# Evaluating the epizootic and zoonotic threat of an H7N9 low pathogenicity avian influenza virus (LPAIV) variant associated with enhanced pathogenicity in turkeys.

Running title: An H7N9 variant with increased pathogenicity in turkeys

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**Abstract**

Between 2013-2017, A/Anhui/1/13-lineage H7N9 low pathogenicity avian influenza virus (LPAIV) was epizootic in chickens in China causing mild disease, with 616 fatal human cases. Despite poultry vaccination, H7N9 has not been eradicated. Previously we demonstrated increased pathogenesis in turkeys infected with H7N9, correlating with the emergence of the L217Q (L226Q H3 numbering) polymorphism in the haemagglutinin (HA) protein. A Q217 containing virus also arose and is now dominant in China following vaccination. Here, we compared infection and transmission of this Q217 containing 'turkey-adapted' (ty-ad) isolate alongside the H7N9 (L217) *wild-type* (*wt)* virus in different poultry species and investigated the zoonotic potential in the ferret model. Both *wt* and ty-ad viruses demonstrated similar shedding and transmission in turkeys and chickens. However, the ty-ad virus was significantly more pathogenic than the *wt* virus in turkeys, causing 86% mortality in turkeys, but none in chickens. Expanded tissue tropism was seen for the ty-ad virus in turkeys but not chickens, yet the viral cell receptor distribution was broadly similar in visceral organs of both species. The ty-ad virus required exogenous trypsin for *in vitro* replication yet had increased replication in primary avian cells. Replication was comparable in mammalian cells and the ty-ad virus replicated successfully in ferrets. The L217Q polymorphism also affected HA antigenicity. Therefore, H7N9 infection in turkeys can generate novel variants with increased risk through altered pathogenicity and potential HA antigenic escape. These findings emphasise the requirement for enhanced surveillance and understanding of A/Anhui/1/13-lineage H7N9 viruses and their risk to different species.

Word Count: 250

39 **INTRODUCTION**

40 In 2013 the first human infection with a novel reassortant H7N9 avian-origin influenza A virus (IAV) was
41 reported in China [1]. To date, there have been 1568 laboratory-confirmed human cases of H7N9 which
42 include 616 fatal cases, giving a case fatality rate of 39% [2, 3], many of which have been associated with
43 human exposure to infected poultry, predominantly at live bird markets [4]. While chickens, pigeons, and
44 ducks have all been reported as positive for H7N9, chickens are considered as the maintenance host [5, 6].
45 H7N9 achieved enzootic levels in chickens across large parts of China, circulating in the absence of clinical
46 disease as a low pathogenicity avian influenza virus (LPAIV) [5, 6]. After almost four years of extensive
47 circulation in China, a high pathogenicity AIV (HPAIV) H7N9 variant was detected among one of the two
48 major clades which had evolved during this period [7, 8]. Vaccination of poultry against H7N9 commenced
49 in China during 2017 during the fifth wave and achieved apparent success in reducing detections of human
50 infections [9]. However, despite the reduction of zoonotic H7N9 cases [9], this virus has not been eradicated
51 from poultry in China, and sporadic detection in poultry and the environment in farms and live bird markets
52 have continued across different provinces of China (Fujian, Guangdong and Henan) [3], suggesting
53 continued circulation in Chinese poultry. Genetic analysis of H7N9 viruses which continue to circulate
54 following the adoption of vaccination have consistently identified the stability of haemagglutinin (HA) gene
55 polymorphisms, namely the change from a Leucine (L) to Glutamine (Q) amino acid residue at position 217
56 in the H7 mature peptide (L217Q: L226Q H3 numbering; L235Q complete protein numbering)) [10, 11].

57 Amino acid position 217 is located in the 220-loop of the HA protein forming part of the receptor
58 binding site. Possession of a L at position 217 has been previously shown to increase the affinity of HA
59 binding to 'human-like', α-2,6 sialic acid (SA), while a Q at position 217 increases affinity of binding to
60 'avian-like', α2,3 SA [12-14]. Thus, the Q217L polymorphism present in wave 1-5 (2013-2017) H7N9
61 viruses, along with other molecular motifs such E627K in the basic polymerase 2 (PB2) protein, have been
62 attributed to the zoonotic potential of these viruses [15, 16]. These human H7N9 cases have followed a
63 seasonal pattern with five successive winter waves [17]. The fifth wave of H7N9 (2016-2017) attained its
64 widest geographical distribution in China [17], reinforcing earlier concerns about the spread of H7N9 to

65 neighbouring countries [18]. Travel-related human cases have resulted in the detection of H7N9 in Canada
66 and Malaysia [19, 20], while confiscated poultry meat produce smuggled from China to Japan was found to
67 be contain H7N9 LPAIV and HPAIV [21]. In addition, H7N9 has been demonstrated to be able to reassort
68 with co-circulating H9N2 virus, producing novel viruses with altered biological properties [22, 23]. Such
69 events may increase the risk of emergence of infection in non-chicken hosts, including other avian species,
70 although whether further adaptation is required is unclear. Experimental investigations of H7N9 LPAIV in
71 poultry have focused almost exclusively on chickens. We previously investigated the effects of H7N9
72 infection and transmission in turkeys [24] and, using the prototypical wild-type (*wt*) H7N9 LPAIV of human
73 origin (A/Anhui/1/2013), demonstrated that direct-infection of turkeys resulted in onward transmission to
74 contact turkeys. Moreover, we observed an unexpectedly dramatic increase in pathogenicity for a LPAIV,
75 resulting in clinical disease and significant turkey mortality [24]. Disease severity correlated with the rapid
76 and consistent emergence of the L217Q polymorphism in the HA gene, leading to the proposal that this
77 genetic change in the isolated turkey-adapted (ty-ad) variant may be influencing susceptibility and virulence
78 in this species [24].

79 Our current study has described separate inoculation of chickens and turkeys, using both *wt* and ty-
80 ad H7N9 LPAIV isolates. The infection outcomes in both poultry hosts considered the different pathogenesis,
81 infectivity, and transmissibility outcomes for the ty-ad and the *wt* viruses. We also explore factors which
82 might impact on the infection outcome, including tissue tropism, receptor distribution and replication
83 kinetics in different avian and mammalian cell types. The zoonotic and reverse-zoonotic potential of the ty-
84 ad variant was also assessed.

85 **METHODS**

86 **Viruses**

87 The H7N9 *wt* (A/Anhui/1/13 [H7N9]) was a kind gift from Professor John Macauley at the Francis Crick
88 Institute, UK, which had undergone three egg passages (EP3) in 9-11 days-old specific pathogen free (SPF)
89 embryonated fowls' eggs (EFEs) [25]. Three further passages in EFEs produced the EP6 stock which served

90 as the inoculum in the *in vivo* experiments (H7N9 *wt*). The EP6 H7N9 was sequenced in its entirely and
91 confirmed as being identical to the original GenBank sequence submission (accession numbers: CY187618-
92 CY187625) except for an amino acid change in the HA, namely N141D (complete gene numbering). The ty-
93 ad H7N9 virus was produced *in vivo* by infection in turkeys by Slomka, Seekings [24] and was grown (EP1)
94 in 12-day-old embryonated turkeys' eggs (ETEs). Both viruses were titrated in EFEs to determine the 50%
95 egg infectious dose ($EID_{50}$) [26] and then diluted. For replication with or without trypsin, a prototypical
96 HPAIV H7N7 virus was used (A/chicken/England/11406/2008) as previously characterised [27]. For all
97 viruses used to assess replication kinetics in cell culture, the virus titre was determined by plaque assay on
98 MDCK cells as described previously [22].

99 **Isolation and sequence analysis of the turkey-adapted (ty-ad) virus**

100 Previous studies demonstrated that infection of turkeys with *wt* A/Anhui/1/13 (H7N9) led to the
101 accumulation of amino acid substitutions [24]. A single amino acid substitution, L217Q (L226Q, H3
102 numbering), was consistently identified in the consensus sequence from all contact turkeys analysed [24]. To
103 evaluate the effect of these substitutions, we isolated and propagated a virus (ty-ad) in ETEs from kidney
104 tissue taken from a contact turkey which exhibited enhanced clinical signs in the last chain of transmission.
105 Both the *wt* and ty-ad H7N9 viruses were characterised as LPAIV by standard intravenous pathogenicity
106 index (IVPI) testing in chickens as described previously [24]. Whole genome sequencing (WGS) of the ty-ad
107 isolate also demonstrated no change in the cleavage site from that of the *wt* H7N9 virus (PEIPKGR'GLF).
108 However, consistent with other virus samples, WGS revealed two amino acid substitutions compared to the
109 *wt* H7N9 virus including the L217Q substitution in the HA gene, and a T10S substitution in the
110 neuraminidase (NA) gene.

111 **Animals**

112 For *in vivo* infection and transmission experiments, both High health status White Holland Turkeys
113 (*Meleagris gallopavo*) and specified-pathogen-free (SPF) White-Leghorn chickens (*Gallus gallus*) were used
114 at three-weeks of age. Birds were housed on solid flooring covered with straw, with food supplied *ad libidum*
115 and drinking water was refreshed daily. High health status ferrets (*Mustela furo*) between 700-1000g (around

116 three months-of-age) were used in zoonotic and reverse zoonotic experiments. All birds and ferrets were
117 confirmed as serologically negative to H7N9 by hemagglutinin inhibition (HI) using homologous antigen on
118 serum collected prior to infection. Oropharyngeal (Op) and cloacal (C) swab (chickens and turkeys) and
119 nasal wash (ferrets) samples collected prior to infection were used to confirm negativity to influenza A virus
120 M-gene RNA by RRT-PCR [23, 28].

