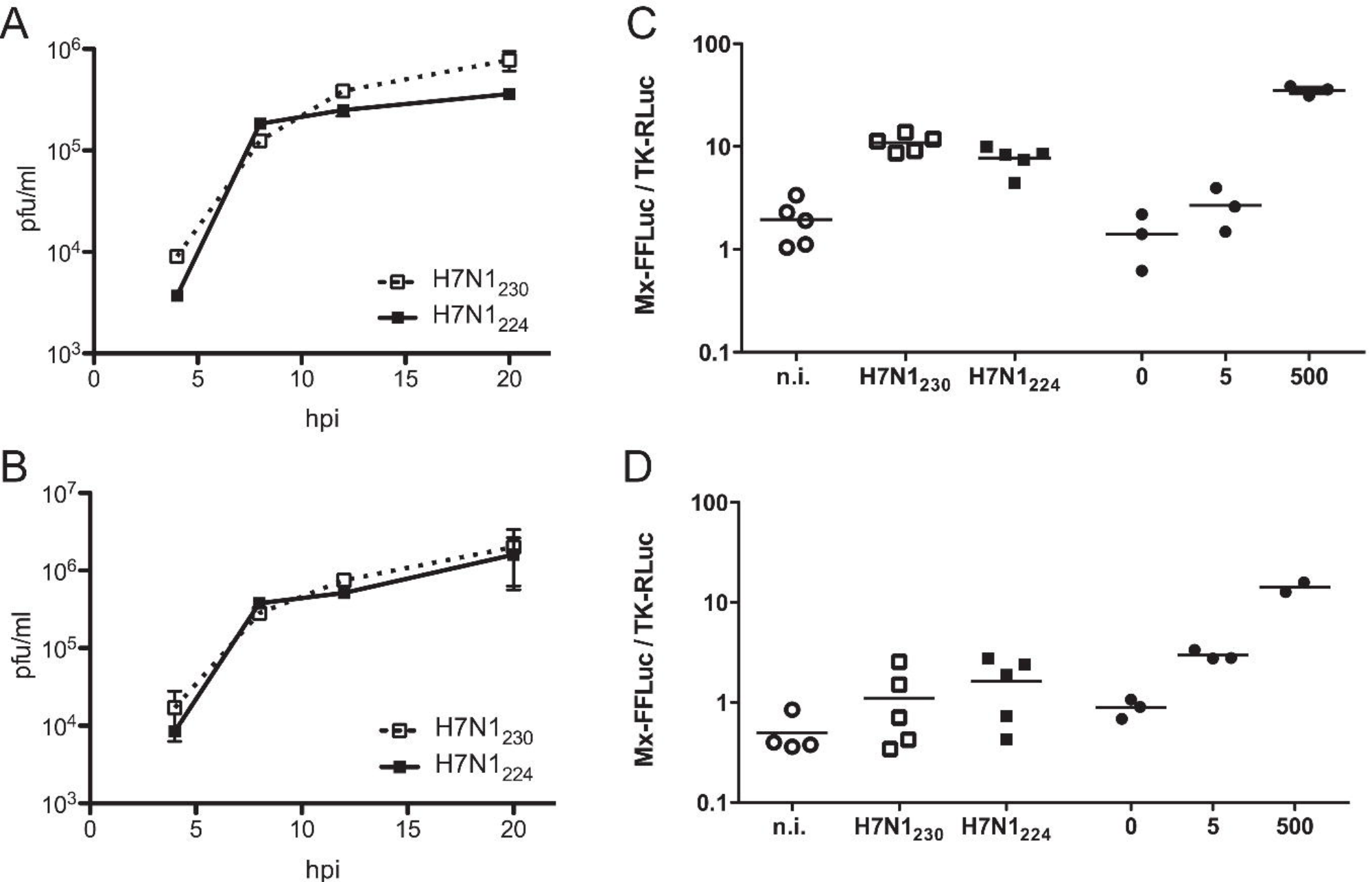
"H7N1-NS1$_{224}$ "

