

1 **PA-X is a virulence factor in avian H9N2 influenza virus**

2

3 Huijie Gao ^{1,†}, Guanlong Xu ^{1,†}, Yipeng Sun ^{1,†}, Lu Qi ¹, Jinliang Wang ¹, Weili Kong ¹,
4 Honglei Sun ¹, Juan Pu ¹, Kin-Chow Chang ², and Jinhua Liu ^{1,#}

5

6 ¹ Key Laboratory of Animal Epidemiology and Zoonosis, Ministry of Agriculture,
7 College of Veterinary Medicine and State Key Laboratory of Agrobiotechnology,
8 China Agricultural University, Beijing, China

9 ² School of Veterinary Medicine and Science, University of Nottingham, Sutton
10 Bonington Campus, United Kingdom

11

12 [#]Corresponding author. Key Laboratory of Animal Epidemiology and Zoonosis of the
13 Ministry of Agriculture, College of Veterinary Medicine and State Key Laboratory of
14 Agrobiotechnology, China Agricultural University, No. 2 Yuanmingyuan West Road,
15 Beijing 100193, China.

16 Tel: +86-10-62733837; Fax: +86-10-62733837; E-mail: ljh@cau.edu.cn

17

18 [†] Huijie Gao, Guanlong Xu and Yipeng Sun contributed equally to this work.

19

20 **Running title:** Contribution of PA-X to the virulence of the H9N2 virus

21 **Content Category:** Animal Viruses - Negative-strand RNA

22 **Word counts:** Summary: 194; Main text: 3716.

23 **Tables and figures:** 5.

24

25

26 **Summary**

27 H9N2 influenza viruses have been circulating worldwide in multiple avian species
28 and regularly infect pigs and humans. Recently, a novel protein PA-X, produced from
29 the PA gene by ribosomal frameshifting, is demonstrated to be an anti-virulence factor
30 in pandemic 2009 H1N1, highly pathogenic avian H5N1 and 1918 H1N1 viruses.
31 However, a similar role of PA-X in the prevalent H9N2 avian influenza viruses has
32 not been established. In this study, we compared the virulence and cytopathogenicity
33 H9N2 wild type virus and H9N2 PA-X deficient virus. Loss of PA-X in H9N2 virus
34 reduced apoptosis and had marginal effect on progeny virus output in human
35 pulmonary adenocarcinoma (A549) cells. Without PA-X, PA was less able to suppress
36 co-expressed green fluorescence protein (GFP) in human 293T cells. Furthermore,
37 absence of PA-X in H9N2 virus attenuated viral pathogenicity in mice which showed
38 no mortality, reduced progeny virus production, mild to normal lung histopathology,
39 and dampened proinflammatory cytokine and chemokine response. Therefore, unlike
40 previously reported H1N1 and H5N1 viruses, we show that PA-X protein in H9N2
41 virus is a pro-virulence factor in facilitating viral pathogenicity, and that the pro- or
42 anti-virulence role of PA-X in influenza viruses is virus-strain dependent.

43

44 **Keywords:** H9N2 influenza virus; PA-X; Pathogenicity

45

46

47 **Introduction**

48 H9N2 influenza viruses have been circulating worldwide in poultry resulting in
49 severe economic losses due to reduced egg production or increased mortality
50 associated with co-infection with secondary pathogens (Banks *et al.*, 2000; Bano *et al.*,
51 2003; Capua & Alexander, 2006). H9N2 influenza viruses have been widely reported
52 to infect mammals, including pigs and humans (Abolnik *et al.*, 2010; Butt *et al.*, 2010;
53 Cong *et al.*, 2007; Sun *et al.*, 2010; Xu *et al.*, 2007); there is evidence that a large
54 number of people have been infected with H9N2 viruses in particular poultry workers
55 (Coman *et al.*, 2013; Jia *et al.*, 2009; Wang *et al.*, 2009). H9N2 virus infections in
56 humans showed typical human flu-like symptoms which can easily go undetected or
57 unreported (Butt *et al.*, 2005; Lin *et al.*, 2000). Recent studies showed that H9N2
58 viruses contributed the six internal genes to the novel H7N9 and H10N8 viruses that
59 are causing severe human infections in China (Chen *et al.*, 2014; Gao *et al.*, 2013;
60 Zhang *et al.*, 2013). H9N2 viruses can be regarded as precursors to emerging subtypes
61 of influenza viruses that are highly infectious to humans. Therefore, it is important to
62 ascertain virulence factors of H9N2 viruses.

63 Recently, PA-X, arising from ribosomal frame-shift in a +1 open reading frame
64 (X-ORF) extension of a growing PA polypeptide, was identified as a protein (Jagger
65 *et al.*, 2012). It was demonstrated that PA-X plays an important role in inhibiting
66 cellular protein synthesis, suggesting that PA-X contributes to host-cell shut off
67 induced by influenza virus (Desmet *et al.*, 2013; Jagger *et al.*, 2012; Katze *et al.*,
68 1986a; Katze *et al.*, 1986b). Jagger *et al.* also showed that PA-X decreased the

69 virulence of the 1918 H1N1 virus in a mouse model, through modulating host
70 inflammatory response, apoptosis, cell differentiation and tissue remodeling (Jagger et
71 al., 2012). We recently reported that loss of PA-X expression in 2009 pandemic H1N1
72 (pH1N1) and highly pathogenic H5N1 viruses increases viral replication and
73 apoptosis in A549 cells and increases virulence and host inflammatory response in
74 mice (Gao et al., 2015). Loss of PA-X expression also increases the virulence and
75 virus replication of H5N1 virus in avian species, and blunts the host innate immune
76 and cell death response (Hu et al., 2015).

77 Here we report that the absence of PA-X in H9N2 virus, contrary to previous
78 findings on pH1N1, highly pathogenic H5N1 and 1918 H1N1 viruses, decreases viral
79 replication and pro-inflammatory response in mice. The absence of PA-X in H9N2
80 virus also reduces virus-induced suppression of cellular protein synthesis.

81

82 **Results**

83 **Generation of PA-X deficient H9N2 virus**

84 In the present study, the use of reverse genetics was based on the
85 A/chicken/Hebei/LC/2008 (H9N2 WT) virus (Sun et al., 2011). To evaluate the effect
86 of loss of PA-X expression on viral function, we generated PA-X deficient virus,
87 H9N2-FS, by altering the frameshifting motif from UCC UUU CGU to AGC UUC
88 AGA in the PA segment to prevent the formation of PA-X (Fig.1a) (Jagger et al.,
89 2012). The mutations did not alter the PA ORF. To show that PA-X expression from
90 H9N2-FS was abolished, Madin Darby Canine Kidney (MDCK) cells were infected

91 with H9N2 PA-X mutant and WT viruses at an MOI of 1, and cell lysates were
92 harvested at 12 hpi. We found that PA-X could be detected in H9N2 WT infected cells
93 but not in H9N2-FS infected cells (Fig.1b).

94 **Decreased apoptosis in A549 cells infected with PA-X deficient H9N2 virus**

95 H9N2 WT and H9N2-FS were used to infect MDCK and human pulmonary
96 adenocarcinoma (A549) cells at an MOI of 0.01, and the supernatants were collected
97 and titrated at 6, 12, 24, 36, 48, 60, 72 and 84 h post infection (hpi). There was no
98 significant difference in the virus output from MDCK cells between H9N2 WT and
99 H9N2-FS viruses (Fig.2a). In A549 cells, H9N2-FS and H9N2WT viruses reached
100 maximum virus output at around the same time (48 hpi) with comparable peak virus
101 titers; viral titers at indicated time points showed no significant difference between
102 H9N2-FS and H9N2 WT virus (Fig.2b).

103 Apoptosis is a contributor to virulence (Roberts & Nichols, 1989; Tumpey *et al.*,
104 2000). Some viral proteins are able to induce apoptosis, such as NS1 and PB1-F2
105 (Chanturiya *et al.*, 2004; Chen *et al.*, 2001; Zhirnov *et al.*, 2002). A549 cells were
106 infected with H9N2-FS and WT viruses at an MOI of 1 for 6 and 12 h and assessed
107 for apoptosis. H9N2-FS virus infection produced less apoptotic cells (1.40% annexin
108 V+ only at 6 hpi and 6.59% at 12 hpi) than H9N2 WT virus (3.60% at 6 hpi and
109 10.73% at 12hpi) ($P < 0.05$) (Fig.2c). Cells that were PI+ only, and annexin V+ PI+
110 showed no significant difference between H9N2-FS and H9N2 WT viruses. Overall,
111 the data show that loss of PA-X in H9N2 virus has little effect on viral replication and
112 produced less apoptosis in A549 cells.

113 **PA-X deficient H9N2 virus is less pathogenic and causes mild inflammatory**
114 **response in mice relative to H9N2 wild type virus**

115 To assess the effect of PA-X on pathogenicity, mice (15 per group) were
116 intranasally inoculated at 10^6 TCID₅₀ with each virus. Clinical signs, mortality and
117 weight loss were monitored over 14 days. Three virus-infected mice a day were
118 humanely killed at 3, 5, and 7 days post infection (dpi), and lungs were collected for
119 virus titration. H9N2-FS virus infection resulted in no death, while H9N2 WT
120 infection caused 33.3% mortality (Fig. 3a). No significant weight loss was observed in
121 the H9N2-FS virus infected group, in contrast to the 15% weight loss of H9N2 WT
122 virus infected mice (Fig. 3b).

123 Histopathologically, H9N2-FS virus infected lung appeared nearly normal.
124 However, in the H9N2 WT virus group, there were extensive vascular congestion, and
125 cellular exudate (Fig. 3c). Viral titers of H9N2-FS virus infected lungs were 8-20 fold
126 lower than those of H9N2 WT virus at 3, 5 and 7 dpi ($P < 0.05$) (Fig. 3d) consistent
127 with the observed pathology.

128 Increased pulmonary cytokine/chemokine expression contributes to the severity of
129 influenza virus infection in humans and animal models (Bermejo-Martin et al., 2010;
130 Hagau et al., 2010; Lam et al., 2010; Perrone et al., 2008). We determined the protein
131 levels of seven cytokines and chemokines in the lungs of H9N2 WT and H9N2-FS
132 virus infected mice at 3 and 5 dpi. Interleukin-1 β (IL-1 β), interleukin-6 (IL-6), the
133 mouse equivalent of human IL-8 (KC), monocyte chemotactic protein 1 (MCP-1),
134 macrophage inflammatory protein-1 α (MIP-1 α or CCL3), tumor necrosis factor-alpha

135 (TNF- α) and interferon gamma (IFN- γ) levels from H9N2-FS virus infected mice
136 were consistently lower than those of H9N2 WT virus infected mice at both time
137 points ($P < 0.05$) (Fig.4). Collectively, these results demonstrate that PA-X in H9N2
138 virus facilitates pathogenicity and up-regulated inflammatory response in mice.