121 **Turkey-to-turkey infection and transmission experimental design**

122 Two separate groups of six, three-week old turkeys were directly infected ('Donor', D0) via the ocular-nasal
123 route with 100 µl of phosphate buffered saline (PBS) containing $1x10^6$ $EID_{50}$ of the *wt* or ty-ad H7N9
124 viruses. At one day-post-infection (dpi), six naïve (contact) turkeys ('first recipient', R1) were introduced
125 into each group of directly infected turkeys. All turkeys were weighed immediately prior to infection and
126 daily post infection.

127 **Intravenous turkey experimental design**

128 The intravenous (IV) inoculation experiment was performed according to the World Organisation for Animal
129 Health (WOAH) protocol for IV pathogenicity index (IVPI) determination [25], using three-week old
130 turkeys, instead of chickens. Two separate groups of ten, three-week old turkeys were directly infected via
131 the IV route with 100 µl of sterile saline containing $1x10^6$ $EID_{50}$ of the *wt* or ty-ad H7N9 viruses.

132 **Chicken infection and transmission chains design**

133 Two separate groups of six chickens were infected via the ocular-nasal route with $1x10^6$ $EID_{50}$ of the *wt* or
134 ty-ad viruses (D0), diluted accordingly from the respective viral stocks in sterile PBS, with 100 µl inoculum
135 administered per bird. At one dpi, six naive chickens were co-housed with the directly infected chickens
136 (R1). At four dpi, the D0 birds were removed, and six additional naive chickens were placed in contact with
137 the R1 chickens ("second recipient", R2) to establish a chain of transmission.

138 **Ferret model of zoonosis and reverse zoonosis**

139 Six ferrets were infected via the intranasal route with 0.5 ml (0.25ml per nare) of PBS containing $1x10^6$
140 $EID_{50}$ of the ty-ad virus. The experiment was conducted in two identical blocks. In each block three ferrets
141 were housed in the middle of three conjoined housing unit, in a linear arrangement, with the central unit
142 separated from its two adjacent units by means of a perforated perspex barrier. One ferret from each block
143 (total of two ferrets) were culled at six dpi for immunohistochemistry (IHC) analysis. For each ferret the
144 external nasal tissues were washed with 1 ml PBS on two, three, four, five, six, seven and eight dpi; 1 ml of
145 PBS was used to wash the internal nasal cavity on days two, four, six and eight dpi as previously described
146 [22, 29]. Blood was collected from all remaining ferrets at the end of the experiment at 14 dpi, for
147 serological testing. Six chickens and six turkeys were housed in the two adjacent cages on three and five dpi
148 for six hours, after which the birds were moved to a new room with a separate pen for each species and
149 group. All birds were swabbed daily and monitored for clinical signs. Blood samples were collected from all
150 birds at 14 dpi.

151 **Sample collection and processing**

152 Turkeys and chickens were swabbed once daily from the Op and C cavities and swabs processed into 1 ml of
153 brain heart infusion broth (BHIB) as previously described [24]. Tissue samples were collected from two
154 turkeys and two chickens pre-destined (random allocation) for culling on four and six dpi. Tissue samples
155 were also taken from five birds which were found dead, or euthanised due to severe infection reaching
156 clinical end points on six or eight dpi. All tissue samples were taken into 1 ml of Brain Heart Infusion broth
157 (BHIB) to achieve around 10 % (w/v) for each sample. Samples of drinking water and faeces were collected
158 from the environment of infected or contact animals and processed as previously described [30].

159 **RNA extraction and AIV reverse transcription Real-Time PCR (RRT-PCR)**

160 RNA was extracted from swabs, tissues and environmental samples as previously described [24] and
161 extracted RNA was tested by the M-gene RRT-PCR which featured the primers and probe designed by Nagy,
162 Vostinakova [28], as described previously to detect AIV viral RNA (vRNA) [24]. Ct values were titrated

163 against a ten-fold dilution series of H7N9 *wt* RNA of a known titre and were converted to relative equivalent
164 units (r.e.u.) as described previously [23]. Ct values ≥ 36 were considered negative for influenza A virus.

165 **Whole genome sequencing (WGS)**

166 Whole-genome sequencing was undertaken on the RNA extracted from inocula, and clinical samples as
167 previously described [24].

168 **Serology**

169 Serological analysis was undertaken as previously described for sera of turkey [24] and chicken origin [23].
170 Ferret sera were treated with four volumes of receptor-destroying enzyme (APHA Scientific, Weybridge,
171 UK) as previously described [22]. Detection of seroconversion to subtype-specific (homologous) HA
172 antigens was conducted using the haemagglutination inhibition (HAI) assay, using four hemagglutination
173 units of the corresponding H7N9 LPAIV as the antigen [25].

174 **Replication kinetics and plaque assay**

175 Replication kinetics were assessed in chicken, turkey, or duck embryo fibroblasts (CEF, TEF, DEF
176 respectively), MDCK (canine), CACO2 (human) and NPTr (swine) cells. Cells (80-90% confluency) were
177 used in six well plates; after removal of media and wash steps with PBS, the cells were infected with 400 µl
178 of virus at an MOI of 0.01. Each plate was then placed in an incubator at 37 ºC for one hour, gently rocking
179 the plate every 20 minutes. The viral inoculum was then removed, and cells washed with PBS. Dulbecco's
180 Modified Eagle Medium (DMEM) containing TPCK trypsin (SIGMA) with a final concentration of 1 µg/ml
181 was then added to each well. The plates were incubated at 37 ºC and samples collected at indicated time
182 points and stored at -80 ºC for further use. For plaque assays, 6 well plates of TEFs or MDCKs were
183 inoculated with 10-fold serially diluted virus in DMEM and added to the plates as above. Flu overlay
184 (immunodiffusion grade melted agarose, Sigma-Aldrich) containing TPCK trypsin with a final concentration
185 between 1 µg/ml was added to each well, 2 ml of this mixture was then added to each well. The plates were
186 incubated at 37 ºC for a period of four days, placed inverted to prevent condensation build up in the wells.
187 Viruses were then harvested from the infection foci for use in determining individual viral growth kinetics.

188 **Immunohistochemistry (IHC) and characterising α-2,3 and α-2,6 receptor distribution in avian tissues**

189 Tissue samples were fixed in 10 % (v/v) neutral buffered formalin (VWR, East Grinstead, UK) and
190 processed routinely through graded alcohols and chloroform and embedded in paraffin wax. Four-
191 micrometre-thick sections, cut on a rotary microtome, were stained with haematoxylin and eosin (H&E) or
192 used for immunohistochemical (IHC) detection of influenza A nucleoprotein as previously described [24].
193 For lectin staining of virus receptor distribution in tissues, slides were dewaxed in xylene and passed through
194 graded alcohols to Tris-buffered saline solution with 0·05% Tween (TBSt) (0·005M Tris, pH 7·6, 0·85% w/v
195 NaCl). Samples were subsequently incubated with biotinylated *Maackia amurensis* lectin II (1/100) (Vector
196 Laboratories, Peterborough, UK) for the α2,3 'avian receptor', or biotinylated *Sambucus nigra* (Elderberry)
197 bark lectin (1/1000) (Vector Laboratories) for the α2,6 'human receptor' for 1 hour, and VECTASTAIN Elite
198 ABC-peroxidase reagent (Vector Laboratories) for 30 minutes, at room temperature. Sections were washed
199 three times with TBSt between incubations. The IHC signal was visualised using 3,3 diaminobenzidine
200 (Sigma-Aldrich, Poole, UK), and sections were counterstained in Mayer's haematoxylin (Surgipath,
201 Peterborough, UK), dehydrated in absolute alcohol, cleared in xylene and mounted using dibutyl phthalate
202 xylene (DPX) and glass coverslips. Samples were processed and images captured in a double-blinded fashion
203 and scored semi-quantitatively by trained veterinary pathologists based on the intensity and distribution of
204 the staining (-, absent; +/-, minimal; +, mild; ++, moderate).

205 **Statistical analysis**

206 To assess disparities among various groups, one-way analysis of variance (ANOVA), Two-way ANOVA, or
207 paired students t-tests were carried out (GraphPad Prism v8). A significance threshold of P values (<0.05)
208 was set. Area under the curve (AUC) analysis was performed on total shedding as previously described [30].

