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

3

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

5

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25

26 **Abstract:**

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

42

43 **Introduction:**

44

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

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

60 Kochs *et al.*, 2007).

61 Several epizootics due to HPAI viruses of the H7N1 subtype occurred in Italy
62 between 1999 and 2001 (Capua & Alexander, 2004). Interestingly, all HPAI viruses
63 isolated had a C-terminally truncated NS1 protein, resulting from a point mutation
64 that introduced a premature stop codon at position 225 (Dundon *et al.*, 2006). Of
65 note, the full-length NS1 protein could only be detected in LPAI viruses circulating at
66 that time, suggesting that the C-terminal truncation of NS1 occurred during the
67 evolution from a LPAI H7N1 virus to a HPAI virus. As a consequence, the HPAI
68 viruses lacked the C-terminal ESEV domain in the NS1 protein that had been
69 previously shown to modulate virulence in a way that appears to be dependent both
70 on the host species and on the virus isolate (Jackson *et al.*, 2008; Soubies *et al.*,
71 2010; Zielecki *et al.*, 2010). The consequences of the truncation on the virulence of
72 this isolate are unknown. In addition, whether the truncation of NS1 contributes to
73 the adaptation of avian influenza viruses from LPAI viruses' reservoir species, such
74 as ducks, to poultry is unknown.

75 In order to address these questions, we generated by reverse genetics H7N1 viruses
76 containing a full-length NS1 protein or a C-terminally truncated NS1 lacking the
77 amino acids RVESEV. We compared the replication of these viruses in duck and
78 chicken embryonic fibroblasts. In addition, we assessed their replication and
79 pathogenicity in ducks and chickens.

80

81

82 **Results**

83

84 **Rescue of recombinant H7N1 viruses**

85 Plasmid-driven reverse genetics was used to recover a wild-type virus that contains
86 a 230 amino acids long NS1 protein with an ESEV C-terminal domain. We called this
87 virus H7N1₂₃₀. In a previously published paper, this virus was referred to as "ESEV"
88 virus (Soubies *et al.*, 2010). In parallel, we mutated the NS segment to obtain a
89 truncated NS1 protein of 224 amino acids. Through a single C to U substitution, we
90 introduced a stop codon at position 225, as observed in the HPAI viruses isolated
91 during the 1999-2001 Italian epizootics (Figure 1). The recovered mutant virus has a
92 NS1 protein of 224 amino acids and was designated as H7N1₂₂₄. In a previously
93 published paper, this virus was referred to as "NEP S70" virus (Soubies *et al.*, 2010).

94 Importantly, the introduced mutation does not alter the amino acid sequence of the
95 nuclear export protein (NEP). The identity of the amplified viruses was verified by
96 sequencing of amplicons of each viral gene.

97

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

99 We compared the growth properties of H7N1₂₃₀ and H7N1₂₂₄ in duck embryonic
100 fibroblasts (DEF) and chicken embryonic fibroblasts (CEF). In order to assess
101 multicycle growth, we infected DEF and CEF at a multiplicity of infection (MOI) of
102 0.001 and cultured the cells in the presence of trypsin. H7N1₂₃₀ and H7N1₂₂₄
103 replicated with a similar kinetics and reached similar titers at 33 hours post-infection
104 (hpi) (Figure 2). At 33hpi, the majority of the H7N1₂₃₀ and H7N1₂₂₄ infected cells
105 were dead and the multicycle growth analysis was therefore terminated. To assess
106 virus production during single-cycle growth, we infected DEF and CEF at a MOI=3.
107 We did not detect any difference between the viral titers of H7N1₂₃₀ and H7N1₂₂₄
108 (Figure 3A&B). We also measured type I IFN production in the supernatant of DEF
109 and CEF infected at a MOI=3. Both viruses induced a detectable type I IFN
110 production (Figure 3C&D). However, in both DEF and CEF, no significant difference
111 could be detected between H7N1₂₃₀ and H7N1₂₂₄. Taken together, these findings
112 indicate that the truncation of NS1 has no impact on virus replication or on type I IFN
113 production in duck and chicken cells.