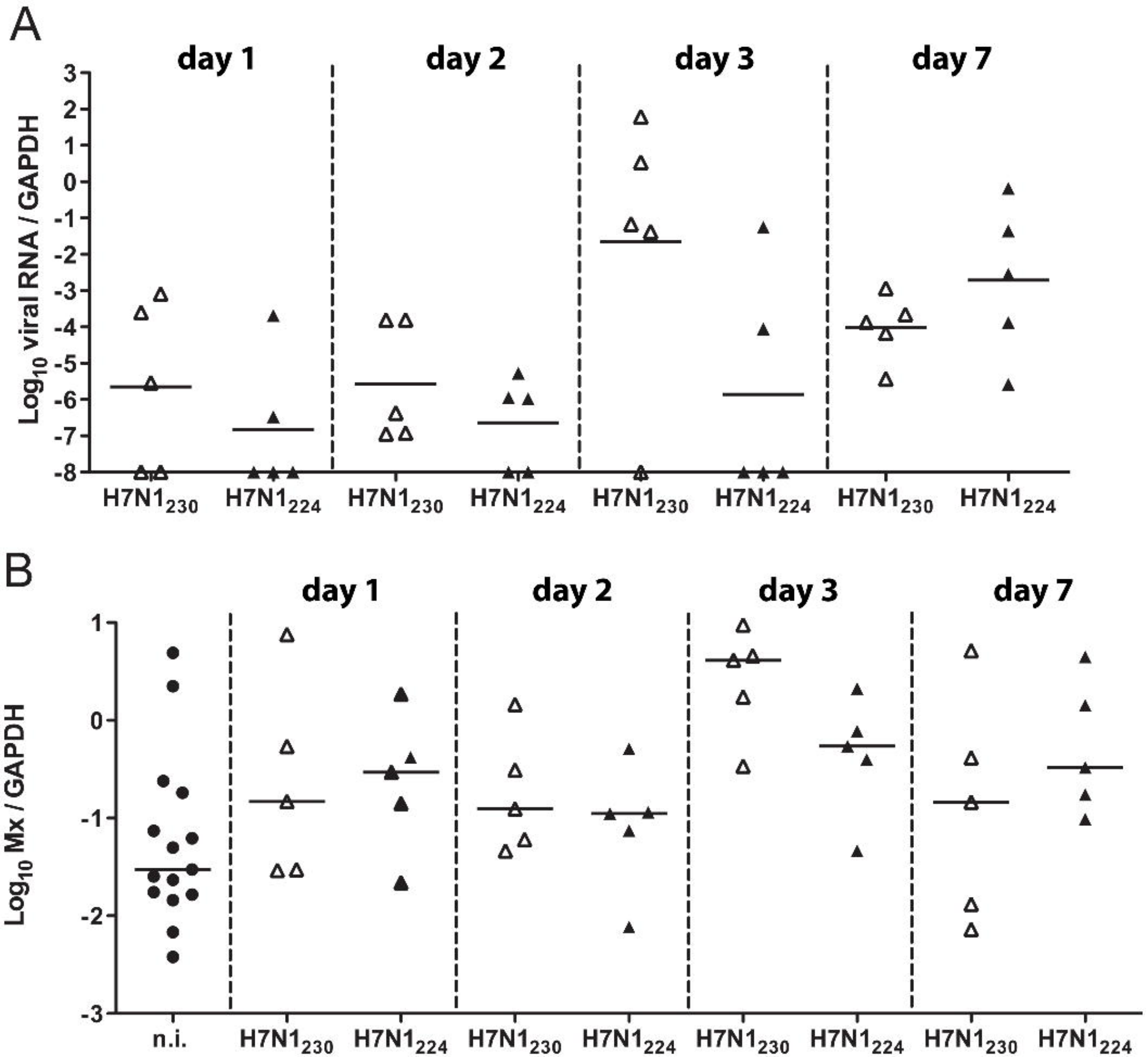
mRNA sequence GAUGAGUUGAGUCAGAAGUUU

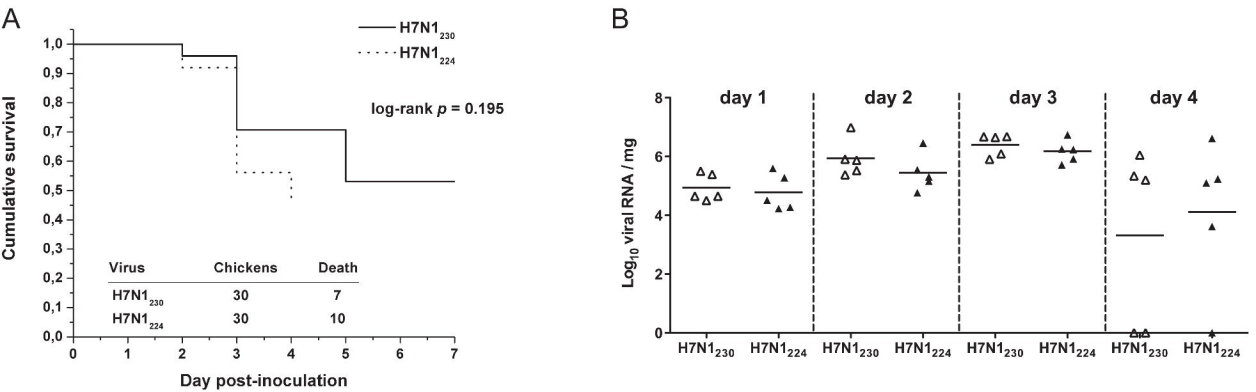