209 **RESULTS**

210 **The ty-ad H7N9 virus has increased pathogenicity for turkeys but not chickens.**

211 Following direct ocular-nasal infection of turkeys, the *wt* virus caused 33 % mortality in the D0 turkeys
212 between 8 and 8.5 dpi (Fig. 1A). Comparatively, the ty-ad virus caused 100 % mortality in D0 turkeys

213 between 6 and 8 dpi. Similarly, following transmission to naive contact turkeys, the *wt* virus caused 0 %
214 mortality, yet the ty-ad virus caused 100 % mortality (Fig. 1A). Importantly, all surviving *wt* virus infected
215 turkeys demonstrated seroconversion to H7N9 when tested by HAI at 14 dpi which confirmed infection (data
216 not shown). A similar difference in pathogenicity was observed when turkeys were inoculated with either
217 virus intravenously; the *wt* virus caused 60 % mortality by 8 dpi, whereas the ty-ad virus caused 100 %
218 mortality within 5 dpi (Fig. 1B). The ty-ad virus infected turkeys also exhibited reduced, but not statistically
219 significant weight gain compared to the *wt* infected turkeys, for both D0 and R1 groups (Fig. S1). By
220 contrast, chickens (both D0 and R1 groups) exhibited 0 % mortality (Fig. 1C) with either virus. Taken
221 together, these data indicate that the ty-ad virus is more pathogenic than the *wt* virus in turkeys, but not in
222 chickens.

223 **Both the *wt* and ty-ad virus infected birds shed similar levels of vRNA and transmitted infection**
224 **equivalently in both turkeys and chickens.**

225 Both the *wt* and ty-ad viruses successfully infected all six of the respective D0 turkeys or chickens. For both
226 groups, vRNA was detected in both the Op and cloacal C swabs taken from turkeys (Fig. 2A and B) and
227 chickens (Fig. 3). Directly infected (D0) turkeys and chickens shed peak levels of vRNA of between $1x10^5$ to
228 $1x10^6$ r.e.u. from the Op cavity following infection with either virus (Fig. 2A and B and Fig. 3A and C).
229 based on AUC analysis, there was no significant difference in total amount of viral shedding during the
230 shedding period (Fig. 2A and E). The levels of vRNA detected in the water and environmental faecal sources
231 was largely comparable between turkeys and chickens infected with either virus, although slightly greater
232 vRNA was detected in the ty-ad group between 4 and 8 dpi (Fig. S2).

233 The transmission efficiency was 100 % for both viruses in both species; all R1 turkeys and chickens
234 became infected and shed vRNA from the Op and C cavities (Fig. 2C & D and Fig. 3). For the turkeys,
235 positive vRNA levels were first detected for both *wt* and ty-ad R1 groups at 3 days post-contact (dpc),
236 indicating transmission within a similar timeframe. Op and C vRNA shedding profiles for the contact turkeys
237 were similar to those of the directly infected turkeys (Fig. 2). For the chickens, a chain of transmission was
238 established from D0 to R1 and subsequently to R2 chickens, with 100 % successful transmission throughout

239 the chain. However, the ty-ad vRNA was detected in swab material from the R1 chickens a day earlier than
240 the *wt* virus (Fig. 3A and C).

241 All chickens and turkeys which survived until the end of the study (19 dpi) were seropositive by the
242 HAI against the H7N9 antigen (data not shown). Interestingly, using post infection anti-sera raised against
243 the *wt* or ty-ad viruses, there was a statistically significant drop in reciprocal HAI titres between the L217
244 and Q217 antigens, for homologous compared to heterologous sera (Fig. S3).

245 **Differences in systemic H7N9 distribution between turkeys and chickens, with increased tissue**
246 **dissemination of ty-ad in turkeys compared to *wt* virus.**

247 The tropism of the *wt* and ty-ad viruses was investigated by vRNA quantification and viral antigen detection
248 by IHC. Tropism was investigated in tissues collected from at least two turkeys and two chickens per group -
249 culled while apparently healthy on 4 and 6 dpi (Fig. 4A, B, D and E). In the turkeys, both the *wt* and ty-ad
250 vRNA were detected in a range of organs, including respiratory (nasal turbinates, trachea lung and air sacs),
251 and other visceral (intestine, spleen, heart, liver and brain) organs (Fig. 4A, B, Fig. S4 and Table S1).
252 However, the ty-ad virus was detected more frequently and with higher vRNA and IHC levels than the *wt*
253 virus. The vRNA levels were also quantified from the organs of five turkeys that died as a result of *wt* and ty-
254 ad virus infections on 6 and 8 dpi, with the three ty-ad virus group mortalities exhibiting a more widespread
255 virus detection in all sampled organs (Fig. 4C). By contrast, chickens exhibited considerably lower vRNA
256 levels in all organs, with limited dissemination beyond the respiratory and enteric tracts, at both 4 and 6 dpi
257 (Fig. 4A-C compared to D and E). There were also no major differences in virus tropism between the *wt* and
258 ty-ad virus in the chickens (Fig. 4D and E).

259 **Turkeys and chickens have broadly different sialic acid receptor distributions in their respiratory**
260 **tracts, but similar distributions in visceral organs.**

261 The L217Q mutation has been previously shown to change virus receptor preference from α-2,6-
262 sialyllactosamine (α-2,6) to α-2,3-sialyllactosamine (α2,3) [12]. The ty-ad virus possesses a Q at amino acid
263 position 217, therefore we hypothesised that differences in α-2,3 and α-2,6 linked SA might explain the

264 expanded tissue tropism of the ty-ad virus in turkeys. Using lectins which bind to α-2,3 (Maackia Amurensis
265 Lectin II [MALII]) or α-2,6 (sambucus nigra [SNA]), the host receptor distribution was assessed in turkey
266 and chicken tissues where the highest levels of vRNA were detected (nasal turbinates, trachea, lung, pancreas
267 and kidney) (Fig. 4 and S5, Table 1). Turkey and chicken tissues had similar α-2,3 and α-2,6 receptor
268 staining distribution although some differences were observed. Turkey tissues had more intense MALII
269 (α2,3) labelling in the nasal cavity and trachea compared to SNA (α2,6) staining (Table 1). Further, in
270 turkeys staining in the nasal cavity and trachea was more intense for MALII (α-2,3) compared to that
271 observed in the same tissues from chickens (Table 1). Interestingly, whilst SNA (α-2,6) staining in the
272 trachea of turkeys was less pronounced than that seen in chickens, the SNA (α-2,6) staining in turkey
273 kidneys was more intense than observed in chickens (Table 1).

274 **Both *wt* and ty-ad viruses require trypsin for efficient replication, but the ty-ad virus replicates to**
275 **higher titres in avian cells.**

276 vRNA from Op swab samples collected at six dpi from all six turkeys directly infected with the ty-ad virus
277 was sequenced. Sequence analysis revealed a single basic cleavage site (CS) sequence present in HA
278 (PEIPKGR'GLF) in all sequences, identical to the CS sequence in the wt and ty-ad viruses used for the
279 inocula. In cell culture, both the *wt* and ty-ad H7N9 viruses required the presence of trypsin to replicate; both
280 viruses formed plaques with trypsin yet failed to form plaques without trypsin (Fig. 5A). In comparison, a
281 HPAIV H7N7 virus did form plaques without trypsin (Fig. 5A). The *wt* and ty-ad viruses were also compared
282 for replication in primary duck, chicken and turkey embryonic fibroblast (DEF, CEF and TEF) cells as well
283 as human (CACO-2), swine (NPTr) and canine (MDCK) cells (a cell line highly susceptible and permissive
284 to IAV replication). In all three of the different avian cells, the ty-ad virus replicated to statistically
285 significantly higher titres compared to the *wt* virus (Fig. 5B-D). In the different mammalian cells however,
286 there was no statistically significant difference in virus replication between the *wt* and ty-ad viruses over the
287 72-hour culture period (Fig. 5E-G).

288 **The ty-ad virus represents a zoonotic threat but does not exhibit reverse zoonotic transmission**
289 **potential.**

290 We next investigated the zoonotic consequence of the ty-ad virus by investigating its ability to infect and
291 replicate in ferrets, a model for human influenza A virus infection. Four ferrets (two per block) were directly
292 inoculated intranasally with $1x10^6$ $EID_{50}$ of the ty-ad virus and successfully became infected, with vRNA
293 being detected in interior nasal wash samples from 2 until 6 dpi (Fig. 6A). Viral RNA was also detected
294 (above the threshold for assay positivity) from 2 to 6 dpi from exterior nasal wash samples. The viruses
295 present in the interior nasal wash samples from all ferrets at 4 or 6 dpi was sequenced (Fig. 6; yellow filled
296 circles). Sequence analysis revealed that viruses present in all ferrets retained the Q217 polymorphism of the
297 input virus at a consensus level (data not shown). Influenza viral NP was also detected in the respiratory
298 turbinates, trachea and lungs of the two ferrets pre-destined for IHC analysis at 6 dpi (Fig. S6 and Table S2).
299 All four of the remaining ferrets bled at study-end (14 dpi) had seroconverted to homologous H7N9 antigen
300 by HAI (Fig. 6B). To mimic reverse zoonoses the ability of infected ferrets to transmit virus to naive turkeys
301 and chickens was investigated. No vRNA was detected in either Op or C avian swabs collected daily for 14
302 days post exposure (dpe) from either block (data not shown). In addition, none of the chickens or turkeys
303 seroconverted to homologous H7N9 antigen by HAI by the same time-point from either block (Fig. 6B).