114

115 **Consequences of NS1 truncation on viral replication and pathogenicity in** 116 **ducks.**

117 We infected Pekin ducks via the oral and intratracheal routes with 10⁷ egg infectious
118 dose 50 (EID₅₀) of H7N1₂₃₀ or H7N1₂₂₄. Infected animals did not show any clinical
119 signs, as classically observed for LPAI infection in ducks (Kida *et al.*, 1980; Pantin-
120 Jackwood & Swayne, 2009; Webster *et al.*, 1978). Histologically, very mild lesions
121 were only observed in trachea and caecum samples of most H7N1₂₃₀ and H7N1₂₂₄-
122 infected ducks, showing some minimal to mild infiltration of the submucosal chorion
123 mainly composed of heterophilic polynuclear cells from day 2 to 4 pi (data not
124 shown).

125 We assessed the level of virus replication by measuring the level of viral RNA by
126 quantitative RT-PCR (qRT-PCR) in the colon, which had previously been shown to
127 be the major site of influenza A virus replication in ducks (Kida *et al.*, 1980). Viral

128 titers were maximal at day 3 post-infection (pi) for H7N1₂₃₀ and at day 7 pi for
129 H7N1₂₂₄ (Figure 4A). However, no significant difference in the level of virus
130 replication in the colon was detected between H7N1₂₃₀ and H7N1₂₂₄ infected ducks.
131 Finally, we quantified the intensity of the type I IFN induced-immune response by
132 measuring Mx transcripts. Mx is an interferon-stimulated gene and a good indicator
133 of the level of type I IFN produced *in situ* (Holzinger *et al.*, 2007; Sommereyns *et al.*,
134 2008). The level of Mx transcripts was increased in both groups of virus-infected
135 ducks compared to non-infected ducks (Figure 4B). However, Mx levels were not
136 significantly different between H7N1₂₃₀ and H7N1₂₂₄ infected ducks.

137

138 **Consequences of NS1 truncation on viral replication and pathogenicity in** 139 **chickens.**

140 We infected four-week old White Leghorn chickens with 6.10^4 EID₅₀ via the
141 intratracheal route, along with 6.10^5 EID₅₀ via the choanal route. Infection with
142 H7N1₂₃₀ and H7N1₂₂₄ caused severe respiratory symptoms and was associated with
143 a significant mortality: out of the 30 inoculated animals in each group, seven died in
144 the H7N1₂₃₀ group and ten died in the H7N1₂₂₄ group during the monitoring week, in
145 addition to the birds that were euthanized for tissue collection. Kaplan-Meier
146 estimate and log-rank test didn't show any difference in survival between H7N1₂₃₀
147 and H7N1₂₂₄ inoculated chickens (4.4 ± 0.2 days vs 3.5 ± 0.1 , $p = 0.195$, Figure 5A).
148 At day 1, 2, 3 and 4 pi, tissue samples were taken from euthanized birds for viral
149 RNA quantification, cytokine mRNA quantification, and histopathology. Due to the
150 high rate of mortality, no bird survived until day 7 pi in the H7N1₂₂₄ infected group,
151 while only two birds survived in the H7N1₂₃₀ infected group.

152 Virus replication was assessed through RT-qPCR-based quantification of viral RNA.
153 Viral RNA was detected in the lungs from day 1 pi to day 4 pi with a peak day at 3 pi
154 (Figure 5B). Lower levels of viral RNA (1 to 10% of those measured in the lungs)
155 were also detected regularly in the kidney and brain, and less regularly in the
156 caecum, between day 2 and 4 pi (data not shown). The presence of virus in kidney
157 and brain probably reflects a systemic transport of the virus. We observed no
158 significant differences in the viral load between the two viruses. Viral sequence
159 analysis performed on four chickens per group at day 3 or 4 pi revealed that no virus
160 had acquired mutations affecting the coding sequence of NS1 (data not shown). The
161 virus was completely cleared from the two surviving H7N1₂₃₀ infected chickens at

162 day 7 pi (data not shown).