NS1 protein $Stop_{225}$

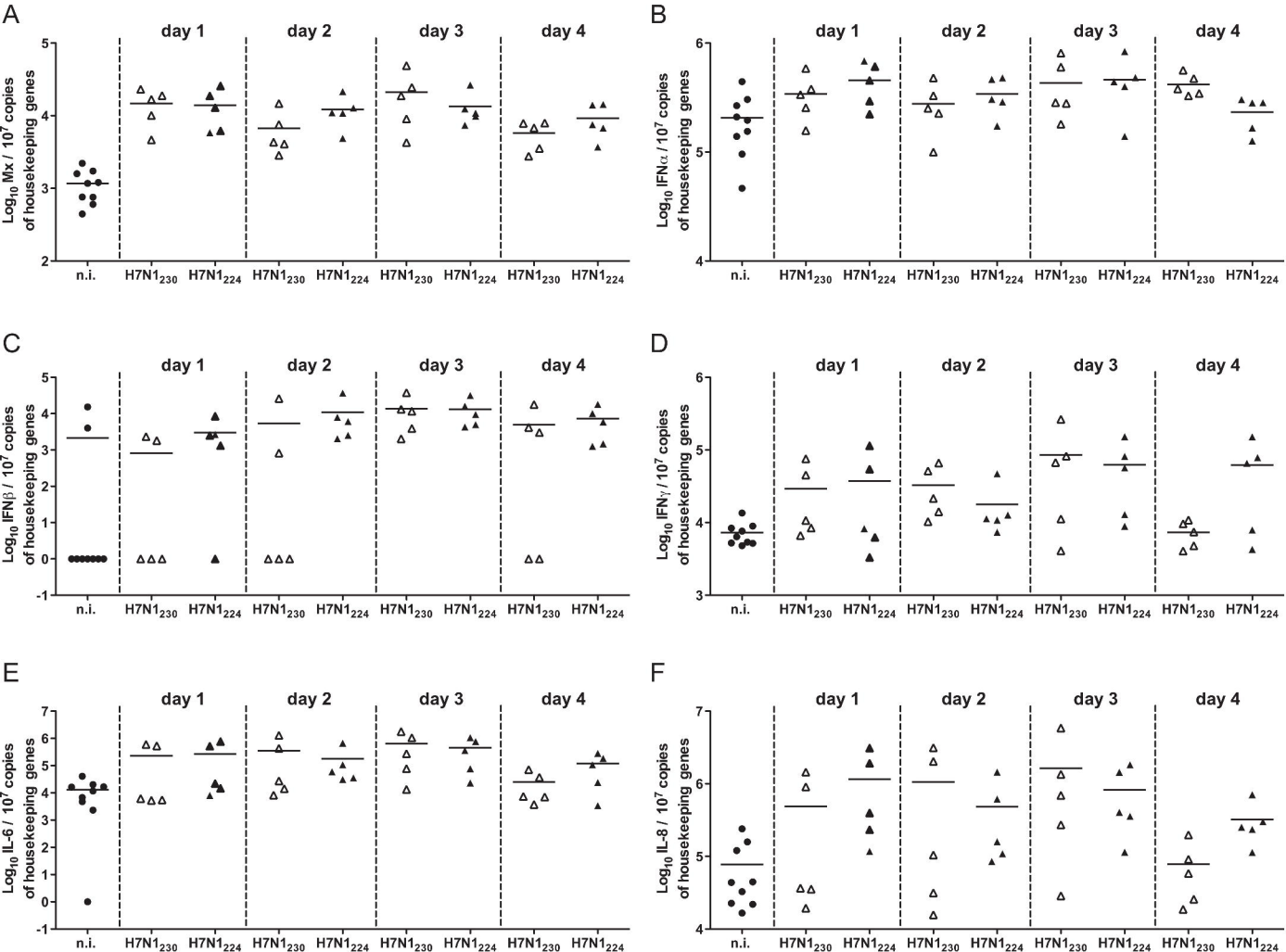
NEP protein D_{67} E_{68} L_{69} S_{70} Q_{71} K_{72} F_{73}