304 **DISCUSSION**

305 Since its emergence in 2013 in China, A/Anhui/1/13-lineage H7N9 LPAIV has continued to circulate in
306 poultry and threatens human health. Currently H7N9 is located exclusively in China [31] where poultry
307 production consists largely of chickens and ducks with almost negligible turkey production [32]. Despite
308 widespread poultry vaccination in China, H7N9 continues to be detected in poultry [3], with an ever present
309 concern that geographical expansion of H7N9 could increase its zoonotic and epizootic risk through
310 reassortment with indigenous AIVs, or access to novel hosts [22].

311 Previously, the risk of A/Anhui/1/13-lineage H7N9 infection in turkeys was investigated, modelling
312 a scenario in which H7N9 virus expands into neighbouring countries with a larger turkey poultry sector [24].

313 Infection and transmission of H7N9 LPAIV in turkeys was associated with the 217 L217Q (H7 numbering of
314 the mature protein) polymorphism in the HA gene [24]. An H7N9 virus from a turkey with severe clinical
315 disease (termed the 'ty-ad' virus) was isolated that contained two amino acid substitutions, one in the HA
316 (L217Q) and one in the neuraminidase (NA; T10I) proteins. In this study we explored the outcome of
317 infection for two poultry species (chicken and turkey) with this 'ty-ad' virus variant compared to the *wt* virus.
318 We further investigated potential mechanisms which might underpin the differences in observed infection
319 outcome and explored the zoonotic and reverse zoonotic risk of these viruses.

320 *In vivo,* when inoculated intravenously or intranasally, the ty-ad virus caused greater, and more rapid,
321 mortality in turkeys than the *wt* H7N9 virus strain. This disparity in clinical disease between the *wt* and ty-ad
322 virus was not observed in experimentally infected chickens. Post-mortem investigation of the turkeys in the
323 'ty-ad' virus infected group revealed a systemic tissue dissemination of vRNA with higher levels of viral
324 RNA being detected in a wider range of visceral organs compared to the *wt* virus. By contrast, following
325 chicken infection, this difference in vRNA levels and its systemic distribution was not observed between the
326 two virus variants. However, both viruses displayed comparable shedding profiles from both the Op and C
327 cavities. Different pathogenic outcomes between chickens and turkeys infected with H7 AIVs has been
328 reported previously [33]. However, the genetic determinants for virulence and pathogenesis of HPAIV in
329 turkeys are largely unknown. For AIVs, tropism is determined by a combination of factors including (i) the
330 distribution of proteases required to cleave HA0 to HA1 and HA2 required for infectivity, (ii) the distribution
331 of the sialic acid (SA) receptors to which the viral hemagglutinin (HA) binds, and (iii) cells being permissive
332 (allowing viral replication) for viral infection. We therefore explored these factors which might underpin the
333 observed differences in pathogenicity and tropism, between the turkey and chicken hosts following infection
334 with the two H7N9 LPAIV variants.

335 The major genetic determinant of AIV tropism is the presence of a multiple basic amino acids
336 proximal to the cleavage site in the HA protein, termed a multibasic cleavage site. This multibasic cleavage
337 site facilitates the cleavage of HA0 to HA1 and HA2 (required for infectivity) by proteases found
338 ubiquitously in the animal tissues [34]. Sequence analysis of the *wt* and ty-ad virus inocula confirmed a

339 single basic cleavage site typical of a LPAIV. Moreover, both viruses required TPCK trypsin for growth in
340 turkey cells (TEFs), affirming a LPAIV phenotype. Therefore, the expanded tropism of the ty-ad virus in
341 turkey tissues may be independent of the restriction imposed by the tissue localisation of proteases, which
342 typically restricts the tropism of most LPAIVs to mainly the respiratory and / or enteric tracts of chickens and
343 turkeys [34]. Proteolytic activation of LPAIV HA occurs extracellularly driven by airway proteases, the
344 trypsin-like serine proteases human airway trypsin-like protease (HAT) and transmembrane protease serine 2
345 (TMPRSS2), albeit a minority of viruses possess HA cleavage site motifs that are processed by other
346 proteases (reviewed by Kido, Okumura [34]). These 'atypical' viruses, including the 1918 influenza A virus
347 [35], can utilise proteases found ubiquitously in tissues of multiple organs for cleavage, and therefore can
348 potentially increase tissues dissemination and pathogenicity. For H9N2 infections, changes in the cleavage
349 site have been shown to alter the clinical outcome in turkeys [36]. The predominant proteases present in
350 other cell types and how they may differ in distribution in turkeys compared to chickens, along with which
351 proteases may differentially cleave the HA of the *wt* and ty-ad viruses, remain to be identified. However,
352 conserved expression and functionality of furin protease has been observed between chickens and ducks
353 [37]. Therefore, considering that turkeys and chickens are closely related, with ~90% genome identity [38], it
354 is unlikely that separate protease compositions exist between the two species. However, non-basic amino
355 acid variations in H9N2 viruses have been shown to bestow a HPAIV phenotype in turkeys compared to
356 chickens [36]. Although the L217Q substitution, present in the HA of the ty-ad virus, is not located near to
357 HA1/HA2 cleavage site, and is therefore unlikely to alter the protease preference.

358 The L217Q polymorphism has been previously shown, for various IAV strains including H7N9, to
359 change the receptor preference by increasing the affinity of haemagglutinin to α-2,6 SA [12-14]. The
360 A/Anhui/1/13 isolate being a human derived virus has a naturally high affinity to α2,6, but is also able to
361 bind α-2,3 SA with a relatively high affinity [14, 39-41]. Recent receptor preference analyses for H7N9 have
362 demonstrated that Q217 increases affinity to α2,3 SA [14]. Consequently, we investigated whether the
363 expanded tropism could be due to a greater density of α2,3 SA in turkey tissues. Previously we did not
364 observe any altered pathogenicity following ty-ad virus infection in chickens [24], we compared the receptor
365 distributions between turkey and chicken tissues. Using lectins which bind preferentially to α2,3 or α2,6 SA,

366 we found that turkeys have a high abundance of α2,3 SA in the nasal turbinates and trachea, comparatively
367 higher than those found in equivalent chicken tissues. The nasal turbinates and trachea are the major site of
368 H7N9 replication, and therefore might explain the rapid emergence of the L217Q polymorphism reported
369 previously [24]. However, analysis of the turkey visceral organs revealed comparatively lower α2,3 levels
370 than α2,6 SA as observed by others [42], suggesting that differences in receptor distribution alone are not
371 likely responsible for the increased tropism or pathogenicity observed. Receptor distribution in chicken and
372 turkey tissues performed by lectin binding served to assess the overall distribution of the α2,3 and α2,6
373 sialosides, and did not identify other receptor derivatives, such as those possessing specific modifications,
374 e.g. sulphated α2,3 sialosides which appear to be important as receptors for other AIVs [43, 44]. It has also
375 been recently reported that the abundance of sialic acid receptors does not necessarily correlate with the
376 preference shared by AIVs [45]. Therefore, a more detailed investigation into the glycome of different avian
377 species is required to fully explore this mechanism, and its possible role in effecting H7N9 LPAIV binding to
378 different turkey tissues, and how this may differ for the H7N9 wt and ty-ad LPAIV variants.

379 We also compared the replication kinetics of both the *wt* and ty-ad viruses in a range of avian and
380 mammalian cells. In turkey, chicken and duck primary cells the ty-ad virus showed a statistically increased
381 level of replication, compared to the *wt*. However, no significant differences in replication were observed in
382 either human, swine, or the highly sensitive and permissive MDCK (canine) cell line. Together, these
383 observations indicated that the ty-ad virus confers a generic replicative advantage in avian, but not
384 mammalian cells. This similar level of replication in mammalian cells with Q217 has also been previously
385 observed [14]. Clearly, multiple factors determine the replication kinetics of AIVs. Considering that the
386 polymorphisms in the ty-ad virus are outside the polymerase, the dynamism of vRNA replication is unlikely
387 to be a contributory factor. This observation is more likely due to different receptor utilisation, or possibly
388 related to a different pH of HA activation. The optimal pH of HA activation, or pH of fusion, is species
389 dependent, and pH has been demonstrated to alter replication kinetics in *ex vivo* organ cultures [46] and for
390 an H5N1 HPAIV isolate *in vivo* [47]. The Q217 slightly decreases the pH of fusion when assessed by
391 syncytia formation assays [14]. Interestingly, it has been suggested that AIVs tend to favour a lower pH of
392 fusion upon infection and circulation in turkeys, in comparison to those isolated from other avian hosts [48].

393 Another consideration, not explored in this study, is the differential host response induced by the *wt*
394 and ty-ad viruses. Indeed, turkeys appear to mount a different host response than chickens in response to H7
395 infection, particularly from genes involved in RNA metabolism and the innate immune response [33].
396 However, it is uncertain whether single amino acid changes in the HA and NA proteins would contribute
397 directly to different immune responses or enhance immune evasion of these viruses. The immune responses
398 to influenza A virus infection in different avian species, including different galliforme species, is
399 understudied and requires further investigation.

400 Alongside the Q217 polymorphism identified in the ty-ad virus, a threonine to isoleucine
401 polymorphism was also detected in the NA protein at residue position 10 (T10I). The function of this
402 mutation has not been documented in the literature, however, an isoleucine at this position is frequently
403 detected in a number of other NA subtypes. This polymorphism is located in the transmembrane domain of
404 NA, away from the functional site and stalk, as such, this polymorphism is unlikely to contribute to the
405 different pathogenicity observed across the two species.