139 **PA protein is less effective at suppressing protein expression without PA-X**

140 Inhibition of host protein synthesis by influenza virus can hinder host anti-viral
141 response and promote virus replication (Katze *et al.*, 1986a; Katze *et al.*, 1986b). PA
142 gene plays a major role in the suppression of host protein synthesis, which is partly
143 mediated by PA-X (Desmet *et al.*, 2013; Jagger *et al.*, 2012). We compared the ability
144 of PA of H9N2 WT and H9N2-FS viruses to suppress non-viral protein synthesis by
145 co-transfections of Human embryonic kidney (293T) cells for 24 h with H9N2 WT PA
146 or H9N2-FS PA, and pEGFP expression plasmids. eGFP expression was significantly
147 higher by more than 20% when co-transfected with H9N2-FS PA, compared with
148 H9N2 WT PA co-transfection (Fig.5a & b). Although the expression level of PA
149 protein of H9N2-FS was higher than that of H9N2 WT, H9N2-FS PA plasmid was less
150 effective in suppressing eGFP expression than H9N2 WT PA (Fig.5b). These results
151 suggest that loss of PA-X in H9N2 virus reduces the host shut off ability of the virus
152 in 293T cells.

153

154 **Discussion**

155 In the present study, we assessed the pathogenic capability of PA-X in avian
156 H9N2 virus. PA-X deficient H9N2 (H9N2-FS) virus was less virulent than its H9N2

157 WT counterpart. Absence of PA-X attenuated the H9N2 virus manifested as decreased
158 viral replication, reduced apoptosis and dampened pro-inflammatory response.
159 Furthermore, PA without PA-X was less able to suppress host protein synthesis.
160 Therefore, we propose that PA-X in H9N2 virus is a virulence factor.

161 Previous studies, however, have shown that PA-X deficient-H1N1 and -H5N1
162 viruses are more pathogenic than their corresponding WT counterparts (Gao et al.,
163 2015; Hu et al., 2015; Jagger et al., 2012). Enhanced virulence of the 1918 H1N1
164 virus deficient in PA-X might be due to alterations in the kinetics of host response
165 (Jagger et al., 2012). Jagger *et al.* (2012) found that loss of PA-X expression in the
166 1918 pandemic virus up-regulated inflammatory, apoptotic and T-lymphocyte
167 signaling pathways. Elevated virulence manifested as increased PA expression,
168 ribonucleoprotein polymerase activity and inflammatory response were the effects of
169 PA-X deletion in pH1N1 and highly pathogenic H5N1 avian influenza viruses (Gao et
170 al., 2015). PA-X is also an anti-virulence factor of avian H5N1 virus in avian species
171 as well as in mice (Hu et al., 2015). H5N1 PA-X blunted the global host response in
172 chicken lungs, which included markedly down-regulated genes associated with
173 inflammation and cell death, and promoted anti-apoptotic activity in chicken and duck
174 fibroblasts (Hu et al., 2015). In the present study, we found that PA-X played an
175 opposite role in H9N2 virus. Apoptotic and inflammatory responses were decreased
176 with H9N2 PA-X deficient virus. These observations indicate that the pro- or
177 anti-virulence role of PA-X in influenza viruses is virus strain specific.

178 Loss of PA-X expression decreased viral replication of H9N2 virus *in vivo*. H9N2

179 PA-X deficient virus showed lower replication levels in mice than H9N2 WT at 3, 5
180 and 7 dpi. The poor replication in murine lungs was directly related to the lower
181 pathogenicity and reduced expression of inflammatory cytokines from H9N2 PA-X
182 deficient virus infection. Hu J *et al.* (2015) showed that PA-X decreases the virulence
183 of H5N1 virus through inhibiting viral replication and the host innate immune
184 response. Jagger *et al.* (2012) showed that loss of PA-X in 1918 H1N1 virus did not
185 affect viral replication in mice but increased pathogenicity through enhanced host
186 immune response.

187 Influenza virus infection can induce host shut off with rapid decline of host protein
188 synthesis (Katze *et al.*, 1986a; Katze *et al.*, 1986b) to divert host resources towards
189 viral replication. Inhibition of host protein synthesis also aids in dampening the
190 anti-viral response. Therefore, virus induced host shut off is closely related to viral
191 replication and pathogenicity. Recently, the roles of PA and PA-X in the inhibition of
192 cellular protein synthesis were demonstrated (Desmet *et al.*, 2013; Jagger *et al.*, 2012).
193 The N-terminal domain of PA, which includes the endonuclease active site, is
194 sufficient to suppress protein expression, and PA-X showed a stronger effect than the
195 corresponding N-terminal domain of PA. We previously showed that the absence of
196 PA-X made PA less able to suppress co-transfected gene expression for pH1N1 and
197 H5N1 viruses (Gao *et al.*, 2015). Conceivably, loss of PA-X in these viruses could less
198 effectively inhibit host protein synthesis, which would result in reduced viral
199 replication and virulence. However, loss of PA-X does enhance the virulence of 1918
200 H1N1, pH1N1 and H5N1 viruses (Gao *et al.*, 2015; Hu *et al.*, 2015; Jagger *et al.*,

201 2012). We speculate that the decrease in suppression of host protein synthesis
202 exacerbates host inflammatory response and enhances apoptosis. As 1918 H1N1,
203 pH1N1 and highly pathogenic H5N1 viruses could elicit significantly high levels of
204 pro-inflammatory cytokines, loss of PA-X in such viruses could lead to more severe
205 lung injury and contribute to the enhanced virulence (Kang et al., 2011; Ma et al.,
206 2011; Perrone et al., 2008). In the present study, H9N2 virus is largely a low
207 pathogenicity virus and does not typically induce high levels of cytokines. PA protein
208 in H9N2 virus was less able to suppress GFP expression in the absence of PA-X,
209 suggesting that PA-X also plays a role in the inhibition of host protein synthesis. The
210 level of host shut off by H9N2-FS could be less effective in promoting viral
211 replication but more effective in eliciting an antiviral response. In summary, our
212 results show that PA-X of H9N2 virus, unlike the more virulent H5N1, pH1N1 and
213 1918 H1N1 viruses, is a pro-virulence factor in the facilitation of viral replication and
214 pathogenicity, and that function of PA-X is virus strain specific. Therefore, the role of
215 PA-X in other influenza viruses needs to be investigated.

216 **Methods**

217 **Viruses and cells**

218 A/chicken/Hebei/LC/2008 (HB/08, H9N2) virus was isolated from a diseased
219 chicken in Hebei province, China, in January 2008 and propagated in 10 day-old
220 specific-pathogen-free (SPF) embryonated chicken eggs (Sun et al., 2011). 293T,
221 MDCK, and A549 cells were maintained in Dulbecco's modified Eagle's medium
222 (DMEM; Life Technologies, Foster City, CA, USA) supplemented with 10% fetal

223 bovine serum (FBS; Life Technologies), 100 units/ml of penicillin and 100 g/ml of
224 streptomycin.

225 **Generation of recombinant viruses by reverse genetics**

226 All eight gene segments have been previously amplified by reverse
227 transcription-PCR (RT-PCR) from HB/08 virus and cloned into the dual-promoter
228 plasmid, pHW2000 (Sun et al., 2011). PA-X deficient virus, H9N2-FS, was created by
229 site-directed mutagenesis (QuikChange mutagenesis kit, Agilent) on the
230 corresponding PA gene of H9N2 WT virus, which converted the frameshifting motif
231 from UCC UUU CGU to AGC UUC AGA (U592A, C593G, U597C, C598A and
232 U600A) to prevent the formation of PA-X (Jagger et al., 2012). PCR primer sequences
233 used are available upon request. PA ORF was unaltered in H9N2-FS. Rescued viruses
234 were detected using hemagglutination assays. The viruses were purified by sucrose
235 density gradient centrifugation, and viral RNA was extracted and analyzed by
236 RT-PCR, and each viral segment was sequenced to confirm sequence identity.

237 **Viral titration and replication kinetics**

238 Fifty % tissue culture infective dose (TCID₅₀) was determined in MDCK cells
239 using 10-fold serially diluted virus inoculated at 37°C and cultured for 72 h. The
240 TCID₅₀ values were calculated by the method of Reed and Muench (Reed & Muench,
241 1938). MDCK and A549 cells were infected with viruses at an MOI of 0.01, overlaid
242 with serum-free DMEM containing 2µg/ml TPCK-trypsin (Sigma–Aldrich) and
243 incubated at 37°C. Supernatants of infected MDCK and A549 cells were harvested at
244 6, 12, 24, 36, 48, 60, 72 and 84 hpi. Virus titers were determined by TCID₅₀ in MDCK

245 cells. Three independent experiments were performed.

246 **Mouse infections**

247 Fifteen mice (six week-old female BALB/c; Vital River Laboratory, Beijing,
248 China) per group were anesthetized with Zoletil (tiletamine-zolazepam; Virbac S.A.,
249 Carros, France; 20 µg/g) and inoculated intranasally with 50 µl of 10⁶ TCID₅₀ of
250 H9N2 diluted in phosphate-buffered saline (PBS). All mice were monitored daily for
251 14 days, and mice losing 30% of their original body weight were humanely
252 euthanized. Three mice were euthanized on 3, 5 and 7 dpi for the determination of
253 lung virus titers, histopathology and cytokine levels. Lungs were collected and
254 homogenized in cold PBS. Virus titers were determined by TCID₅₀. All animal
255 research was approved by the Beijing Association for Science and Technology and
256 complied with Beijing Laboratory Animal Welfare and Ethical Guidelines as issued
257 by the Beijing Administration Committee of Laboratory Animals.

258 **Histopathology**

259 A portion of the lung from each euthanized mouse at 5 dpi was fixed in 10%
260 phosphate-buffered formalin and processed for paraffin embedding. Each 5 µm
261 section was stained with hematoxylin and eosin and examined for histopathological
262 changes. Images were captured with a Zeiss Axioplan 2IE epifluorescence
263 microscope.

264 **Quantification of cytokine/chemokine protein levels in mouse lungs**

265 Levels of cytokines/chemokines including IFN-γ, IL-1β, IL-6, KC, TNF-α,
266 MIP-1α or CCL3 and MCP-1 in lungs were determined by cytometric bead array

267 assays (BD Cytometric BEAD Array Mouse Inflammation Kit; BD Bioscience, San
268 Diego, CA, USA). Briefly, 50 μ l mouse inflammation capture bead suspension and 50
269 μ l detection reagent were added to an equal amount of sample and incubated in the
270 dark for 2 h at room temperature. Subsequently, each sample was washed with 1 ml
271 wash buffer and then centrifuged at $200 \times g$ at room temperature for 5 min.
272 Supernatants were discarded and a further 300 μ l wash buffer was added. Samples
273 were analyzed on a BD FACS Array bioanalyzer (BD Bioscience). Data were
274 analyzed using BD CBA Software (BD Bioscience). Each chemokine or cytokine was
275 computed as pg/ml of homogenate.