163 The levels of various cytokines and Mx mRNAs were measured by qRT-PCR in the
164 lungs of chickens euthanized at day 1, 2, 3, and 4 pi. Mx mRNA levels were
165 significantly upregulated from day 1 to day 4 pi in both H7N1₂₃₀ and H7N1₂₂₄
166 inoculated chickens compared to non-infected animals ($p<0.01$) (Figure 6A). We did
167 not detect any significant upregulation of IFN- α mRNA in infected chickens (Figure
168 6B), whereas IFN- β mRNA levels were significantly upregulated in both H7N1₂₃₀ and
169 H7N1₂₂₄ inoculated chickens compared to non-infected animals at day 3 pi ($p<0.05$)
170 (Figure 6C). However, IFN- β mRNA levels were similar between H7N1₂₃₀ and
171 H7N1₂₂₄ inoculated chickens. Thus, our results indicate that the intensity of the type I
172 IFN immune response does not significantly differ between H7N1₂₃₀ and H7N1₂₂₄
173 infected chickens. We detected an upregulation of IFN- γ (Figure 6D), IL-6 (Figure
174 6E) and IL-8 (Figure 6F) in infected chickens. At day 3 pi, these mRNAs were
175 significantly increased compared to controls in both H7N1₂₃₀ and H7N1₂₂₄ infected
176 chickens ($p<0.05$). However, IFN- γ , IL-6 and IL-8 mRNA levels did not significantly
177 differ between H7N1₂₃₀ and H7N1₂₂₄ infected chickens.

178 Histopathological analysis of the lungs revealed a broncho-interstitial pneumonia in
179 infected chickens from day 2 to 4 pi. (Table 1). The intensity of the lesions was
180 significantly higher in H7N1₂₃₀-infected chickens than in H7N1₂₂₄-infected chickens.
181 The nature of the lesion was roughly similar, associating mononuclear cells around
182 parabronchi and some heterophils in the parabronchial lumen (Figure 7A&B).
183 However, at day 2 and 3 pi, an increased tendency to epithelial necrosis of bronchi
184 and parabronchi was observed in H7N1₂₃₀ infected chickens compared to H7N1₂₂₄
185 infected chickens. This was associated with the accumulation of cellular debris
186 occluding respiratory duct lumens in H7N1₂₃₀ infected chickens compared to
187 essentially fibrinous exudation in parabronchi of H7N1₂₂₄ infected chickens (Figure
188 7C&D). Atrial emphysema recognized by severe enlargement of air spaces with
189 surrounding atelectasis was frequently observed secondary to respiratory airway
190 occlusion in H7N1₂₃₀ infected chickens. To specifically detect cells with replicating
191 virus, we performed a NS1 immunolabelling on frozen lung sections (Figure 7E&F).
192 At day 2 pi, NS1-positive cells (Figure 7F) localized at sites with a high cellularity
193 compared to the normal lung parenchyma cellularity observed in controls (Figure
194 7E). This focal increase in cell density is most likely due to the influx of inflammatory
195 cells in the infected lung areas suggesting that inflammatory cells are recruited to the

196 site of infection.

197

198

199 **Discussion**

200

201 Our results show that the truncation of the C-terminal RVESEV amino acids of NS1
202 has no impact on the ability of a LPAI H7N1 virus to replicate in duck cells or chicken
203 cells *in vitro*, as well as *in vivo*. We further provide evidence that the truncation of
204 NS1 has no impact on the ability of NS1 to antagonize the host immune response.
205 Indeed, the H7N1₂₃₀ and H7N1₂₂₄ viruses were shown to induce similar levels of type
206 I IFN and of pro-inflammatory cytokines and IL-8. Infected ducks had no detectable
207 symptoms and no histological lesions, in accordance with the well reported low
208 morbidity observed in LPAI virus infected ducks (Kida *et al.*, 1980; Webster *et al.*,
209 1978). By contrast, respiratory symptoms are more commonly observed in chickens
210 infected with LPAI viruses, including LPAI H7 viruses (Spackman *et al.*, 2010). In this
211 study, infected chickens suffered from severe respiratory signs and a high rate of
212 mortality, in accordance with the results from a previous study using the same wild-
213 type virus and a similar inoculation protocol (Hoffmann *et al.*, 2012).
214 Histopathological analysis of the lungs from infected chickens revealed an interstitial
215 pneumonia that was significantly more severe in H7N1₂₃₀ than in H7N1₂₂₄ infected
216 chickens. Given the similar viral load and immune response observed with the two
217 viruses, the cause for the severity of the histological lesions observed in H7N1₂₃₀-
218 infected chickens remains unclear. The C-terminal ESEV domain of NS1 was shown
219 to bind to PDZ domains containing cellular proteins (Obenauer *et al.*, 2006; Tonikian
220 *et al.*, 2008). NS1 proteins with a PDZ-protein binding domain have been shown to
221 disrupt cellular tight junctions by binding to Scribble and Dlg1 (Golebiewski *et al.*,
222 2011). The PDZ-protein binding of H7N1₂₃₀ NS1 could damage tight junctions,
223 thereby causing increased lung pathology compared to the H7N1₂₂₄ virus.