406 Antigenic comparisons between viruses possessing either Q217 or L217 induced 23- and 8-fold
407 reductions in HAI titre with ferret and chicken antisera, respectively [49]. In this study we saw similar
408 antigenic differences between the ty-ad (Q217) and *wt* (L217) H7N9 viruses, reinforcing this finding.
409 However, the differential receptor binding of the L217Q mutation has been postulated to reduce the avidity
410 of binding to chicken red blood cells, thereby over exaggerating antigenic changes in HAI assays. Despite
411 the possible effect of different avidities, the L217Q polymorphism has previously been shown to emerge as
412 an antigenic 'escape' mutant upon incubation with H7N9 A/Anhui/1/13 antisera, indicating a genuine role of
413 Q217 in the HA antigenicity of H7N9 [49]. In addition, since 2017, China has implemented a mass
414 vaccination program of poultry against H7N9. This campaign has successfully reduced zoonotic cases yet
415 has not eradicated H7N9 from poultry in China. Genetic analysis of H7N9 viruses detected in poultry after
416 the introduction of the vaccine has highlighted an increased detection of Q217 in the sequenced HA gene
417 [10, 11]. The vaccine consists of the HA antigen derived from an A/Anhui/1/13-like candidate vaccine virus
418 [49]. As such, this work suggested that Q217 emergence through antigenic evolution may afford greater

419 pathogenic outcomes in some susceptible avian species, such as turkeys. Through *in vitro* characterisation,
420 Q217 appears to have reduced zoonotic ability [14]. However, in this study we observed that the ty-ad virus
421 was still capable of infecting ferrets, producing similar levels of vRNA in nasal wash samples, when
422 compared to that of H7N9 *wt* virus ferret infections conducted in the same animal facilities [22, 29]. This
423 observation suggested that while the ty-ad virus possess no greater zoonotic risk to *wt*, it still likely retains its
424 ability to infect humans. We also explored the potential for this virus to spread from infected ferrets to
425 chickens or turkeys, in an attempt to mimic reverse zoonotic transmission. No evidence of transmission to
426 naïve birds which shared the same airspace could be detected.

427 In conclusion, this study has demonstrated that the ty-ad virus emerged rapidly following infection
428 of turkeys, possibly due to high levels of α2,3 SA in the major sites of virus replication in turkeys. When this
429 adaptation occurred, it enabled the virus to replicate systemically in a wider range of organs, and at implied
430 higher titres. This enhanced tropism conferred increased pathogenesis and mortality to infected turkeys. The
431 enhanced tissue tropism was not due to any change in the ability of the virus to replicate without trypsin, or
432 due to any obviously different receptor distributions in the turkey organs. However, the adaptation did confer
433 a statistically significant increase in replication in avian cells, including turkey cells, but not mammalian
434 cells. The exact mechanism for the systemic tropism of ty-ad virus and its associated pathogenesis in turkeys
435 remains elusive. However, this work highlighted the requirement for continued surveillance of low
436 pathogenicity H7N9, particularly as vaccination programmes continue.

437

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445 **Author contribution**

446 M.J.S., A.H.S., S.M.B. and J.J. conceived and designed the studies. J.J., A.H.S., S.M., A.N., S.T., M.J.S.
447 performed the experimental work. J.J., S.M., S.T., M.J.S., A.C.B interpreted the data. J.J. and A.C.B, drafted
448 the manuscript. All authors reviewed and edited the manuscript.

449 **Conflict of interest**

450 The authors declare no competing interests.

451 **Ethical and safety statement**

452 All animal experiments and procedures required approval from the local APHA Animal Welfare and Ethical
453 Review Body to comply with the relevant European and UK legislation and were carried-out in accord with
454 the Home Office (UK) Project Licences 70/8332 and 70/8692. Any infected poultry which began to display
455 severe clinical signs were euthanised and were recorded as a mortality. UK regulations categorise the H7N9
456 LPAIV as a SAPO-4 and ACDP-3 pathogen because it is a notifiable animal disease agent and presents a
457 zoonotic risk. All laboratory and containment work with H7N9 specimens, including infected poultry, was
458 done in licenced BSL3 facilities.

459

460 **FIGURE LEGENDS**

461 **Fig. 1. Survival of turkeys infected with H7N9 *wt* and ty-ad virus variants.**

462 **A and C.** The survival rates of six turkeys (**A**) or chickens (**C**) directly inoculated (D0) (solid lines) with
463 $1x10^6$ $EID_{50}$ of the *wt* (blue lines) or ty-ad (red lines) virus variants. The survival of six turkeys (**A**) or
464 chickens (**C**) co-housed with either group at 1 day post infection (R1) (dashed lines) or six contact chickens

465 added at 4 dpi (dotted lines) is also shown. **B.** The survival rates of 10 turkeys inoculated intravenously with
466 $1x10^6$ $EID_{50}$ of *wt* (blue lines) or ty-ad (red lines) virus variants.

467 **Fig. 2. Viral RNA shedding from *wt* and ty-ad virus infected or contact turkeys.**

468 Viral RNA shedding profiles from turkeys directly inoculated with $1x10^6$ $EID_{50}$ with *wt* (blue lines) or ty-ad
469 (red lines) virus variants (D0) (**A & B**) or turkeys co-housed with either group at 1 day post infection (R1) (**C**
470 **& D**). Symbols show individual bird vRNA levels and lines indicate the mean vRNA levels for all live birds
471 in a given group, quantified as r.e.u. (see Methods for details). Viral RNA shedding was quantified from
472 swabs collected from the oropharyngeal (Op) (**A & C**) or cloacal (C) cavities (**B & D**). Shedding profiles
473 from individual birds were used to calculate area under the curve (AUC) and graphically displayed for the
474 directly infected (D0) (**E**) or contact (R1) (**F**) birds. One-way ANOVA with multiple analysis was performed
475 comparing the Op and C AUC shedding for both the D0 and R1 groups, ns (not significant) indicates P-value
476 >0.05.

477 **Fig. 3. Viral RNA shedding from *wt* and ty-ad virus infected or contact chickens.**

478 Viral RNA shedding profiles from chickens directly infected with $1x10^6$ $EID_{50}$ with *wt* (**A & B**) or ty-ad (**C**
479 **& D**) virus variants (D0) (black lines) or chickens co-housed with either group at 1 dpi (R1, red lines) or 4
480 dpi (R2, teal lines). The D0 chickens were removed prior to the addition of second contact (R2) chickens at 4
481 dpi to facilitate an extended transmission chain. Symbols relate to individual birds and lines indicate the
482 mean virus titre (r.e.u.), the pink shaded areas indicate the window of detectable viral RNA being shed from
483 the R1 chickens. Viral RNA shedding was quantified in from swabs collected from the oropharyngeal (Op)
484 (**A & C**) or cloacal (C) cavities (**B & D**).

485 **Fig. 4. Viral RNA levels in different organs of directly infected turkeys and chickens.**

486 Viral RNA was quantified in organs collected from turkeys (**A, B & C**) or chickens (**D & E**) directly infected
487 with $1x10^6$ $EID_{50}$ of the *wt* or ty-ad virus variants. Organs were taken from 2 birds in each group which were
488 culled at 4 dpi (**A & D**) or 6 dpi (**B & E**). Organs were taken from turkeys which were found dead or

489 euthanised following onset of severe clinical signs at 6 or 8 dpi (**C**). Detection of viral RNA is shown
490 graphically in a heat map ranging from zero to $1x10^9$ relative equivalency units (r.e.u.)**.**

491 **Fig. 5. Replication kinetics of *wt* and ty-ad virus variants in cells from different species.**

492 **A.** Representative well visualising plaques on turkey embryo fibroblasts (TEF) cells from three independent
493 replicates with the same dilution of wt, ty-ad or A/chicken/England/11406/2008 (H7N7 HPAIV) viruses in
494 the presence (+) (2µg/ml) or absence (-) of TPCK trypsin. Plaque assays were stained with crystal violet. **B-**
495 **G.** Virus replication of *wt* (blue) and the ty-ad (red) virus variants in chicken, turkey or duck embryo
496 fibroblasts (CEF, TEF, DEF), MDCK (canine), CACO2 (human), NPTr (swine) cells. Cells were infected at a
497 MOI of 0.01 with each virus and cell supernatant was harvested at 24, 48 or 72 hours post infection. Viral
498 titres were determined based by plaque forming units per ml ($PFU.ml^{-1}$) using plaque assay. Each time point
499 corresponds to the mean of four biological replicates with standard deviations indicated. Two-way ANOVA
500 with multiple analysis was performed comparing the two strains, * indicates P-value <0.05, ** indicates P-
501 value < 0.005, *** indicates P-value ≤ 0.001.

