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Contribution of segment 3 to the acquisition of virulence in contemporary H9N2 avian influenza viruses

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4   Running title: H9N2 PA mutations and virulence

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14   Abstract word count: 243

15   Main body word count: 6083

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18   **Abstract**

19   H9N2 avian influenza viruses circulate in poultry throughout much of Asia, the Middle

20   East and Africa. These viruses cause huge economic damage to poultry production systems

21   and pose a zoonotic threat both in their own right as well as in the generation of novel

22   zoonotic viruses, for example, H7N9. In recent years it has been observed that H9N2 viruses

23   have further adapted to gallinaceous poultry, becoming more highly transmissible and

24   causing higher morbidity and mortality. Here, we investigate the molecular basis for this

25   increased virulence, comparing a virus from the 1990s and a contemporary field strain. The

26   modern virus replicated to higher titres in various systems and this difference mapped to a

27   single amino acid polymorphism at position 26 of the endonuclease domain shared by the

28   PA and PA-X proteins. This change was responsible for increased replication and higher

29   morbidity and mortality rates along with extended tissue tropism seen in chickens. Although

30   the PA K26E change correlated with increased host cell shutoff activity of the PA-X protein *in*

31   *vitro*, it could not be overridden by frameshift site mutations that block PA-X expression and

32   therefore increased PA-X activity could not explain the differences in replication phenotype.

33   Instead, this indicates these differences are due to subtle effects on PA function. This work

34   gives insight into the ongoing evolution and poultry adaptation of H9N2 and other avian

35   influenza viruses and helps us understand the striking morbidity and mortality rates in the

36   field, as well as rapidly expanding geographical range seen in these viruses.

37

Importance

Avian influenza viruses, such as H9N2, cause huge economic damage to poultry production worldwide and are additionally considered potential pandemic threats. Understanding how these viruses evolve in their natural hosts is key to effective control strategies. In the Middle East and South Asia an older H9N2 virus strain has been replaced by a new reassortant strain with greater fitness. Here we take representative viruses and investigate the genetic basis for this 'fitness'. A single mutation in the virus was responsible for greater fitness, enabling high growth of the contemporary H9N2 virus in cells, as well as in chickens. The genetic mutation that modulates this change is within the viral PA protein, a part of the virus polymerase gene that contributes in viral replication as well as contribute in the virus accessory functions – however, we find that the fitness effect is specifically due to changes in the protein polymerase activity.

Introduction

Influenza A viruses possess a segmented, negative-sense RNA genome which is transcribed and replicated by a tripartite RNA-dependent RNA polymerase (RdRp) composed of the subunits PB2, PB1 and PA. Due to the segmented nature of its genome, influenza viruses can readily swap genes when two virus strains co-infect a single cell, in a process known as reassortment. Reassortment can result in generation of viruses with increased (1) or reduced viral fitness (2).

H9N2 avian influenza viruses (AIVs) are low pathogenicity avian influenza (LPAI) viruses that are enzootic in poultry in many countries across Asia, Africa and the Middle East (3-6). In afflicted countries they cause a constant burden on poultry production systems through

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60   mortality often associated with co-infection, or morbidity that leads to reduced egg

61   production and bird growth rates (7-9). They also pose a zoonotic risk as evidenced by over

62   60 confirmed cases of human infection, with over half of those occurring since 2015 (6) .

63   Due to their extensive geographical range H9N2 AIVs often co-circulate with other AIV

64   subtypes resulting in frequent reassortment events (10). Several viruses have emerged in

65   recent years which contain the internal gene cassette derived from H9N2 AIVs including an

66   avian-origin H7N9 virus which has caused human infections in China. H7N9 possesses the

67   polymerase genes from an enzootic co-circulating H9N2 strain (11, 12). Novel genotypes of

68   H9N2 AIV have also emerged in poultry due to co-circulation and reassortment with local

69   highly pathogenic avian influenza virus strains; we have previously described G1-lineage

70   H9N2 viruses in Pakistan that possess the NS gene segments from H7N3 or H5N1 strains and

71   the polymerase genes from other Indian/Middle East lineage H9N2 viruses (13). These

72   reassortants have replaced previously circulating genotypes of the G1-lineage H9N2 AIVs,

73   and are now the predominant genotype across the Indian subcontinent and Middle East,

74   and display enhanced morbidity and mortality in the field (14-16).

75   The molecular basis for the increased pathogenicity of contemporary reassortant H9N2

76   AIVs has yet to be established. Thus, we set out to understand which genes are responsible

77   for the enhanced virulence of these H9N2 viruses in poultry. We created a panel of reverse

78   genetics reassortants between a pair of G1-lineage viruses A/guinea fowl/Hong

79   Kong/WF10/1999 (WF10), a virus representing G1-lineage viruses circulating in the late

80   1990s, and A/chicken/Pakistan/UDL-01/2008 (UDL-01), representative of novel G1-lineage

81   reassortant H9N2 viruses. UDL-01 contains the HA, NA, NP and M genes related to

82   previously circulating enzootic G1-lineage H9N2 viruses in the region, the polymerase gene

83   cassette from different G1-lineage H9N2 viruses, more predominant in the Middle East, and

84   the NS gene segments from an HPAIV H7N3 (13).