276 **Cell death assays**

277 Virus infection assays were conducted in 6 well plates. Cells were seeded at a
278 density of 1×10^6 cells/well for overnight incubation in infection media (cell growth
279 media with 1% bovine serum albumin was used in place of FBS). Cells were then
280 infected with virus at 1.0 MOI for 12 h. Cells pooled from the supernatant and
281 monolayer were then harvested, washed and stained with FITC labeled annexin V and
282 propidium iodide (PI) (Becton Dickinson, San Jose, CA) for 20 min. After a final
283 wash, cells were resuspended in 100 μ l FACs wash buffer (PBS containing 3% BSA
284 and 0.01% sodium azide) and analyzed on the FACs Calibur (BD Biosciences) with
285 Flow Jo software (version 7.6.1). Cell death (apoptosis and necrosis) was defined as
286 annexin-V⁺ and PI⁺, while apoptotic cells were annexin-V⁺ only. Viable cells were
287 considered as neither annexin-V nor PI positive.

288 **Western blotting**

289 Total cell protein lysates were extracted from transfected 293T cells or infected
290 MDCK cells with CA630 lysis buffer (150 mM NaCl, 1% CA630 detergent, 50 mM
291 Tris base [pH 8.0]). Cellular proteins were separated by 12% sodium dodecyl
292 sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and transferred to a
293 polyvinylidene difluoride (PVDF) membrane (Amersham Biosciences, Germany).
294 Each PVDF membrane was blocked with 0.1% Tween 20 and 5% nonfat dry milk in
295 Tris-buffered saline and subsequently incubated with a primary antibody. Primary
296 antibodies were specific for influenza A virus PA (1:3000, GeneTex, USA), influenza
297 A virus PA-X [diluted 1:2000, polyclonal rabbit antiserum against a H5N1 X-ORF
298 derived peptide(CAGLPTKVSHRTSPA), Genscript, China)], influenza A virus PB1
299 (diluted 1:3000, Thermo Fisher Scientific, USA), GFP (1:1000, Abcam, UK), β -actin
300 (1:1000, Santa Cruz, USA). The secondary antibody used was either horseradish
301 peroxidase (HRP)-conjugated anti-mouse antibody or HRP-conjugated anti-rabbit
302 antibody (diluted 1:10,000 Jackson ImmunoResearch USA), as appropriate. HRP
303 presence was detected using a Western Lightning chemiluminescence kit (Amersham
304 Pharmacia, Freiburg, Germany), following the manufacturer's protocol.

305 **Statistics**

306 All statistical analyses were performed using GraphPad Prism Software Version
307 5.0 (GraphPad Software Inc., San Diego, CA, USA). The two treatment methods were
308 compared by two-tailed Student's t-test, and multiple comparisons were carried out by
309 two-way analysis of variance (ANOVA) considering time and virus as factors.
310 Differences were considered statistically significant at $P < 0.05$. All data are reported

311 as the mean \pm standard deviation (SD).

312

313 **Acknowledgments**

314 This work was supported by the National Natural Science Foundation of China (No.

315 31302102 and No. 31430086), the National Basic Research Program (973 Program)

316 (No. 2011CB504702), and a grant from the Chang Jiang Scholars Program.

317

318 **References**

- 319 **Abolnik, C., Gerdes, G. H., Sinclair, M., Ganzevoort, B. W., Kitching, J. P.,**
320 **Burger, C. E., Romito, M., Dreyer, M., Swanepoel, S., Cumming, G. S. &**
321 **Olivier, A. J. (2010).** Phylogenetic analysis of influenza A viruses (H6N8,
322 H1N8, H4N2, H9N2, H10N7) isolated from wild birds, ducks, and ostriches in
323 South Africa from 2007 to 2009. *Avian Dis* **54**, 313-322.
- 324 **Banks, J., Speidel, E. C., Harris, P. A. & Alexander, D. J. (2000).** Phylogenetic
325 analysis of influenza A viruses of H9 haemagglutinin subtype. *Avian Pathol* **29**,
326 353-359.
- 327 **Bano, S., Naeem, K. & Malik, S. A. (2003).** Evaluation of pathogenic potential of
328 avian influenza virus serotype H9N2 in chickens. *Avian Dis* **47**, 817-822.
- 329 **Bermejo-Martin, J. F., Martin-Loeches, I., Rello, J., Antón, A., Almansa, R., Xu,**
330 **L., Lopez-Campos, G., Pumarola, T., Ran, L. & Ramirez, P. (2010).** Host
331 adaptive immunity deficiency in severe pandemic influenza. *Crit Care* **14**,
332 R167.
- 333 **Butt, A. M., Siddique, S., Idrees, M. & Tong, Y. (2010).** Avian influenza A (H9N2):
334 computational molecular analysis and phylogenetic characterization of viral
335 surface proteins isolated between 1997 and 2009 from the human population.
336 *Virology* **7**, 319.
- 337 **Butt, K. M., Smith, G. J., Chen, H., Zhang, L. J., Leung, Y. H., Xu, K. M., Lim,**
338 **W., Webster, R. G., Yuen, K. Y., Peiris, J. S. & Guan, Y. (2005).** Human
339 infection with an avian H9N2 influenza A virus in Hong Kong in 2003. *J Clin*

340 *Microbiol* **43**, 5760-5767.

341 **Capua, I. & Alexander, D. J. (2006).** The challenge of avian influenza to the
342 veterinary community. *Avian Pathol* **35**, 189-205.

343 **Chanturiya, A. N., Basanez, G., Schubert, U., Henklein, P., Yewdell, J. W. &**
344 **Zimmerberg, J. (2004).** PB1-F2, an influenza A virus-encoded proapoptotic
345 mitochondrial protein, creates variably sized pores in planar lipid membranes.
346 *J Virol* **78**, 6304-6312.

347 **Chen, H., Yuan, H., Gao, R., Zhang, J., Wang, D., Xiong, Y., Fan, G., Yang, F., Li,**
348 **X., Zhou, J., Zou, S., Yang, L., Chen, T., Dong, L., Bo, H., Zhao, X., Zhang,**
349 **Y., Lan, Y., Bai, T., Dong, J., Li, Q., Wang, S., Li, H., Gong, T., Shi, Y., Ni,**
350 **X., Li, J., Fan, J., Wu, J., Zhou, X., Hu, M., Wan, J., Yang, W., Li, D., Wu,**
351 **G., Feng, Z., Gao, G. F., Wang, Y., Jin, Q., Liu, M. & Shu, Y. (2014).**
352 Clinical and epidemiological characteristics of a fatal case of avian influenza A
353 H10N8 virus infection: a descriptive study. *Lancet* **383**, 714-721.

354 **Chen, W., Calvo, P. A., Malide, D., Gibbs, J., Schubert, U., Bacik, I., Basta, S.,**
355 **O'Neill, R., Schickli, J., Palese, P., Henklein, P., Bennink, J. R. & Yewdell,**
356 **J. W. (2001).** A novel influenza A virus mitochondrial protein that induces cell
357 death. *Nat Med* **7**, 1306-1312.

358 **Coman, A., Maftei, D. N., Krueger, W. S., Heil, G. L., Friary, J. A., Chereches, R.**
359 **M., Sirlincan, E., Bria, P., Dragnea, C., Kasler, I. & Gray, G. C. (2013).**
360 Serological evidence for avian H9N2 influenza virus infections among
361 Romanian agriculture workers. *J Infect Public Health* **6**, 438-447.

362 Cong, Y. L., Pu, J., Liu, Q. F., Wang, S., Zhang, G. Z., Zhang, X. L., Fan, W. X.,
363 Brown, E. G. & Liu, J. H. (2007). Antigenic and genetic characterization of
364 H9N2 swine influenza viruses in China. *J Gen Virol* **88**, 2035-2041.

365 Desmet, E. A., Bussey, K. A., Stone, R. & Takimoto, T. (2013). Identification of the
366 N-Terminal Domain of the Influenza Virus PA Responsible for the
367 Suppression of Host Protein Synthesis. *J Virol* **87**, 3108-3118.

368 Gao, H., Sun, Y., Hu, J., Qi, L., Wang, J., Xiong, X., Wang, Y., He, Q., Lin, Y.,
369 Kong, W., Seng, L. G., Sun, H., Pu, J., Chang, K. C., Liu, X. & Liu, J.
370 (2015). The contribution of PA-X to the virulence of pandemic 2009 H1N1
371 and highly pathogenic H5N1 avian influenza viruses. *Sci Rep* **5**, 8262.

372 Gao, R., Cao, B., Hu, Y., Feng, Z., Wang, D., Hu, W., Chen, J., Jie, Z., Qiu, H., Xu,
373 K., Xu, X., Lu, H., Zhu, W., Gao, Z., Xiang, N., Shen, Y., He, Z., Gu, Y.,
374 Zhang, Z., Yang, Y., Zhao, X., Zhou, L., Li, X., Zou, S., Zhang, Y., Yang,
375 L., Guo, J., Dong, J., Li, Q., Dong, L., Zhu, Y., Bai, T., Wang, S., Hao, P.,
376 Yang, W., Han, J., Yu, H., Li, D., Gao, G. F., Wu, G., Wang, Y., Yuan, Z. &
377 Shu, Y. (2013). Human infection with a novel avian-origin influenza A (H7N9)
378 virus. *N Engl J Med* **368**, 1888-1897.

379 Hagau, N., Slavcovici, A., Gonganau, D. N., Oltean, S., Dirzu, D. S., Brezozski, E.
380 S., Maxim, M., Ciuce, C., Mlesnite, M. & Gavrus, R. L. (2010). Clinical
381 aspects and cytokine response in severe H1N1 influenza A virus infection. *Crit*
382 *Care* **14**, R203.

383 Hu, J., Mo, Y., Wang, X., Gu, M., Hu, Z., Zhong, L., Wu, Q., Hao, X., Hu, S., Liu,

384 **W., Liu, H. & Liu, X. (2015).** PA-X Decreases the Pathogenicity of Highly
385 Pathogenic H5N1 Influenza A Virus in Avian Species by Inhibiting Virus
386 replication and Host Response. *J Virol* **89**, 4126-4142.

387 **Jagger, B., Wise, H., Kash, J., Walters, K.-A., Wills, N., Xiao, Y.-L., Dunfee, R.,**
388 **Schwartzman, L., Ozinsky, A. & Bell, G. (2012).** An overlapping
389 protein-coding region in influenza A virus segment 3 modulates the host
390 response. *Science* **337**, 199-204.

391 **Jia, N., de Vlas, S. J., Liu, Y. X., Zhang, J. S., Zhan, L., Dang, R. L., Ma, Y. H.,**
392 **Wang, X. J., Liu, T., Yang, G. P., Wen, Q. L., Richardus, J. H., Lu, S. &**
393 **Cao, W. C. (2009).** Serological reports of human infections of H7 and H9
394 avian influenza viruses in northern China. *J Clin Virol* **44**, 225-229.

395 **Kang, Y. M., Song, B. M., Lee, J. S., Kim, H. S. & Seo, S. H. (2011).** Pandemic
396 H1N1 influenza virus causes a stronger inflammatory response than seasonal
397 H1N1 influenza virus in ferrets. *Arch Virol* **156**, 759-767.

398 **Katze, M. G., DeCorato, D. & Krug, R. M. (1986a).** Cellular mRNA translation is
399 blocked at both initiation and elongation after infection by influenza virus or
400 adenovirus. *J Virol* **60**, 1027-1039.