502 **Fig. 6. Zoonotic and reverse zoonotic infection dynamics for the ty-ad virus.**

503 Infection dynamics of the ty-ad virus in the ferret model and investigation of reserve zoonotic infection from
504 ferrets to chickens and turkeys. **A.** Virus titre estimates (r.e.u.) derived from interior (pink) or exterior (black)
505 nasal wash samples collected from six ferrets (three per block) directly infected intranasally with $1x10^6$
506 $EID_{50}$ of the ty-ad virus. One ferret per block (total of two ferrets) were culled at 6 dpi for IHC analysis.
507 Symbols show an individual ferret's vRNA levels in the washes, while continuous lines indicate the mean
508 vRNA levels. Yellow filled circles indicate the interior nasal wash samples for which sequencing of amino
509 acid position 217 in HA was performed and confirmed as Q217 (samples from five ferrets at four dpi and one
510 ferret at six dpi) **B.** HAIs were performed on sera taken from four directly inoculated ferrets at the end of the
511 experiment (14 dpi). Chickens (n=12) and turkeys (n=12) were similarly bled at 14 dpi, after having been
512 housed in the same air space, yet physically separated, from the infected ferrets for a 6 h period at 3 dpi. HAI
513 assays were performed using homologous H7N9 antigen.

514

515 **SUPPLEMENTARY FIGURE LEGENDS**

516 **Fig. S1. Percent weight change of turkeys infected (D0) with *wt* or ty-ad virus variants, or of turkeys**
517 **housed in contact (R1) with either group.**

518 Six turkeys (D0) were infected via the oronasal route with $1x10^6$ $EID_{50}$ or either *wt* or ty-ad virus variants.
519 Six further turkeys (R1) were co-housed with either group at 1 day post infection. All turkeys were weighted
520 daily, and percentage weight change was calculated from their original weight prior to infection.

521 **Fig. S2. Detection of vRNA in environmental drinking water and faecal samples following infection**
522 **and transmission of *wt* or ty-ad virus variants in turkeys.**

523 vRNA detection in drinking water (**A and C**) or faecal samples (**B and D**) collected from the environment of
524 infected or contact turkeys (**A and B**) or chickens (**C and D**) from **Fig 2** or **Fig 3** respectively.

525 **Fig. S3. Homologous H7 HAI titres from infected turkeys.**

526 HAI assays were performed on serum taken from chickens and turkeys direct infected with *wt* (total n=17;
527 n=8 chickens; n=9 turkeys) or ty-ad (total n=14; n=6 chickens; n=8 turkeys) viruses. Serum was collected
528 between 8- and 19-days post infection. The HAI assays were performed using homologous or heterologous
529 the *wt* and ty-ad viruses. Individual points are shown with lines connecting paired sera. Paired t-tests were
530 performed comparing the L217 and Q217 antigens. *** indicate p-value <0.001. ** indicate p-value <0.01.

531 **Fig. S4. Representative IHC images from two turkeys infected with *wt* or ty-ad H7N9 viruses.**

532 Representative IHC images showing the presence of influenza A virus Nuclear Protein (NP) antigen on the
533 indicated tissues collected from turkeys infected with *wt* or ty-ad H7N9 viruses. Tissues were collected from
534 birds (*wt*, bird E or ty-ad, bird G) which were culled at 6 dpi. NP labelling is shown in brown.

535 **Fig. S5. Representative images showing lectin staining for sialic acid receptor distribution in chicken**
536 **and turkey tissues.**

537 Representative images showing the staining of α2,3 (Mal II) and α2,6 (SNA) sialic acid using lectins.
538 Respective lectin staining of tissues collected from the nasal cavity of uninfected chickens and turkeys
539 shown in brown.

540 **Fig. S6. Representative IHC images from two ferrets infected with *wt* or ty-ad H7N9 viruses.**

541 Representative IHC images showing the presence of influenza A virus NP antigen in of the indicated tissues
542 collected from ferrets infected with the ty-ad H7N9 virus. Tissues were collected from ferrets which were
543 culled at 6 dpi. NP labelling is shown in brown.

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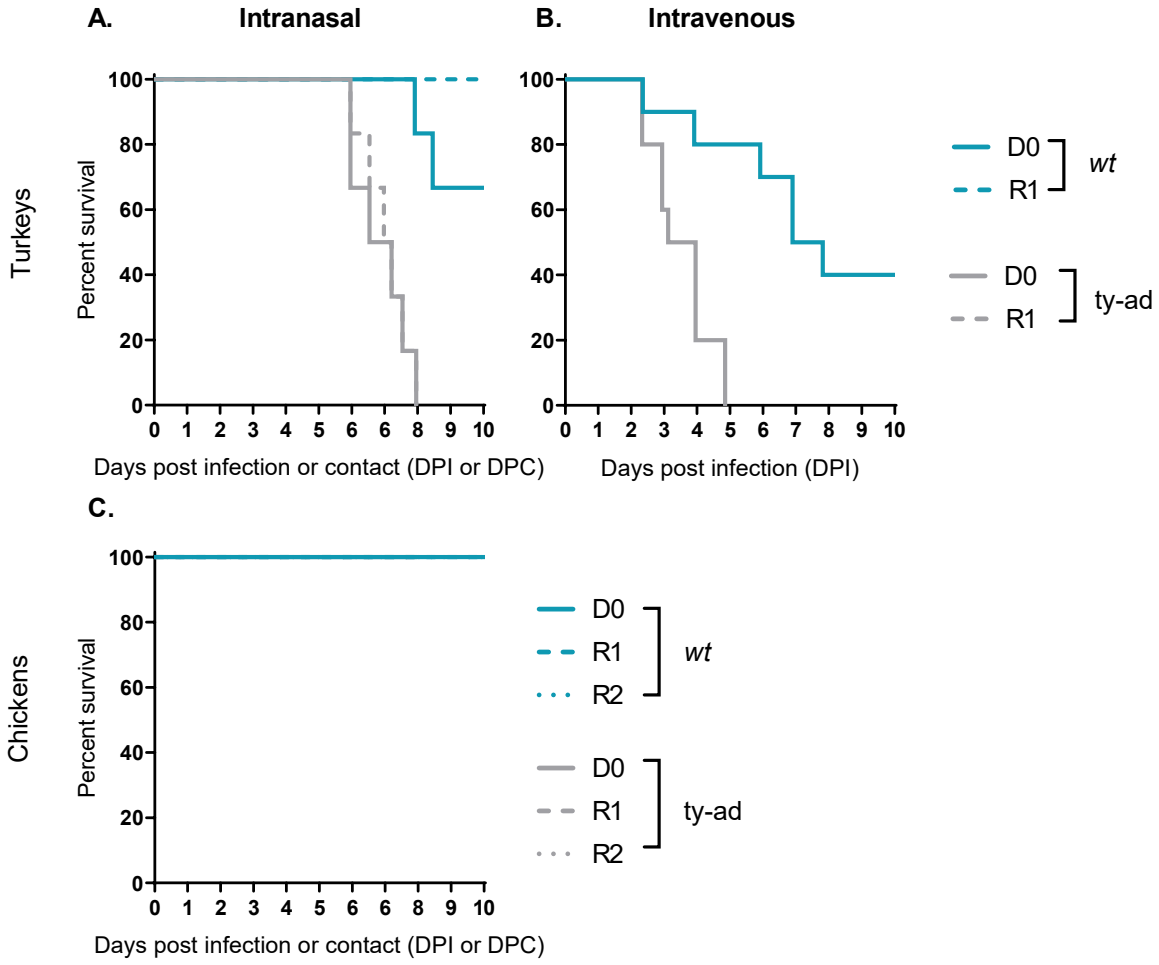
677

**Fig. 1**


A.
Intranasal
B.
Intravenous
Turkeys
Percent survival
100
80
60
40
20
0
0 1 2 3 4 5 6 6 8 9 10
Days post infection or contact (DPI or DPC)
0 1 2 3 4 5 6 7 8 9 10
Days post infection (DPI)
D0
R1
wt
D0
R1
ty-ad
C.
Chickens
Percent survival
Days post infection or contact (DPI or DPC)
D0
R1
R2
wt
D0
R1
R2
ty-ad