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

230 Our results do not support this hypothesis. Indeed, the H7N1₂₂₄ virus with a
231 truncated NS1 has no major phenotypic advantage compared to the wild type virus
232 in chickens. During the Italian avian influenza epizootics from 1999 and 2001, only
233 the HPAI viruses were found to harbor the 1-224 truncated variant of NS1 (Dundon
234 *et al.*, 2006), while our studies were performed with LPAI H7N1 viruses. Thus, we
235 cannot rule out that the truncation of NS1 could have other consequences on the
236 replication and pathogenesis of HPAI H7N1 virus.

237 The C-terminal ESEV domain of NS1 was previously described as a virulence motif
238 in mice by Jackson *et al.* using the H1N1 PR8 strain (Jackson *et al.*, 2008) and by
239 our group using the H7N1 strain used in this work (Soubies *et al.*, 2010). The results
240 of the current study indicate that the C-terminal ESEV domain of NS1 does not
241 significantly increase virulence in ducks and chickens, confirming our previous
242 results in ducks (Soubies *et al.*, 2010) and Zielecki and colleagues' results in
243 chickens (Zielecki *et al.*, 2010). Thus, although it is highly conserved in avian
244 influenza viruses, the ESEV domain of NS1 does not seem to increase virus
245 replication and virulence in bird species. We have previously shown that a virus with
246 a C-terminal ESEV domain replicates to lower levels than a virus with a C-terminal
247 RSKV domain in ducks, but that it is excreted for a longer period (Soubies *et al.*,
248 2010). As a consequence, viruses with an ESEV domain could contaminate more
249 individuals and therefore be selected during virus evolution in birds.

250 Although the vast majority of influenza virus strains have a NS1 of 230 amino acids,
251 a number of strains with a C-terminal truncation in the NS1 protein have been
252 identified (Dundon & Capua, 2009). One remarkable example is the 2009 pandemic
253 influenza A H1N1 virus (pH1N1) which has a NS1 of 219 amino acids due to the
254 acquisition of a stop codon at position 220 (Garten *et al.*, 2009). Mutating the stop
255 codon to obtain a 230 amino acid long NS1 had no impact on the replication of the
256 pH1N1 virus in mice or in ferrets (Hale *et al.*, 2010). The pH1N1 thus seems to
257 tolerate truncations in the C-terminal domain of NS1, as does the LPAI H7N1
258 described in the current study. At present, the mechanisms contributing to the
259 selection of NS1 proteins of different lengths and with different C-terminal domains
260 remain largely unknown.

261

262

263

264 **Methods**

265

266 **Cells and reagents.**

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

272

273 **Virus and reverse genetics.**

274 The low-pathogenicity avian influenza (LPAI) virus A/Turkey/Italy/977/1999 (H7N1)
275 was a kind gift of I. Capua (Istituto Zooprofilattico Sperimentale Delle Venezie,
276 Legnaro, Italy). The recombinant viruses H7N1₂₃₀ was generated as described
277 previously (Soubies *et al.*, 2010). In order to generate the mutant virus H7N1₂₂₄, site-
278 directed mutagenesis was performed on the NS segment by using the QuikChange II
279 kit (Stratagene) according to the manufacturer's protocol. We verified the identity of
280 amplified viruses by sequencing of amplicons of each viral segment using reverse
281 transcription (RT)-PCR.