401 **Katze, M. G., Detjen, B. M., Safer, B. & Krug, R. M. (1986b).** Translational control
402 by influenza virus: suppression of the kinase that phosphorylates the alpha
403 subunit of initiation factor eIF-2 and selective translation of influenza viral
404 mRNAs. *Mol Cell Biol* **6**, 1741-1750.

405 **Lam, W. Y., Yeung, A., Chu, I. & Chan, P. (2010).** Profiles of cytokine and

406 chemokine gene expression in human pulmonary epithelial cells induced by
407 human and avian influenza viruses. *Virology* **7**, 344.

408 **Lin, Y. P., Shaw, M., Gregory, V., Cameron, K., Lim, W., Klimov, A., Subbarao,**
409 **K., Guan, Y., Krauss, S., Shortridge, K., Webster, R., Cox, N. & Hay, A.**
410 **(2000).** Avian-to-human transmission of H9N2 subtype influenza A viruses:
411 relationship between H9N2 and H5N1 human isolates. *Proc Natl Acad Sci U S*
412 *A* **97**, 9654-9658.

413 **Ma, W., Belisle, S. E., Mosier, D., Li, X., Stigger-Rosser, E., Liu, Q., Qiao, C.,**
414 **Elder, J., Webby, R. & Katze, M. G. (2011).** 2009 pandemic H1N1 influenza
415 virus causes disease and upregulation of genes related to inflammatory and
416 immune responses, cell death, and lipid metabolism in pigs. *J Virol* **85**,
417 11626-11637.

418 **Perrone, L. A., Plowden, J. K., García-Sastre, A., Katz, J. M. & Tumpey, T. M.**
419 **(2008).** H5N1 and 1918 pandemic influenza virus infection results in early and
420 excessive infiltration of macrophages and neutrophils in the lungs of mice.
421 *PLoS pathog* **4**, e1000115.

422 **Reed, L. J. & Muench, H. (1938).** A simple method of estimating fifty per cent
423 endpoints. *Am J Epidemiol* **27**, 493-497.

424 **Roberts, N. J., Jr. & Nichols, J. E. (1989).** Regulation of lymphocyte proliferation
425 after influenza virus infection of human mononuclear leukocytes. *J Med Virol*
426 **27**, 179-187.

427 **Sun, Y., Pu, J., Jiang, Z., Guan, T., Xia, Y., Xu, Q., Liu, L., Ma, B., Tian, F.,**

428 **Brown, E. G. & Liu, J. (2010).** Genotypic evolution and antigenic drift of
429 H9N2 influenza viruses in China from 1994 to 2008. *Vet Microbiol* **146**,
430 215-225.

431 **Sun, Y., Qin, K., Wang, J., Pu, J., Tang, Q., Hu, Y., Bi, Y., Zhao, X., Yang, H. &**
432 **Shu, Y. (2011).** High genetic compatibility and increased pathogenicity of
433 reassortants derived from avian H9N2 and pandemic H1N1/2009 influenza
434 viruses. *Proc Natl Acad Sci USA* **108**, 4164-4169.

435 **Tumpey, T. M., Lu, X., Morken, T., Zaki, S. R. & Katz, J. M. (2000).** Depletion of
436 lymphocytes and diminished cytokine production in mice infected with a
437 highly virulent influenza A (H5N1) virus isolated from humans. *J Virol* **74**,
438 6105-6116.

439 **Wang, M., Fu, C. X. & Zheng, B. J. (2009).** Antibodies against H5 and H9 avian
440 influenza among poultry workers in China. *N Engl J Med* **360**, 2583-2584.

441 **Xu, K. M., Smith, G. J., Bahl, J., Duan, L., Tai, H., Vijaykrishna, D., Wang, J.,**
442 **Zhang, J. X., Li, K. S., Fan, X. H., Webster, R. G., Chen, H., Peiris, J. S. &**
443 **Guan, Y. (2007).** The genesis and evolution of H9N2 influenza viruses in
444 poultry from southern China, 2000 to 2005. *J Virol* **81**, 10389-10401.

445 **Zhang, Q., Shi, J., Deng, G., Guo, J., Zeng, X., He, X., Kong, H., Gu, C., Li, X.,**
446 **Liu, J., Wang, G., Chen, Y., Liu, L., Liang, L., Li, Y., Fan, J., Wang, J., Li,**
447 **W., Guan, L., Li, Q., Yang, H., Chen, P., Jiang, L., Guan, Y., Xin, X., Jiang,**
448 **Y., Tian, G., Wang, X., Qiao, C., Li, C., Bu, Z. & Chen, H. (2013).** H7N9
449 influenza viruses are transmissible in ferrets by respiratory droplet. *Science*

450 **341**, 410-414.

451 **Zhirnov, O. P., Konakova, T. E., Wolff, T. & Klenk, H. D. (2002).** NS1 protein of
452 influenza A virus down-regulates apoptosis. *J Virol* **76**, 1617-1625.

453

454

455

456 **Figure legends**

457 **Figure 1. Generation of H9N2 PA-X deficient viruses.** (a) The frameshifting motif
458 of UCC UUU CGU was mutated to AGC UUC AGA (in red) in the PA gene which
459 did not alter the PA ORF but abrogated the expression of PA-X. (b) PA-X protein
460 expression was abolished in H9N2-FS virus infected cells. MDCK cells were infected
461 with H9N2-FS and H9N2 WT virus for 12 h. Western blotting was performed on cell
462 lysates with antibodies against PA-X or PB1 or β -actin, as indicated, followed by
463 AP-conjugated secondary antibodies.

464 **Figure 2. Growth of H9N2 WT and FS viruses and induction of apoptosis.** Virus
465 growth curves of H9N2 WT and H9N2-FS viruses in MDCK cells (a) and A549 (b)
466 cells over 84 h. (c) Cell death was determined in A549 cells by detection of annexin⁺
467 and/or PI⁺ at 6 and 12 hpi of H9N2 WT or H9N2-FS virus at 1.0 MOI. Representative
468 dual-labeled quadrants of bivariate fluorescence dot plots showing the induction of
469 apoptosis in infected cells. Apoptotic cells, positive for annexin V but not PI, were
470 identified in the right lower quadrant. Cells positive for PI but not annexin V
471 (indicative of necrosis) were identified in the right upper quadrant. MOCK =
472 uninfected control cells. Each value represents the mean of three independent
473 experiments performed in triplicates; error bars indicate standard deviations (SD). *
474 indicates significant difference between FS virus and wild type virus ($P < 0.05$).

475 **Figure 3. Pathogenicity of H9N2 WT and H9N2-FS viruses in mice.** (a) Reduced
476 survival (percentage) was found with H9N2 WT virus infected mice over a 14 day
477 period (inoculation at day 0). (b) H9N2 WT virus infected mice showed significant

478 weight loss, unlike H9N2-FS virus and mock infected mice. Any mouse that lost more
479 than 30% of its body weight was euthanized. (c) Histopathology in lung of H9N2-FS
480 virus infected mice was mild to normal compared with corresponding H9N2 WT virus
481 infection which showed vascular congestion and cellular infiltration of bronchioles
482 and alveoli. Scale bars, 100 μ m. (d) Mean of viral lung load \pm SD was based on log₁₀
483 TCID₅₀ determination in MDCK cells. * indicates significant difference between
484 H9N2 WT and H9N2-FS virus ($P < 0.05$). Means of the data of three mice per group
485 are shown, and error bars are SDs.

486 **Figure 4. Detection of cytokine/chemokine proteins in lungs of mice infected with**
487 **H9N2 WT and H9N2-FS viruses.** Mean cytokine/chemokine levels \pm SDs are shown
488 (n=3). * indicates significant difference between H9N2-FS and H9N2 WT ($P < 0.05$).

489 **Figure 5. PA without PA-X is less able to suppress co-expressed GFP in 293T cells.**

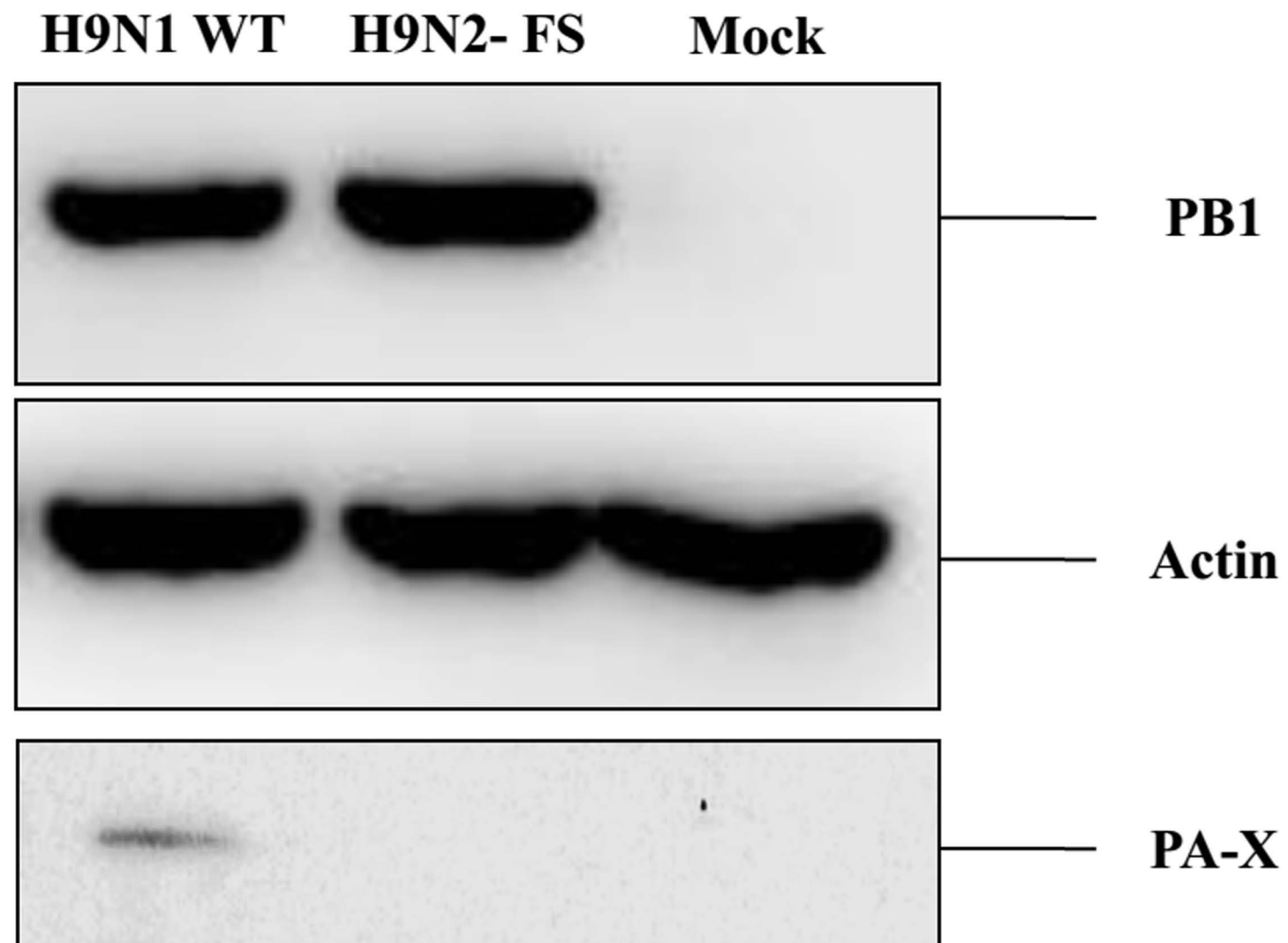
490 (a) eGFP expression plasmid was co-transfected with PA plasmid derived from H9N2
491 WT or H9N2-FS viruses, or with mock plasmid (control pcDNA3.1). eGFP
492 fluorescence was captured at 24 h post transfection. GFP fluorescence (green) is
493 shown in the left panel, and merged with DAPI staining fluorescence is shown in the
494 right panel. The GFP expression levels were quantified by fluorescence intensity. The
495 fluorescence intensities were analyzed with Image-Pro-Plus (Media Cybernetics).
496 Relative fluorescence intensity of each group as compared with control was shown as
497 histograms. Values shown are means of the results of three independent experiments \pm
498 SDs. (b) PA and GFP protein expression were determined by Western blotting analysis
499 using anti-PA and anti-GFP antibody. Anti- β -actin antibody was used as loading