# Fig. 2

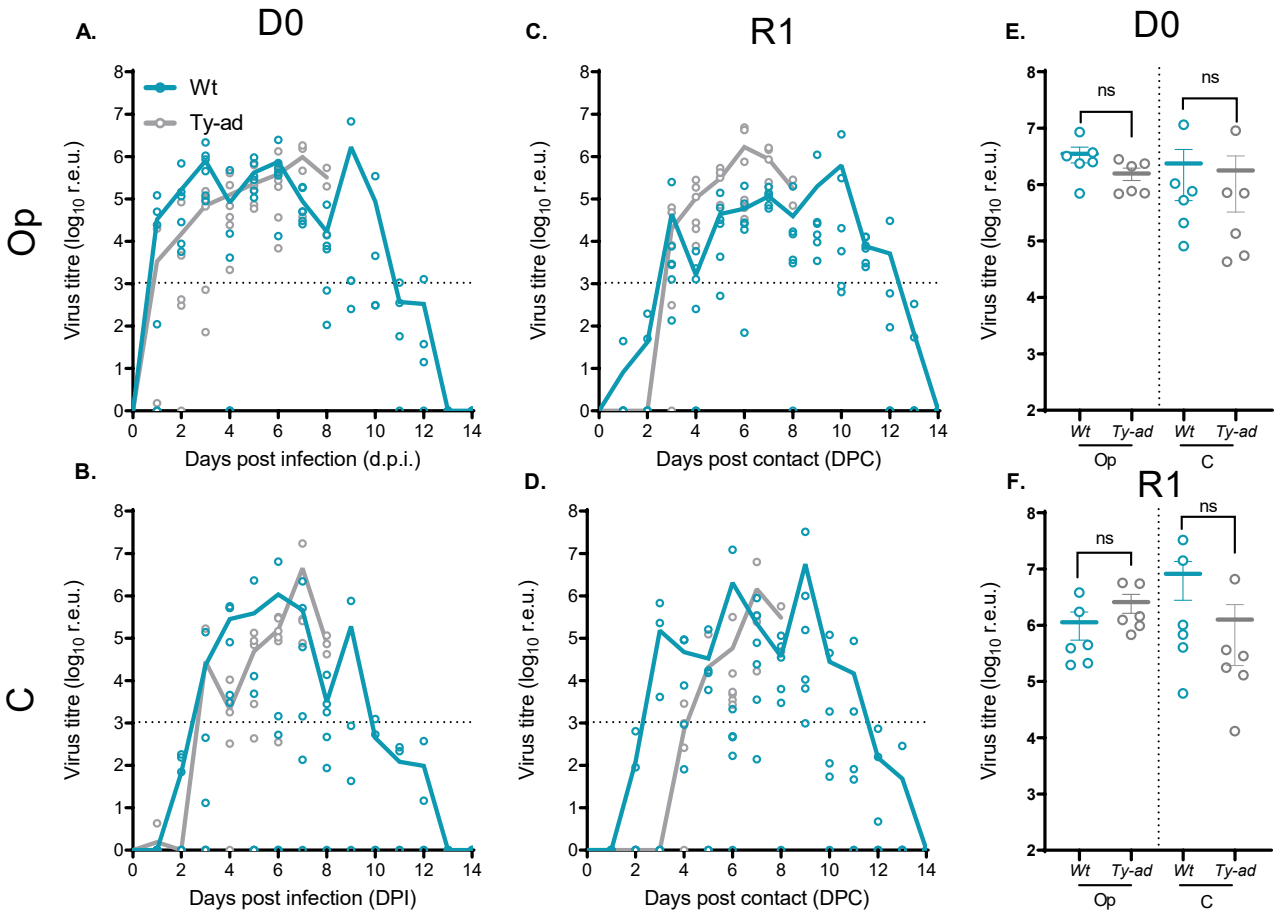

A.
D0
Wt
Ty-ad
Op
Virus titre (log10 r.e.u.)
Days post infection (d.p.i.)
C.
R1
Days post contact (DPC)
E.
D0
ns
ns
Wt
Ty-ad
Wt
Ty-ad
Op
C
B.
C
Days post infection (DPI)
D.
F.
R1

**Fig. 3**

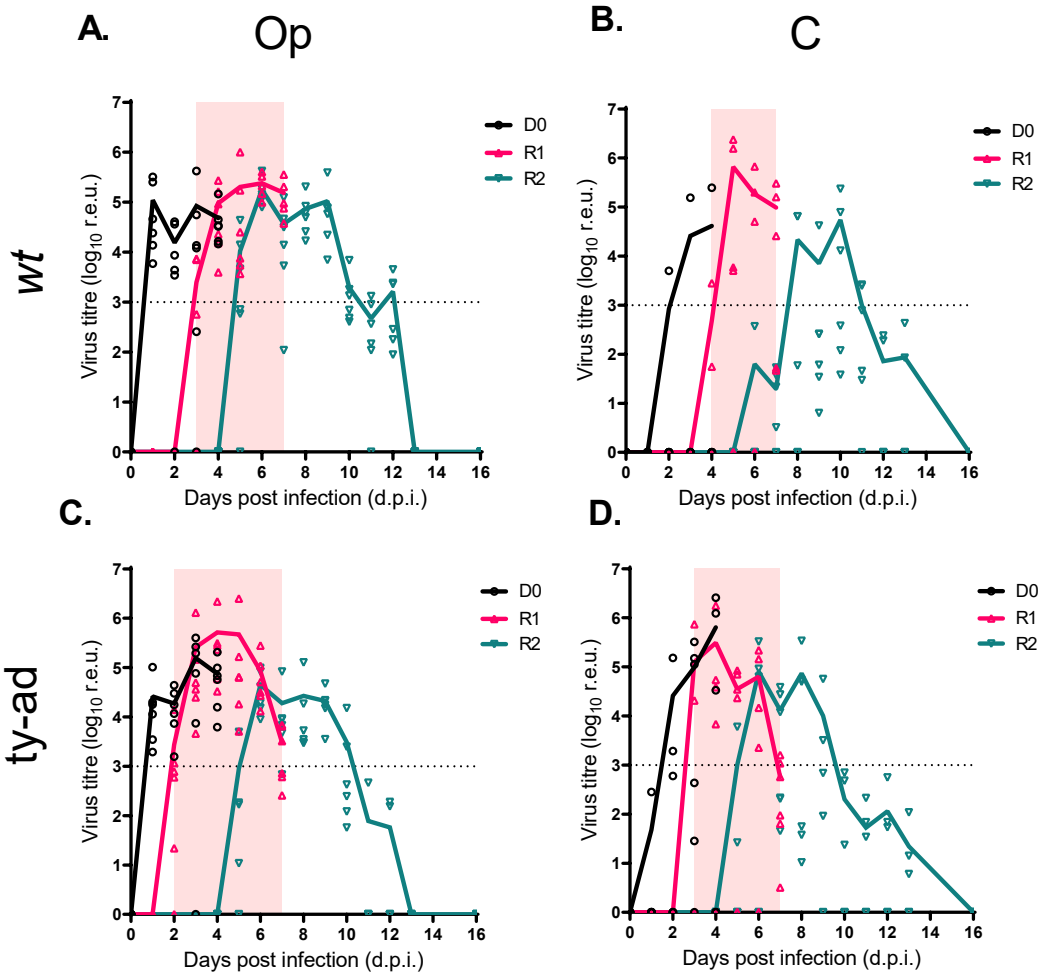
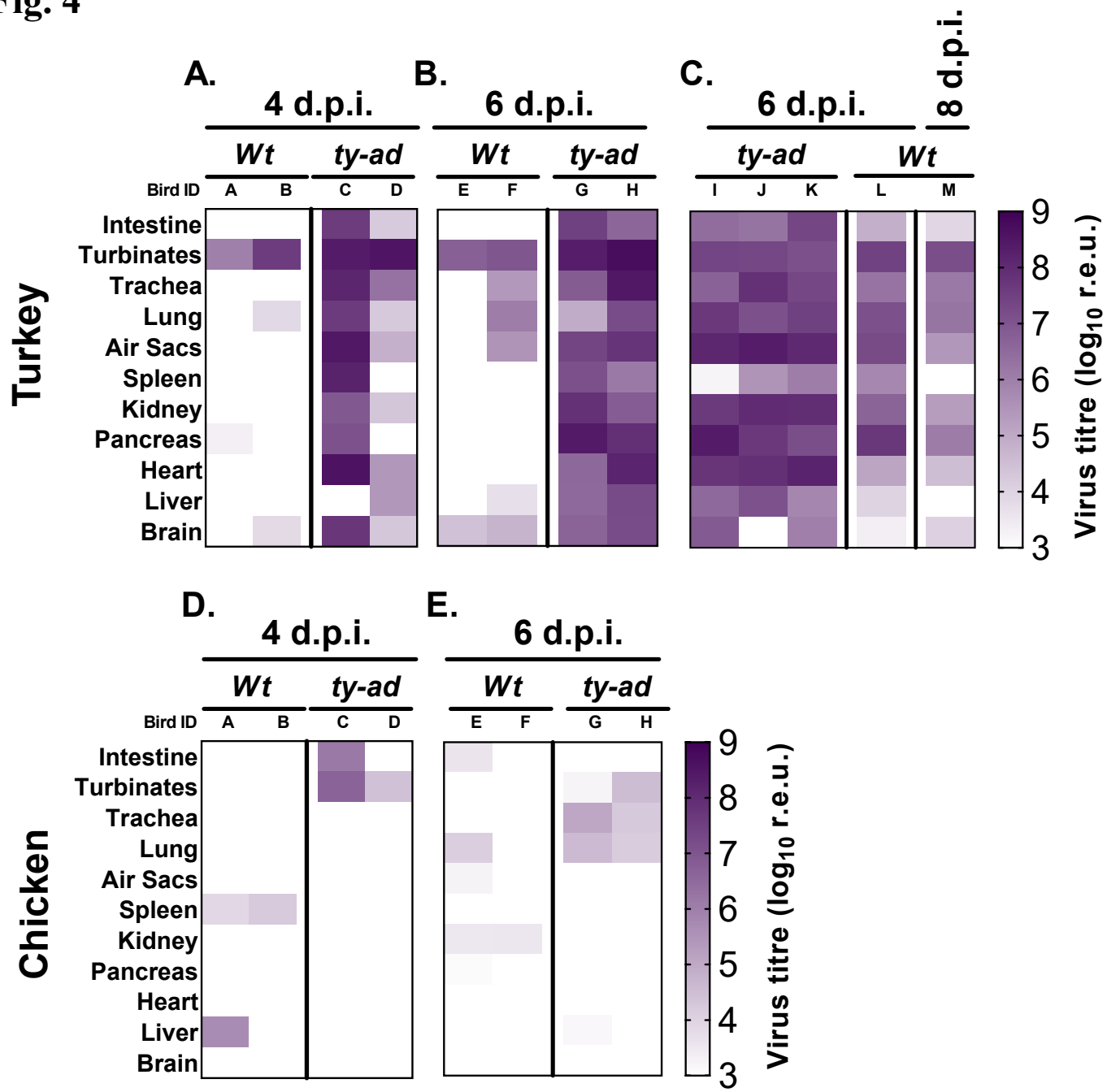