282

283 **Infections and virus titration.**

284 All infections were performed with DMEM supplemented with 0.2% bovine serum
285 albumin (BSA). TPCK trypsin (0.1 µg/ml) was added in the case of multiple-cycle
286 growth analysis. Viral titers were determined by plaque assay with MDCK cells.

287

288 **Chicken and duck type I IFN titration.**

289 Titration of chicken and duck type I IFN was performed by transfecting CEF and
290 DEF, respectively, with a plasmid containing the sequence of firefly luciferase under
291 the control of a chicken Mx promoter (Mx-FFLuc), kindly provided by P. Staeheli
292 (Universität Freiburg, Freiburg, Germany) (38) and TK-RLuc, as previously described
293 (42).

294

295 ***In vivo* experiments.**

296 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 Vieilleville, France). We verified that animals had no anti-H7 antibodies prior to
302 inoculation. Animals were inoculated with 10^7 egg infectious dose 50 (EID₅₀) of virus
303 diluted in PBS to reach a final volume of 500 μ L, of which 250 μ L was administered
304 via the intrachoanal cleft route and 250 μ L 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 EID₅₀
309 in 0.1 mL via the intratracheal route, along with 6.10^5 EID₅₀ 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 μ L 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 μ g
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 μ L of the 3-fold-

331 diluted cDNA sample, 7.5 μ L of 2X iQ Supermix SYBR green (Bio-Rad), 4 μ L of
332 ultrapure water (Invitrogen), and 0.75 μ L of each specific primer (10 μ M). mRNA
333 expression of chicken IFN- α , IFN- β , IFN- γ , Mx, interleukin-6 (IL-6), IL-8, IL-15,
334 GAPDH, G10, and ubiquitin was assessed. The sequence of primers can be
335 provided upon request. For each target sequence, a standard curve was obtained by
336 subjecting 2 to 2.10⁶ copies of a corresponding DNA plasmid to qPCR in parallel.
337 Only PCR products showing a unique temperature of fusion after raising the
338 temperature to 95°C at a transition rate of 0.5°C/s were retained for further
339 quantitative automated analysis with the Opticon Monitor 3 software (Bio-Rad). The
340 copy numbers of cytokine cDNAs determined experimentally were normalized to 10⁷
341 copies of the geometric mean of GAPDH, G10 and ubiquitin cDNA copy numbers, as
342 measured in the same sample.

343

344 **Histopathology and immunohistochemistry.**

345 Lung, caecum, colon, and liver were collected and fixed in 4% neutral-buffered
346 formalin, embedded in paraffin wax, sliced into 5- μ m thick sections, and dropped off
347 on positive-charge slides. Sections were then stained using a routine Hematoxylin-
348 Eosin-Saffron staining (HES). Histopathological analysis was done by a ECVP-
349 certified veterinary pathologist who was blinded to the experimental conditions. Two
350 sections of each sample were analysed. Semi-quantitative evaluations were
351 performed for extension and intensity of lung lesions. Scores were as follows: 0, no
352 lesion ; 1, focal lesion (less than 500 μ m diameter); 2, focal extensive or multifocal
353 coalescent lesions; 3, generalized lesion. NS1 immunolabelling was performed using
354 IG12 anti-NS1 mouse monoclonal antibody on frozen lung samples as described
355 previously (Munier *et al.*, 2010).

356

357 **Data analysis.**

358 Data are presented as means \pm standard errors of the means (SEM) for virus growth
359 and as means and individual values for all other analyses. Statistical significance
360 was assessed by using an unpaired, two-tailed Mann Whitney test. In addition, log-
361 rank test was used to compare survival estimated by Kaplan-Meier analysis between
362 H7N1₂₃₀ and H7N1₂₂₄-inoculated chickens.

363

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369

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373

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461 **Figure legends**

462

463 **Figure 1**

464 **NS segments of H7N1₂₃₀ and H7N1₂₂₄.** The NS segment of influenza A viruses
465 encodes two proteins, named NS1 and NEP. The nucleotide mutated in H7N1₂₂₄ 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 H7N1₂₃₀ and H7N1₂₂₄ 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 H7N1₂₃₀ virus (A) or H7N1₂₂₄ (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 H7N1₂₂₄ inoculated animal. Higher magnification of A and B are presented in C and
518 D respectively. Following H7N1₂₃₀ inoculation (C), airways are obliterated by necrotic
519 debris and heterophils (arrow), disrupting parabronchus wall (dotted lines), whereas
520 H7N1₂₂₄ 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 μ m ; Lower panel : bars = 100 μ m. (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) H7N1₂₃₀ virus-
525 inoculated chicken. The viral NS1 antigen is detected in the centre of areas of higher
526 cellular density.