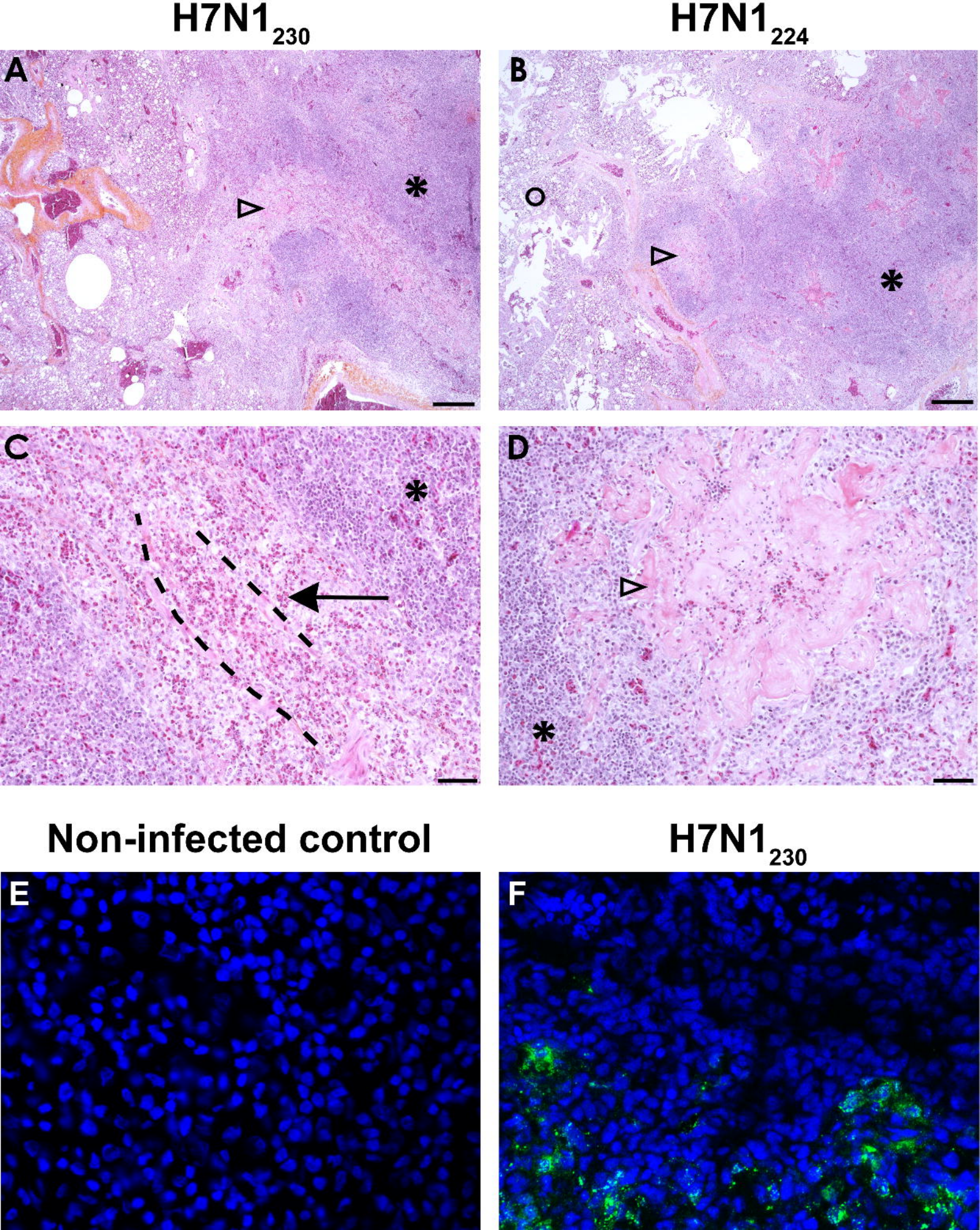

A
10^8
10^7
10^6
10^5
10^4
pfu/ml
0
10
20
30
40
hpi
H7N1$_{230}$
H7N1$_{224}$
B
10^7
10^6
10^5
10^4
10^3
pfu/ml
0
10
20
30
40
hpi
H7N1$_{230}$
H7N1$_{224}$


A
B
C
D
pfu/ml
hpi
H7N1230
H7N1224
Mx-FFLuc / TK-RLuc
n.i.
0
5
500


A
day 1
day 2
day 3
day 7
Log10 viral RNA / GAPDH
3
2
1
0
-1
-2
-3
-4
-5
-6
-7
-8
H7N1230
H7N1224
H7N1230
H7N1224
H7N1230
H7N1224
H7N1230
H7N1224
B
day 1
day 2
day 3
day 7
Log10 Mx / GAPDH
1
0
-1
-2
-3
n.i.
H7N1230
H7N1224
H7N1230
H7N1224
H7N1230
H7N1224
H7N1230
H7N1224


A
H7N1230
H7N1224
log-rank p = 0.195
Cumulative survival
1,0
0,9
0,8
0,7
0,6
0,5
0,4
0,3
0,2
0,1
0,0
0
1
2
3
4
5
6
7
Day post-inoculation
Virus
Chickens
Death
H7N1230
30
7
H7N1224
30
10
B
day 1
day 2
day 3
day 4
Log10 viral RNA / mg
8
6
4
2
0
H7N1230
H7N1224
H7N1230
H7N1224
H7N1230
H7N1224
H7N1230
H7N1224


A
B
C
D
E
F
day 1
day 2
day 3
day 4
n.i.
H7N1230
H7N1224
Log10 Mx / 10^7 copies of housekeeping genes
Log10 IFNα / 10^7 copies of housekeeping genes
Log10 IFNβ / 10^7 copies of housekeeping genes
Log10 IFNγ / 10^7 copies of housekeeping genes
Log10 IL-6 / 10^7 copies of housekeeping genes
Log10 IL-8 / 10^7 copies of housekeeping genes


H7N1 230
H7N1 224
A
B
C
D
Non-infected control
H7N1 230
E
F