A.
Op
B.
C
C.
D.
wt
ty-ad
D0
R1
R2
Virus titre (log10 r.e.u.)
Days post infection (d.p.i.)

**Fig. 4**


A.
4 d.p.i.
B.
6 d.p.i.
C.
6 d.p.i.
8 d.p.i.
Wt
ty-ad
Wt
ty-ad
ty-ad
Wt
Bird ID
A B C D E F G H I J K L M
Turkey
Intestine
Turbinates
Trachea
Lung
Air Sacs
Spleen
Kidney
Pancreas
Heart
Liver
Brain
9
8
7
6
5
4
3
Virus titre (log10 r.e.u.)
D.
4 d.p.i.
E.
6 d.p.i.
Wt
ty-ad
Wt
ty-ad
Bird ID
A B C D E F G H
Chicken
Intestine
Turbinates
Trachea
Lung
Air Sacs
Spleen
Kidney
Pancreas
Heart
Liver
Brain
9
8
7
6
5
4
3
Virus titre (log10 r.e.u.)

# Fig. 5

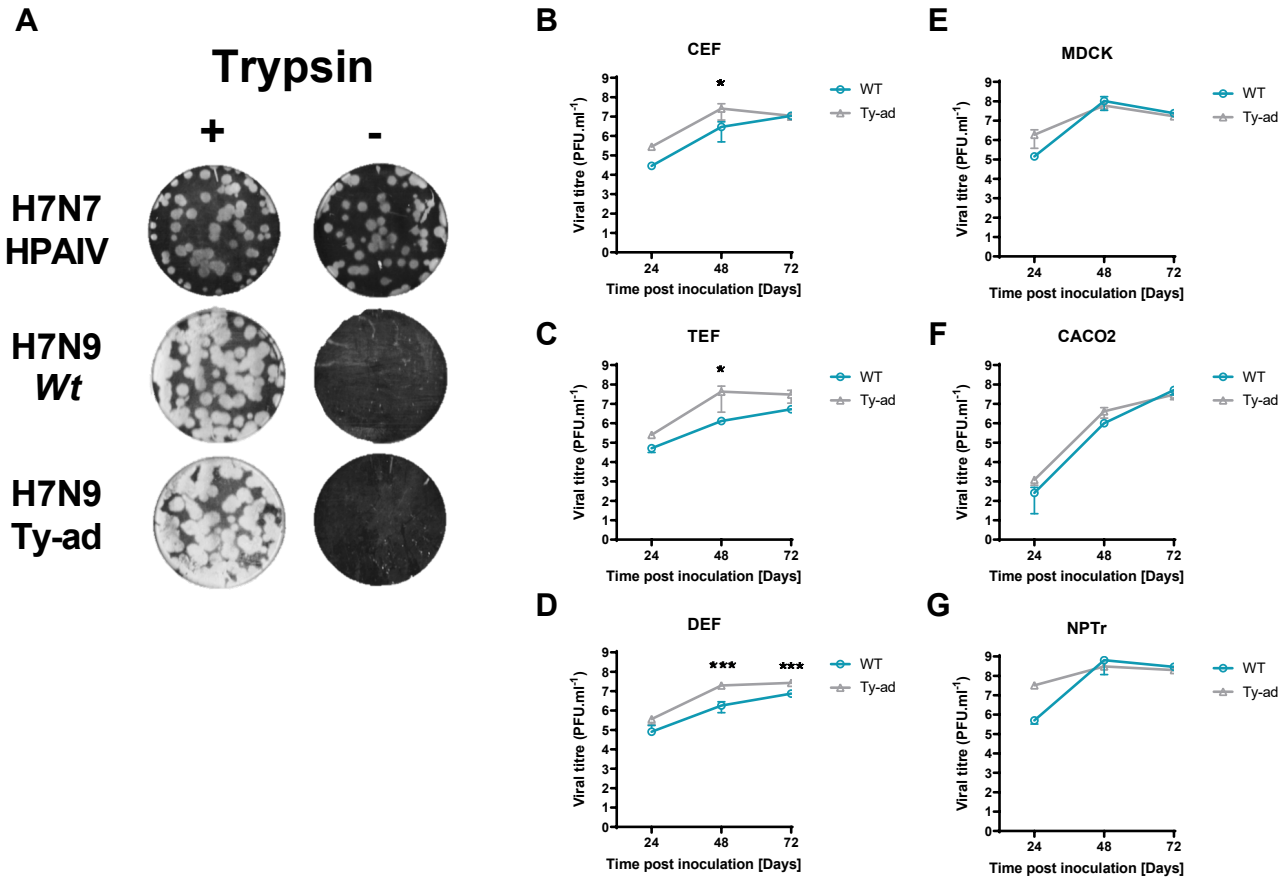
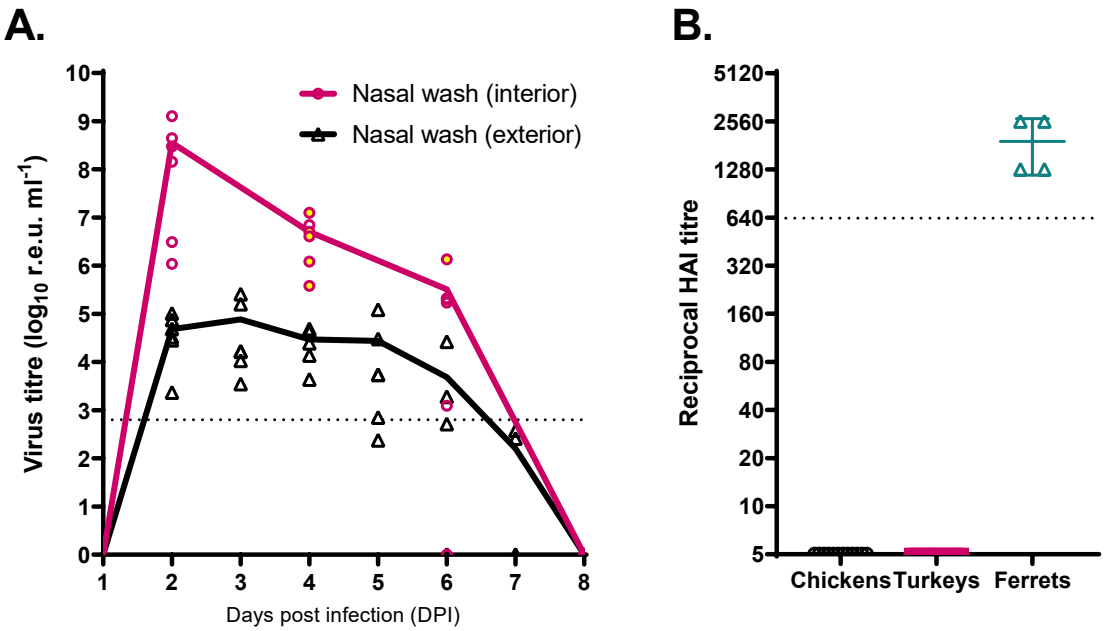

A
Trypsin
+
-
H7N7 HPAIV
H7N9 *Wt*
H7N9 Ty-ad
B
CEF
C
TEF
D
DEF
E
MDCK
F
CACO2
G
NPTr
WT
Ty-ad
Viral titre (PFU.ml$^{-1}$)
Time post inoculation [Days]
24
48
72
*
***

**Fig. 6**


A.
Nasal wash (interior)
Nasal wash (exterior)
Virus titre (log10 r.e.u. ml-1)
Days post infection (DPI)
B.
Reciprocal HAI titre
Chickens
Turkeys
Ferrets

# Table 1

**Table 1.** α2,3 and α2,6 linked sialic acid distribution in chicken and turkey tissues

| Lectin (receptor preference) | Tissue | Chicken | | | Turkey | | | Average Chicken | Average Turkey |
|---|---|---|---|---|---|---|---|---|---|
| | | A | B | C | A | B | C | | |
| MAL II (α2,3) | Nasal cavity | +/- | + | + | ++ | ++ | ++ | + | ++ |
| | Trachea | +/- | + | + | ++ | ++ | ++ | + | ++ |
| | Lung | + | + | + | + | + | + | + | + |
| | Pancreas | + | + | + | + | + | + | + | + |
| | Kidney | ++ | ++ | ++ | ++ | ++ | ++ | ++ | ++ |
| SNA (α2,6) | Nasal cavity | + | ++ | + | +/- | + | + | + | + |
| | Trachea | + | ++ | ++ | + | ++ | + | ++ | + |
| | Lung | + | + | + | + | + | + | + | + |
| | Pancreas | +/- | + | + | ++ | + | + | + | + |
| | Kidney | + | + | + | ++ | ++ | ++ | + | ++ |

Semi-quantitative scoring was performed in blinded fashion; -, absent; +/-, minimal; +, mild; ++, moderate