527

528 **Table 1**

529

530 **Histological lesions in chickens inoculated with wild type or mutant H7N1**

531 **virus.** Each lesion intensity was semi quantitatively evaluated (0=no lesion, 1=mild,

532 2=marked, 3=severe) and a mean score was calculated for each batch of animals.

533 Frequency of lesions is reported into brackets. Histological scores from day 2 + 4

534 (grouping day 2 and day 4 animals) were significantly higher in H7N1₂₃₀ virus

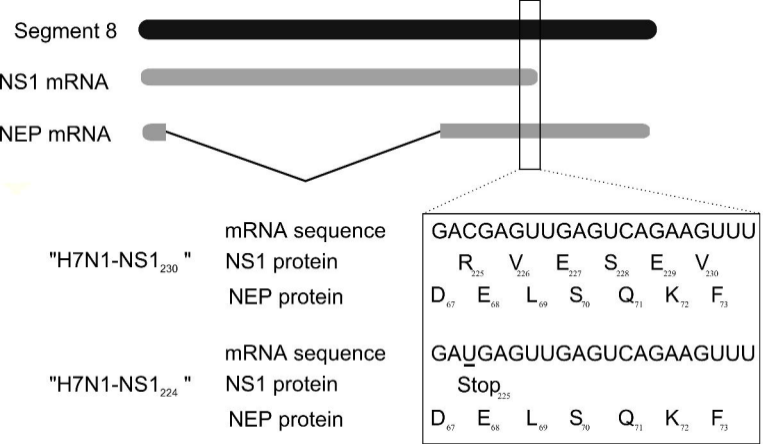
535 chickens than in H7N1₂₂₄ infected chickens ($p<0.05$).

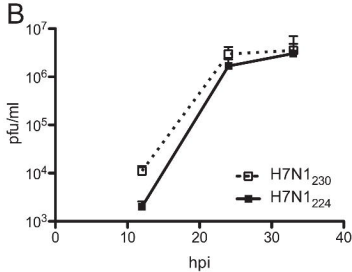
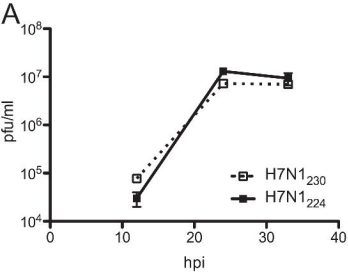
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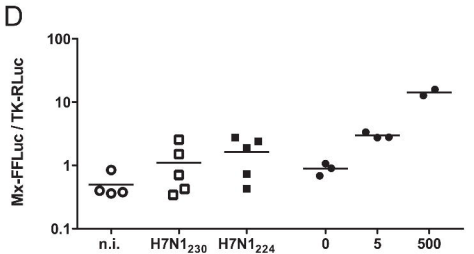
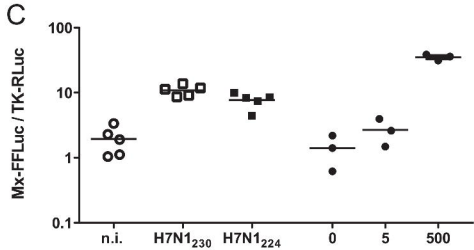
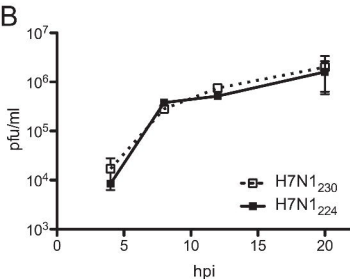
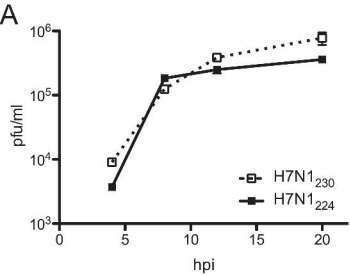
bronchial lesions		
dpi	NS1₂₃₀	NS1₂₂₄
2	2.7 (n=3/3)	0.7 (n=1/3)
4	2.3 (n=3/3)	0.7 (n=1/3)
2 + 4	2.5 (n=6/6)	0.7 (n=2/6)