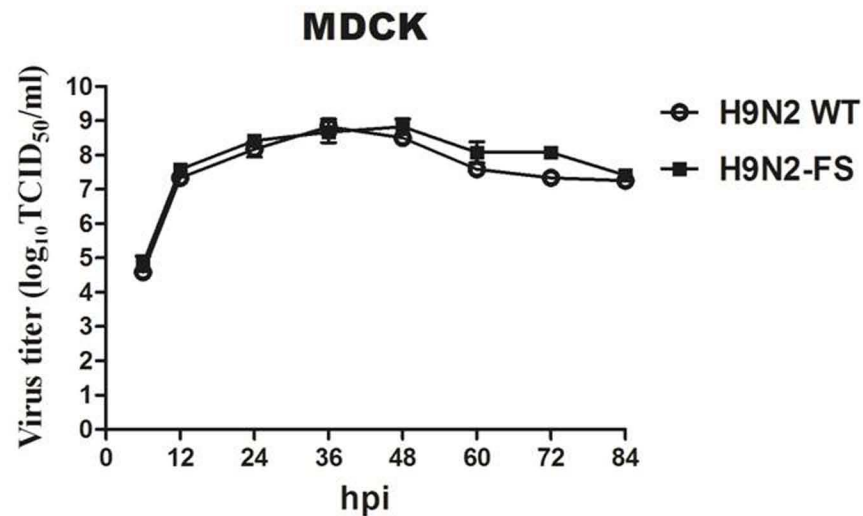
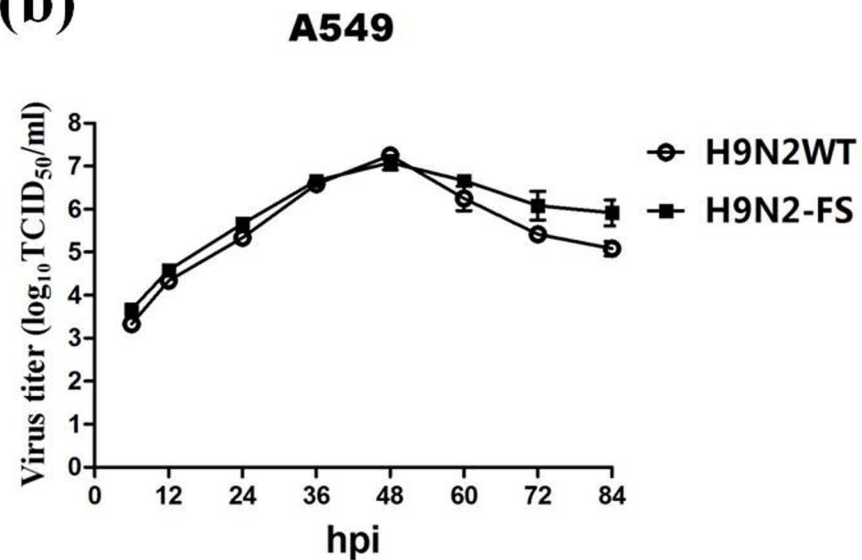
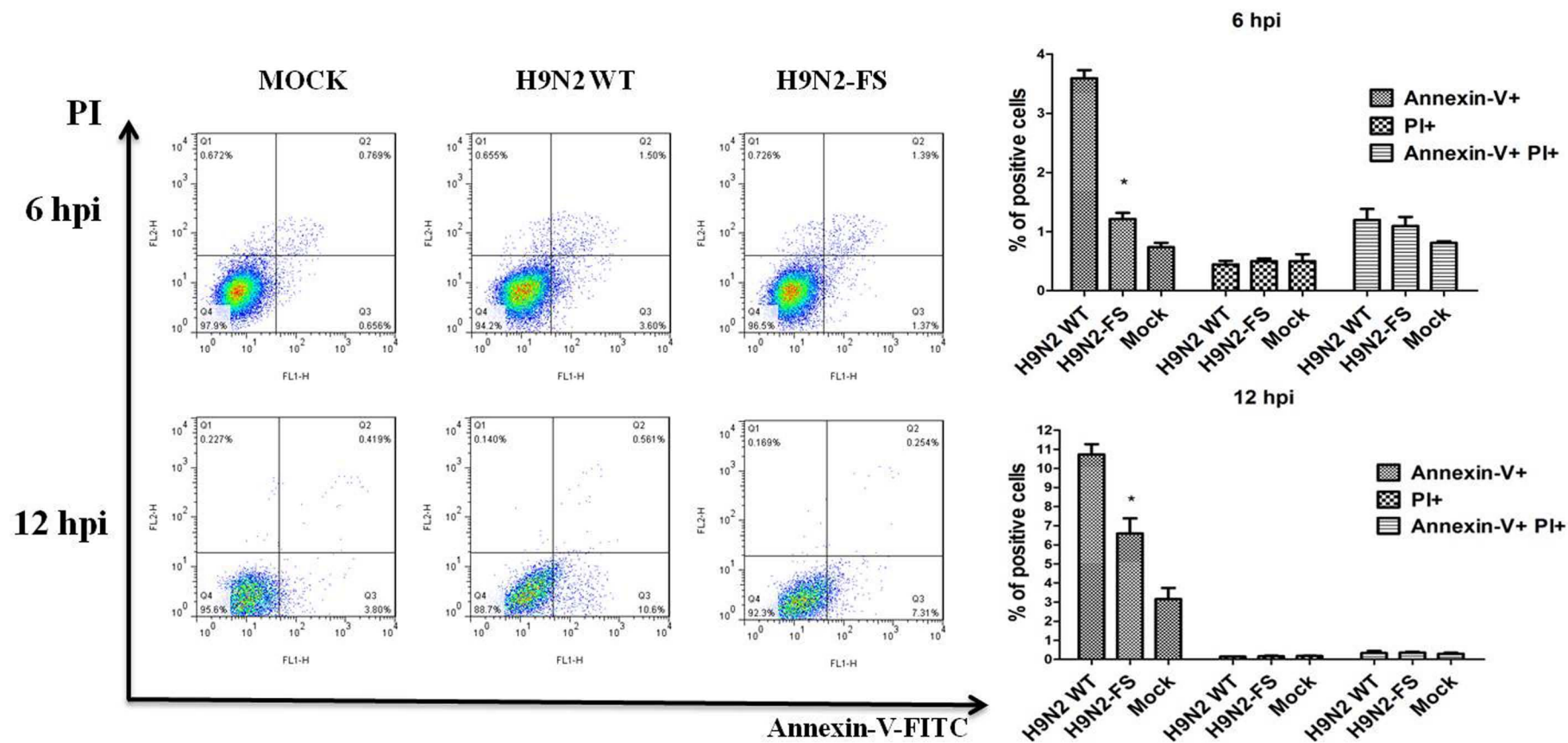
500 control. Protein bands were quantified by densitometry. Protein levels of PA and GFP
501 relative to β -actin are shown as histograms. Stronger expression of PA, in the absence
502 of PA-X, derived from H9N2-FS virus did not lead greater inhibition of GFP
503 expression. The results shown are representative data from three independent
504 experiments. Error bars indicate SDs. * indicates significant difference between H9N2
505 WT and H9N2-FS virus ($P < 0.05$).