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

537

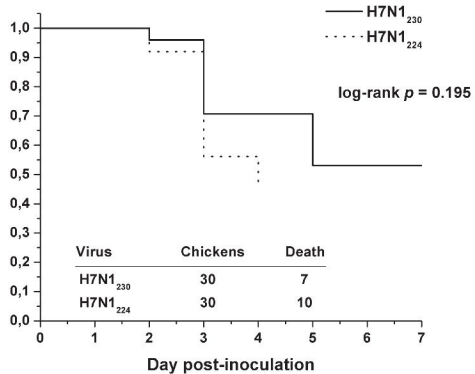
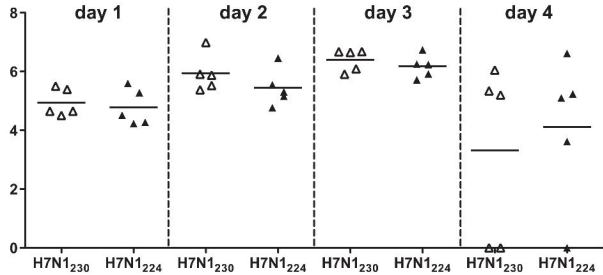


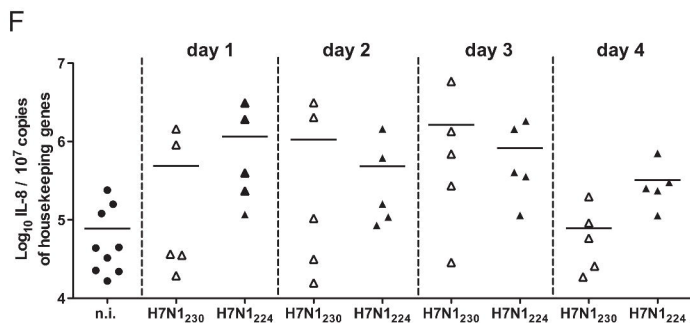
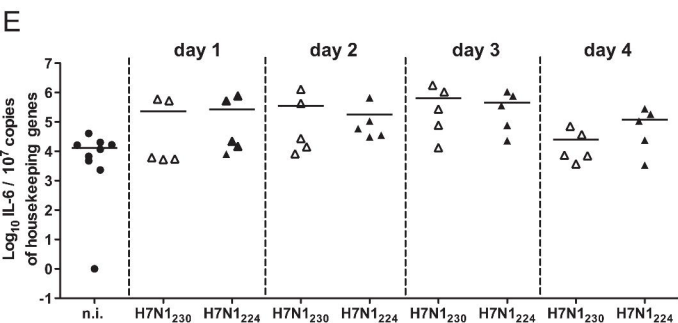
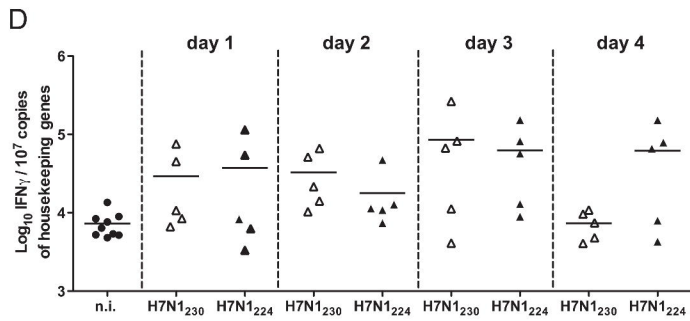
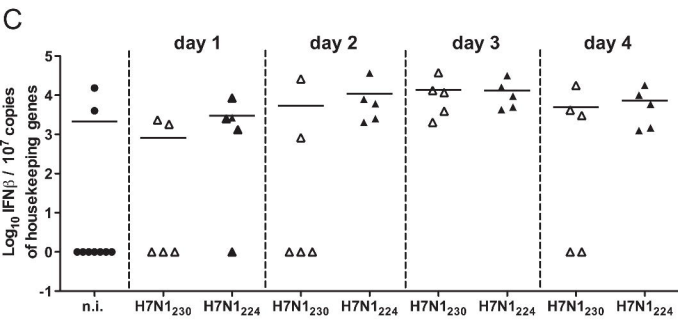
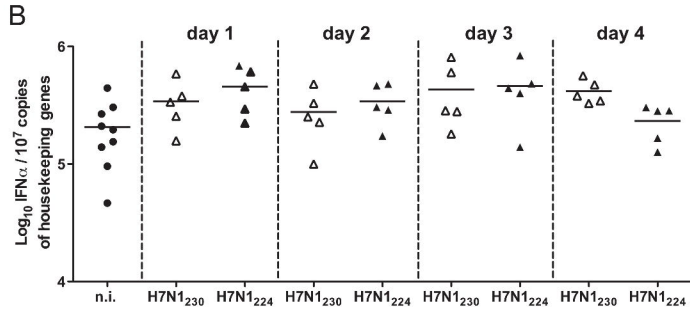
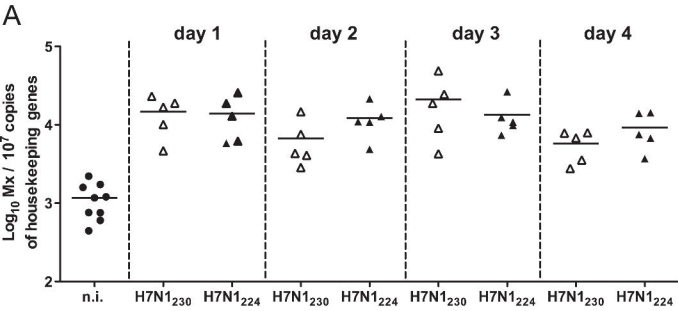