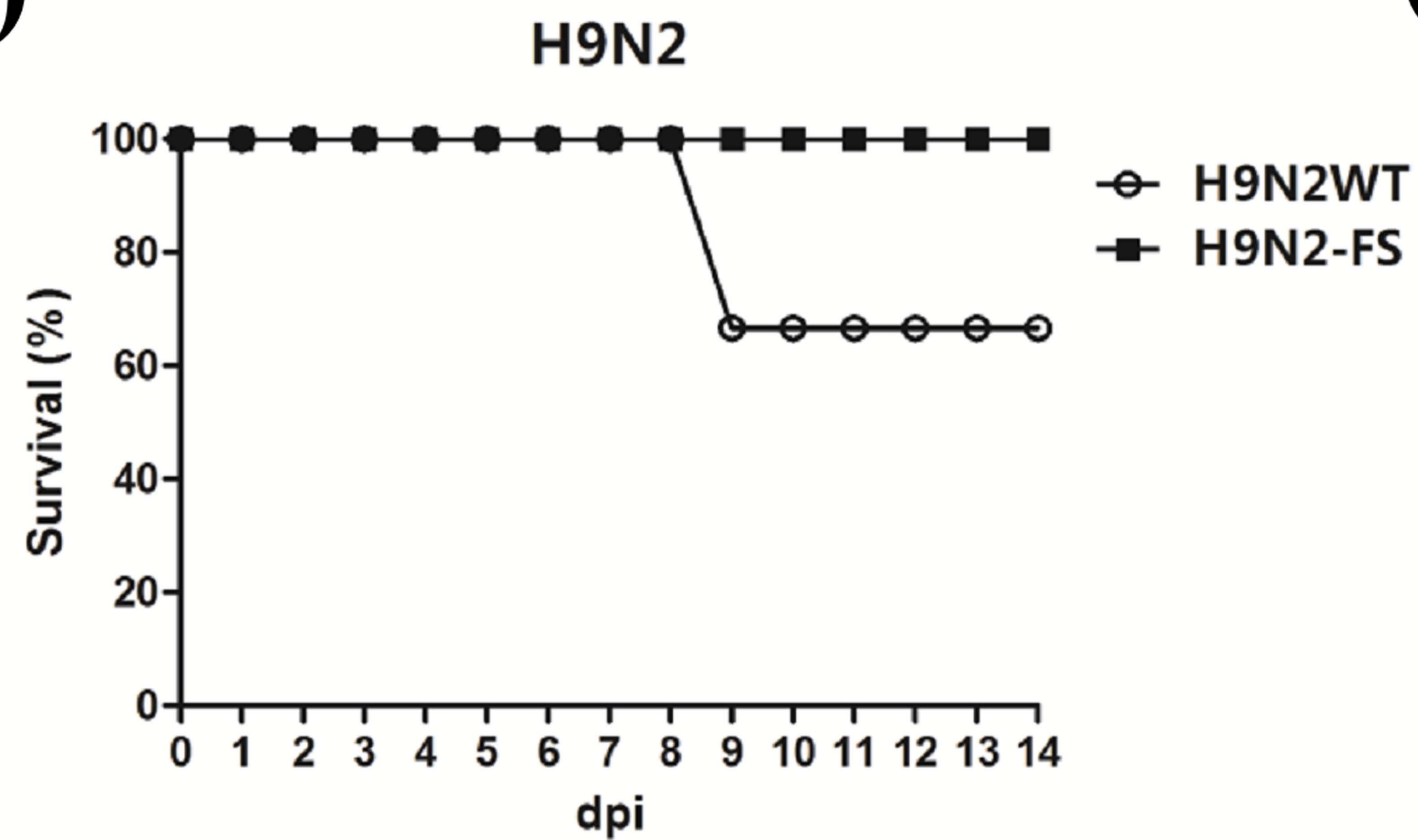
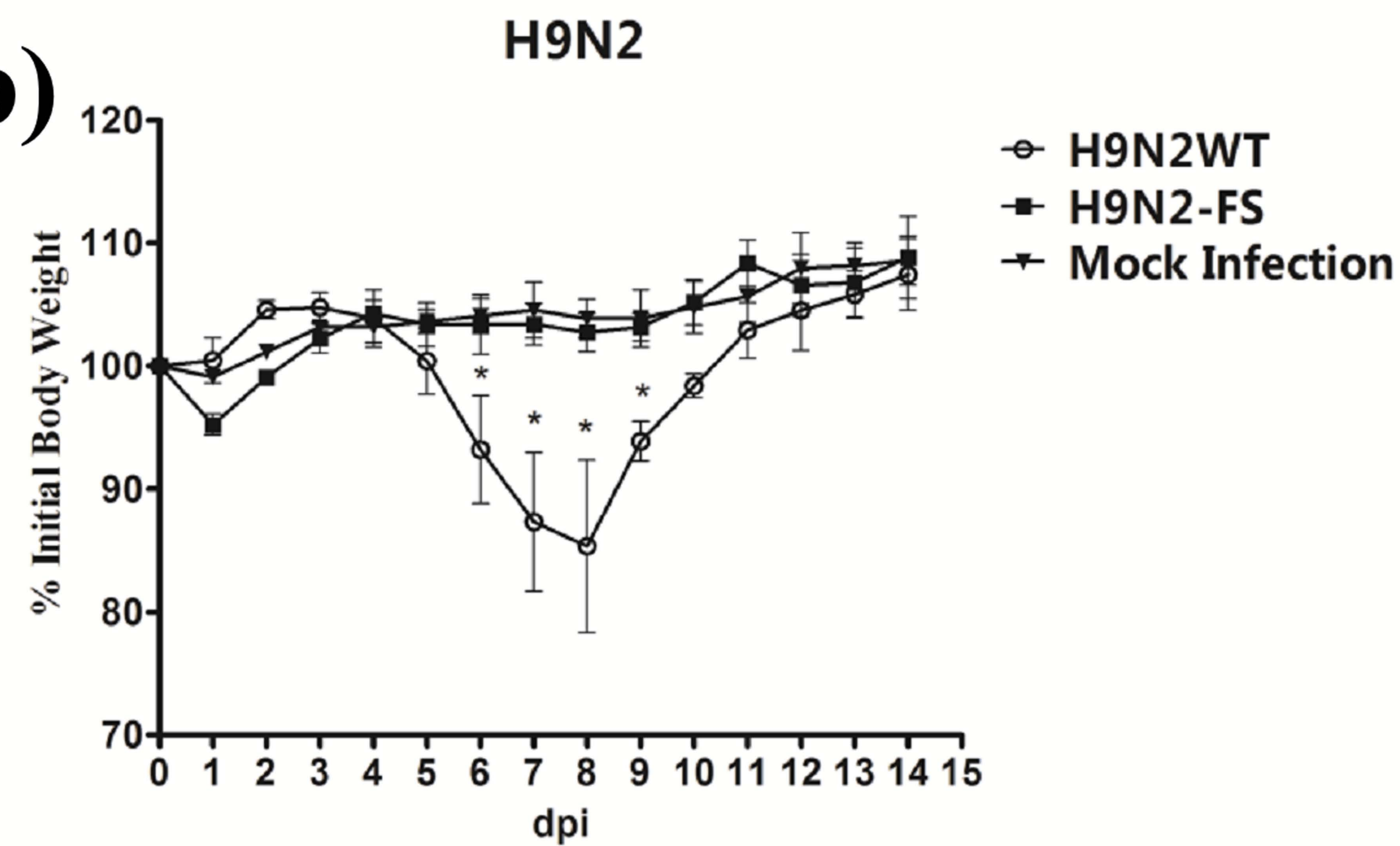
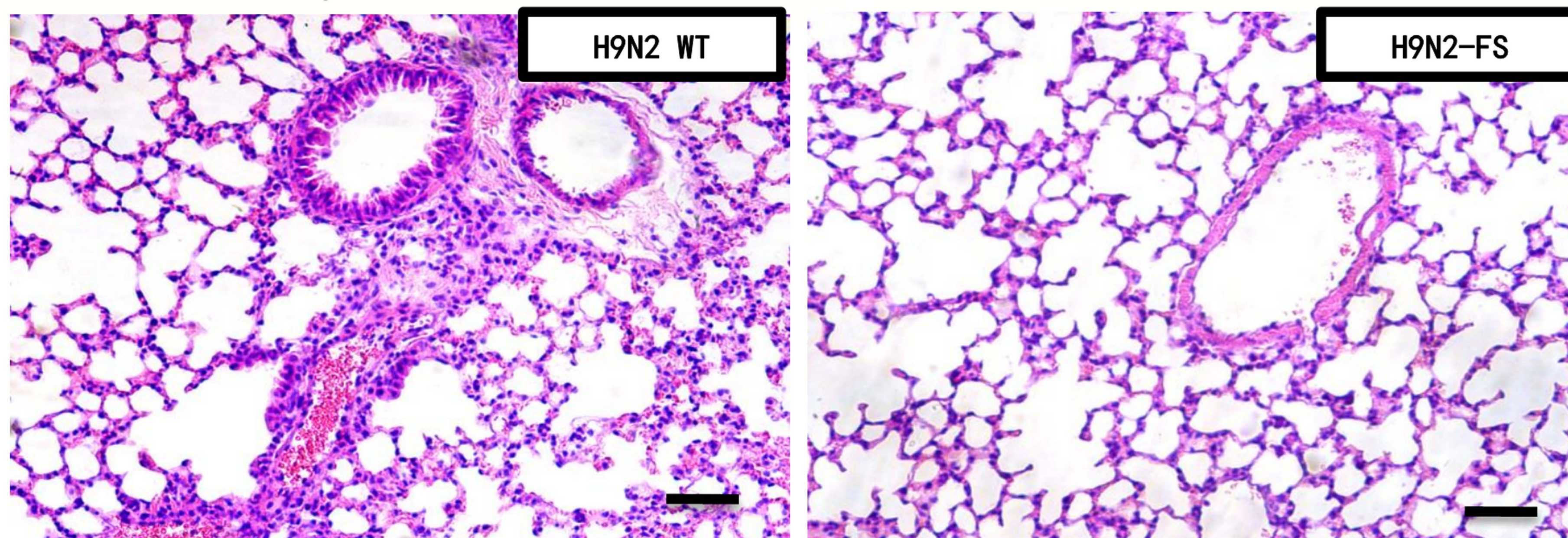
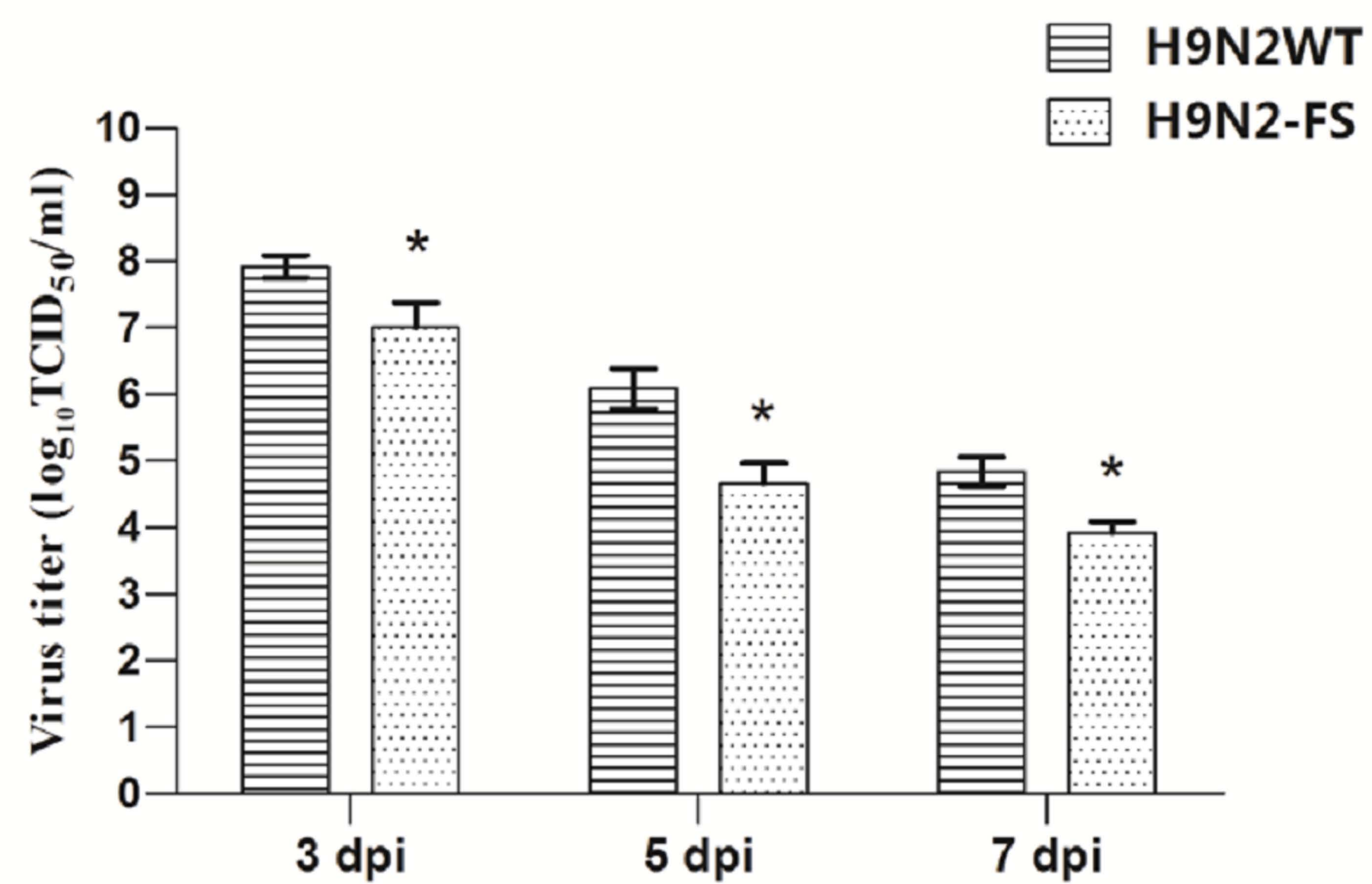
(a)