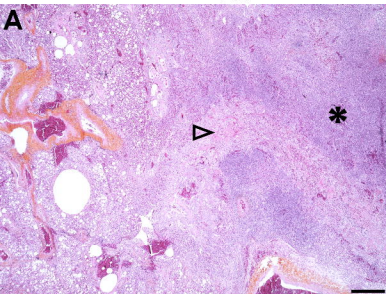
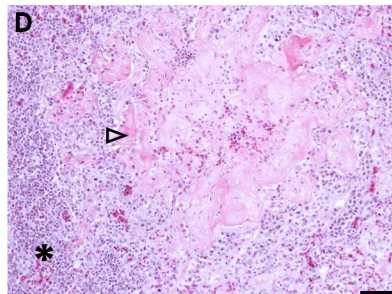
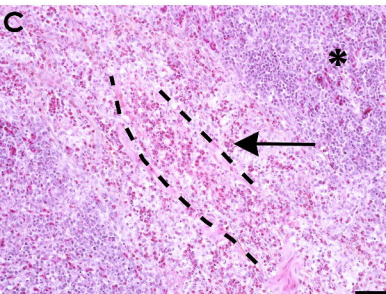
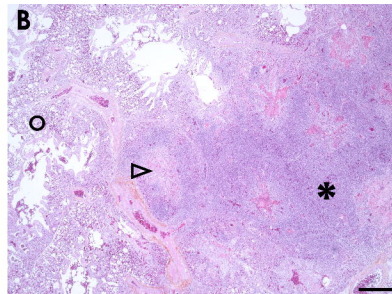
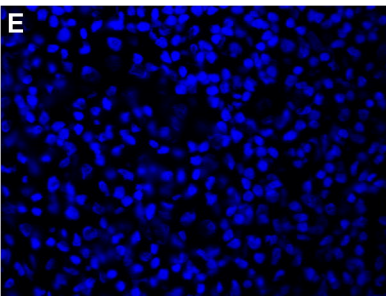


A

Cumulative survival

**B**Log₁₀ viral RNA / mg



H7N1₂₃₀**H7N1₂₂₄****Non-infected control****H7N1₂₃₀**