Nucleotide Sequence Of PA	WT	592		600
	FS	UCC	UUU	CGU
Amino Acids Of PA		AGC	UUC	AGA
		190	191	192
		S	F	R

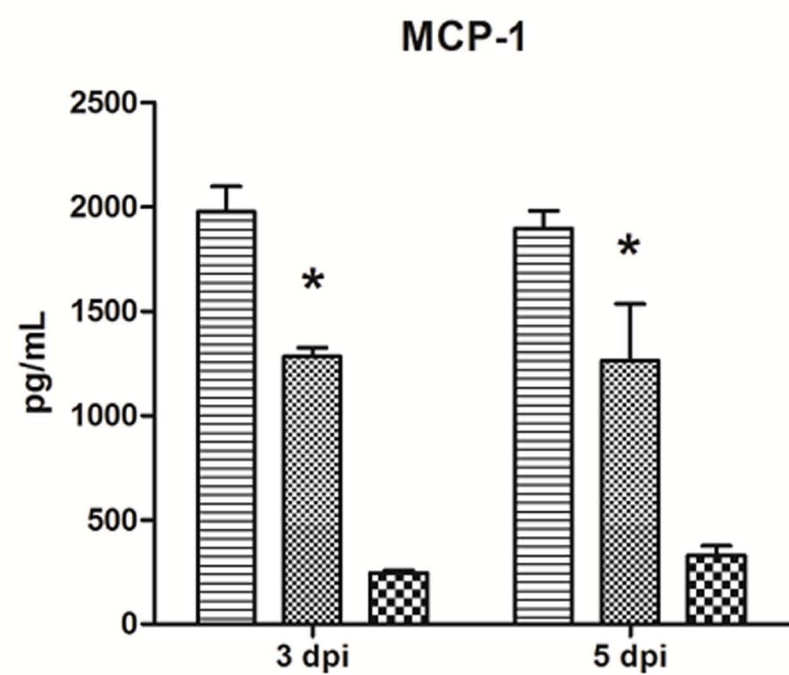
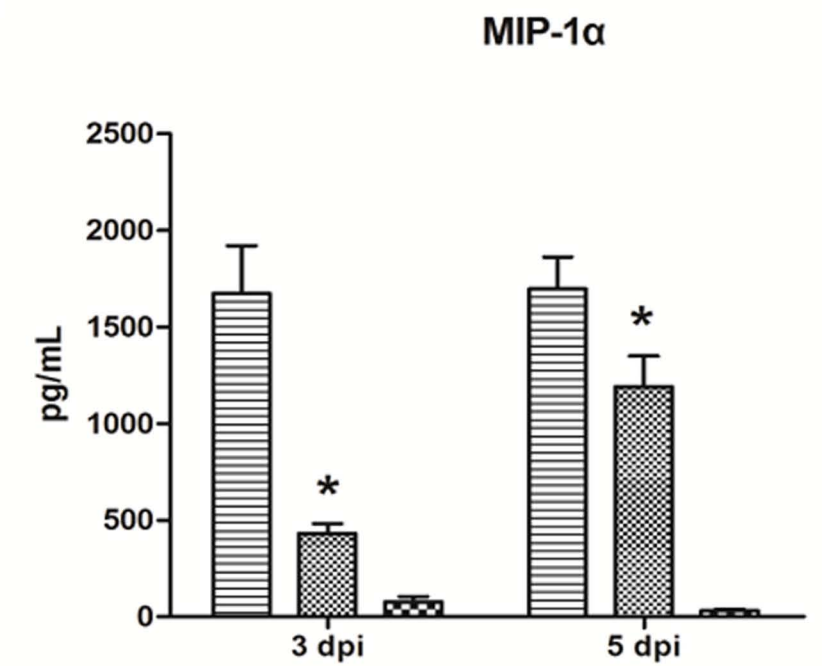
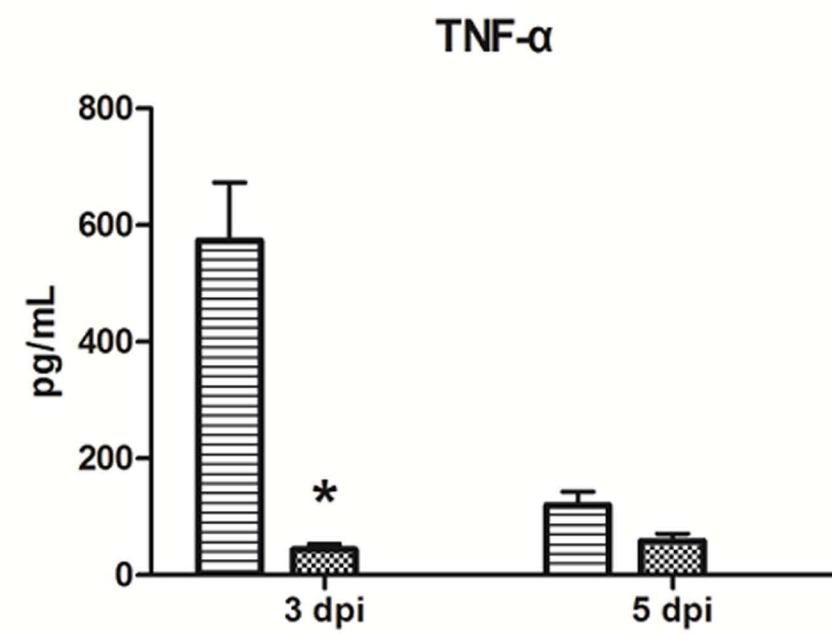
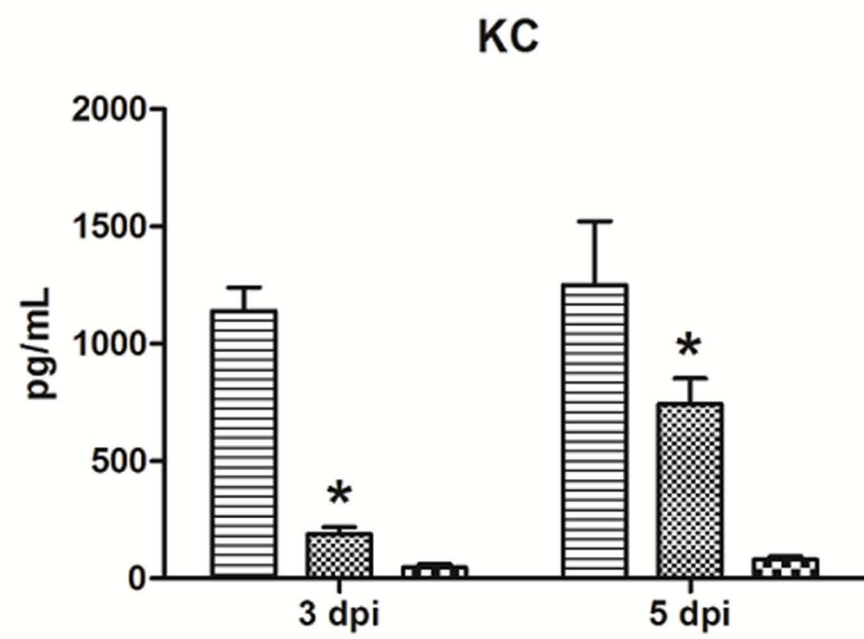
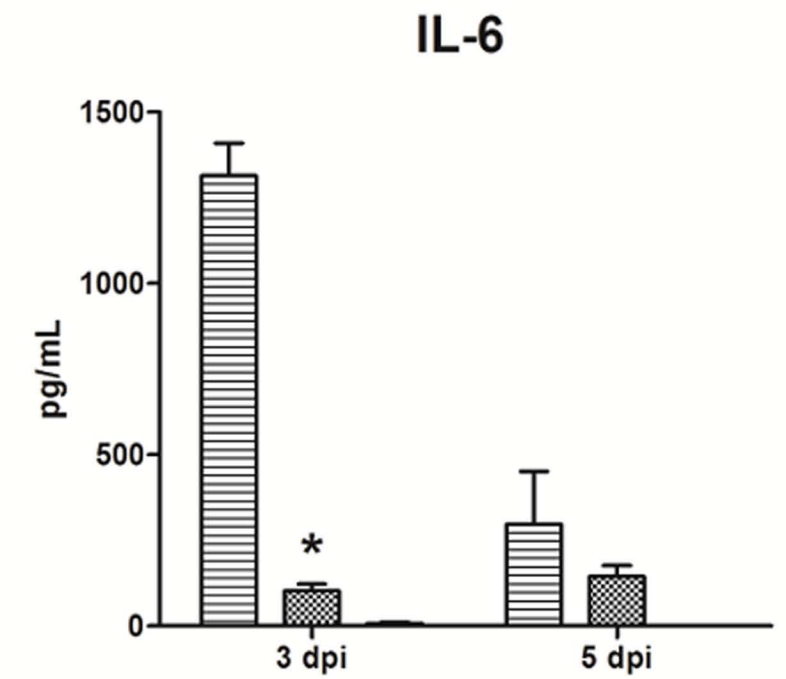
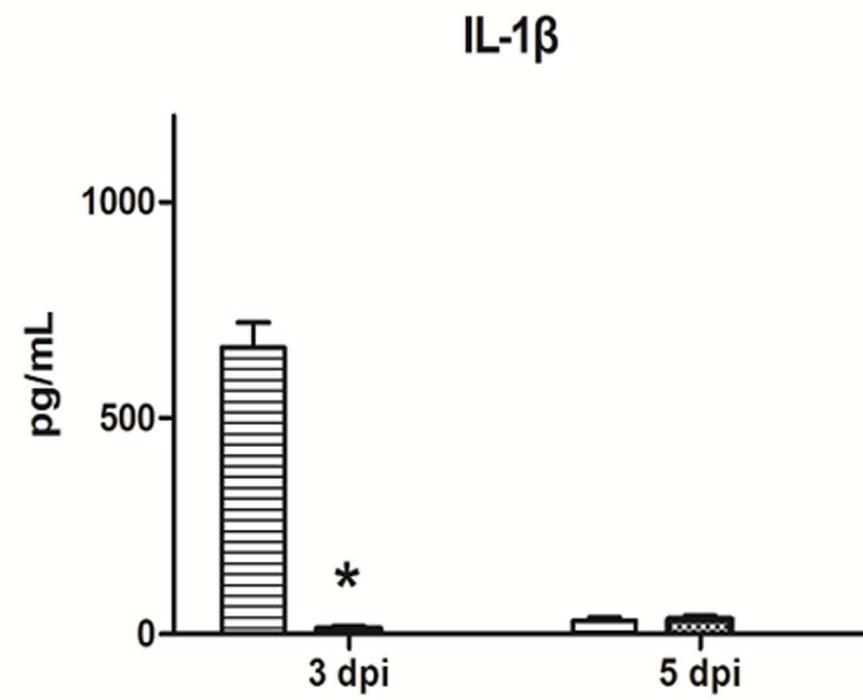
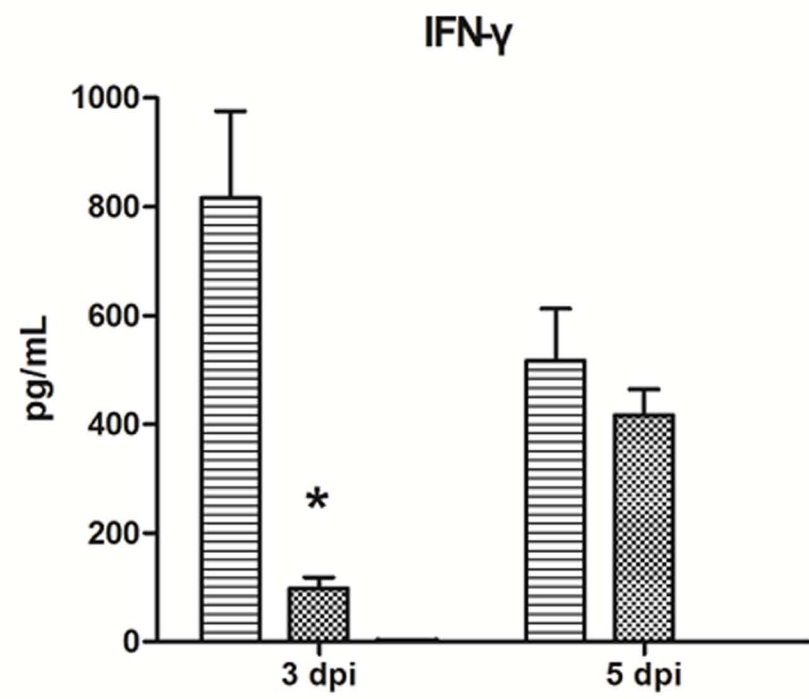
(b)



(a)**(b)****(c)**

(a)**(b)****(c)****(d)**

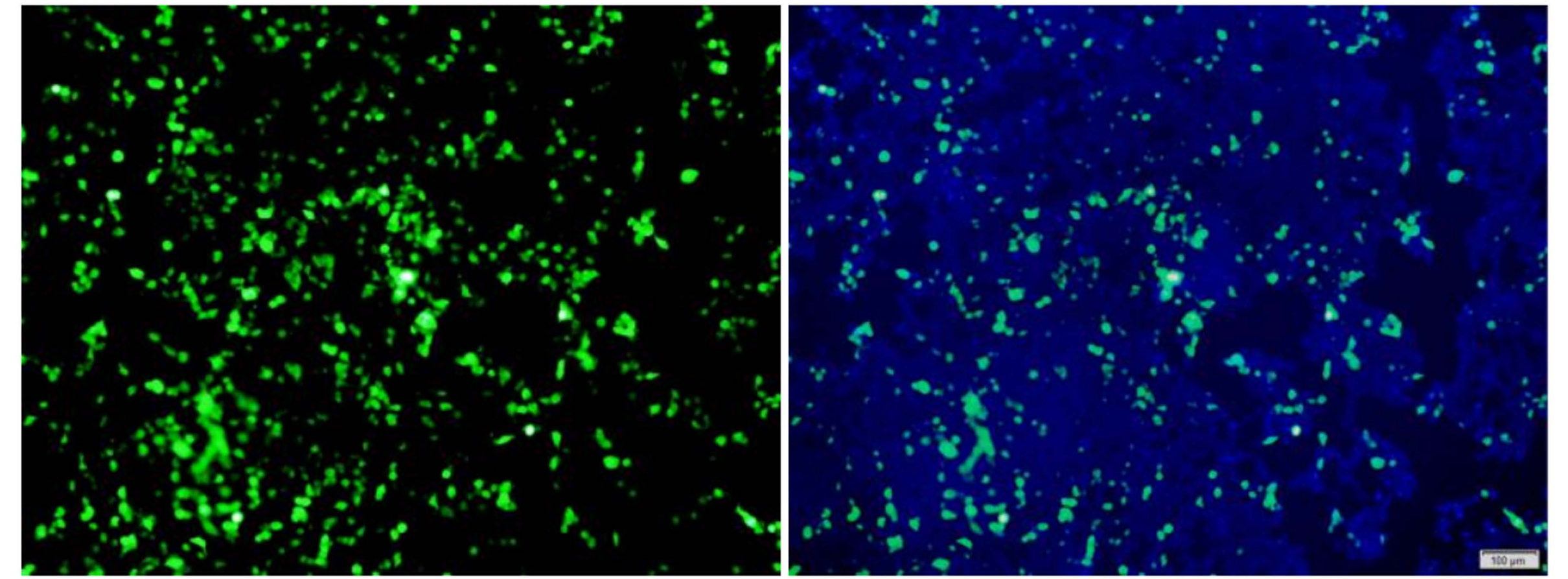
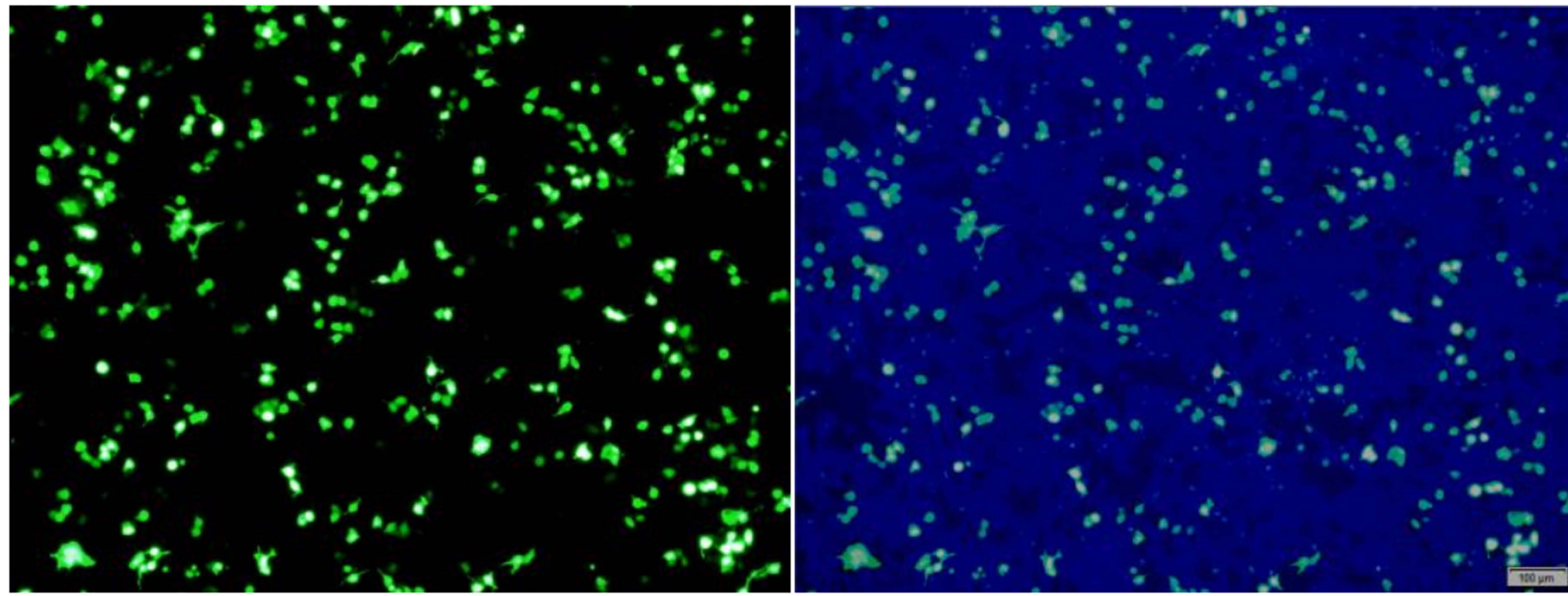
▨ H9N2WT
▩ H9N2-FS
▣ Mock Infection



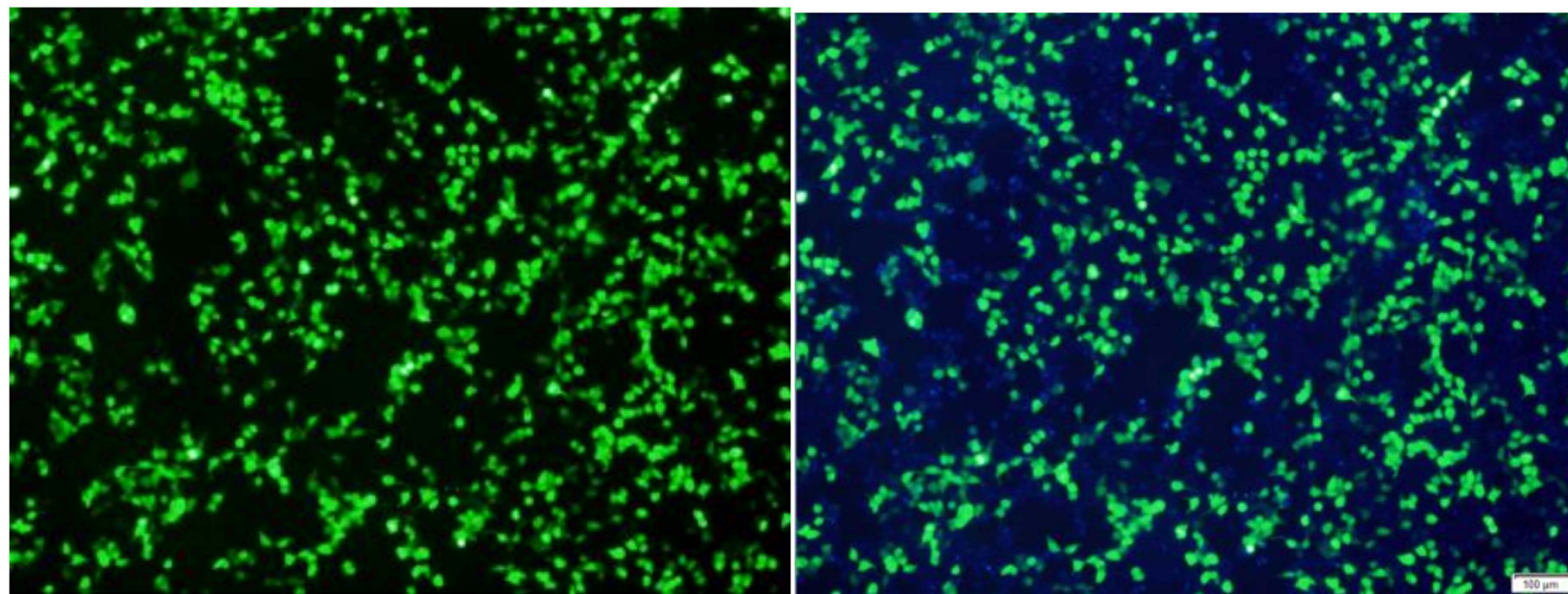
(a)

H9N2 WT PA

H9N2- FS PA



Control



(b)

H9N2 WT
PA

H9N2-FS
PA

Control