85   In this study we find that the contemporary H9N2 virus, UDL-01, showed an enhanced

86   replication phenotype *in vitro* when compared to ancestral H9N2 WF10 virus. This

87   phenotypic difference mapped to a single amino acid residue in the PA endonuclease

88   domain (position 26) within segment 3. This single residue also determined the replicative

89   fitness and virulence of the virus *in vivo* and was further shown to modulate the activity of

90   PA in a PA-X independent manner.

91   **Results**

92   **Differences in plaque phenotype between two H9N2 AIV strains maps to the N-**

93   **terminal half of segment 3**

94   We generated a panel of reciprocal reassortant viruses between the full reverse

95   genetics systems of WF10, a virus representing G1-lineage H9N2 AIVs that circulated in the

96   late 1990s, and UDL-01, representative of a novel reassortant G1-lineage H9N2 with genes

97   from several previously enzootic G1-lineage H9N2 viruses and HPAIV H7N3 viruses (see

98   Figure 1A and B for phylogenetic trees of HA and PA genes). Wild-type (WT) WF10 virus

99   generated small hazy plaques in MDCK cells whereas WT UDL-01 generated significantly

100   larger, clearer plaques (Figure 1C, D). We tested the plaque phenotype of all reassortants

101   and identified segment 3 as capable of reciprocating plaque phenotype between WF10 and

102   UDL-01 (Figure 1C, D; data for non-segment 3 reassortants not shown). UDL-01 virus

103   containing segment 3 of WF10 presented significantly smaller plaques, while reassortant

104   WF10 presented significantly larger plaques, relative to WT viruses (Figure 1C, D).

105   To identify the region of segment 3 responsible for this alteration, two chimeric

106   segments 3s were generated by Gibson assembly: one chimera encoded the N-terminus

107   (amino acids 1-367) from PA of UDL-01 and the C-terminus (amino acids 368-716) of WF10

108   (U/W), while the other was *vice versa* (W/U). These chimeric segments were rescued by

109   reverse genetics in the background of UDL-01 and WF10 viruses. Determination of the

110   plaque phenotypes showed that, regardless of the rest of the virus genes, viruses containing

111   a UDL-01 PA N-terminal coding region had significantly larger plaques that those without

112   (Figure 1D, E). Furthermore, UDL-01 virus, which typically presents a large plaque

113   phenotype, presented significantly smaller plaques when given a WF10 N-terminus (Figure

114   1D, E). These results show that the small plaque phenotype could be mapped specifically to

115   the N-terminal half of WF10 PA.

116   **Amino acid residue 26 in PA modulates plaque phenotype**

117   PA is composed of two major domains, an N-terminal endonuclease (endo) domain and

118   a C-terminal domain (PA-C) connected by a linker region (17). The PA endo domain is a

119   flexible appendage that hangs away from the catalytic core of the RdRp and is involved in

120   cleaving host capped RNAs to be fed into the RdRp active site to be used as primers for viral

121   transcription. The PA-C domain is packed close to PB1 and makes up part of the catalytic

122   core of the viral RdRp (18).

123   The first 191 amino acids of PA, incorporating the endo domain, are also shared with

124   the accessory protein PA-X. PA-X is expressed due to a ribosomal frame shift site in PA,

125   during segment 3 translation a small proportion of ribosomes, when encountering a rare

126   tRNA codon slip into the +2 open reading frame (ORF) of segment 3 and express a fusion

127   protein comprised of the PA endo domain and an X-ORF from the +2 reading frame (19, 20).

128   PA-X dampens the innate immune response though its host cell shutoff activity, mediated

129   through degradation of cellular mRNAs and disruption of mRNA processing machinery (19,

130   21). The evidence for PA-X playing a role as a virulence factor in avian influenza viruses is

131   unclear, with several studies showing either attenuation or promotion of virulence *in vivo*

132   (22-26).

133   To identify amino acid substitutions responsible for modulating virus plaque

134   phenotype, we compared amino acids in the N-terminal half of PA between UDL-01 and

135   WF10, as well as between the X-ORFs of PA-X. We identified a total of twenty amino acid

136   differences between PA and 2 unique to the X-ORF (Table 1). Mapping the residues onto the

137   crystal structure of influenza PA within the context of the polymerase trimer bound to vRNA

138   showed that they lay in the PA-endo domain, the linker region and at the N-terminus of the

139   PA-C domain (27) (Figure 1F, Table 1). This enabled us to speculate if any of the

140   substitutions had a direct effect due to their proximity to known functional regions.

141   Substitution I118T is specifically located within the endonuclease active site, while A20T,

142   E26K and I100V/D101E lie proximal to the active site and could potentially interfere with

143   endonuclease activity. Therefore, these mutations, alongside the X-ORF polymorphisms and

144   several other mutants at residues shown previously to modulate polymerase activity (28),

145   were selected for further testing.

146   A panel of viruses was made carrying reciprocal single amino acid substitutions at the

147   sites identified and viruses were rescued using reverse genetics. Within the UDL-01 panel of

148   PA mutant viruses, two mutants gave significantly smaller plaque sizes than UDL-01 WT: the

149   E26K mutant produced comparably-sized plaques to that of the WF10 WT virus (Figure

150   2A,B,C), while the double mutant I100V/E101D also had a small plaque phenotype, though

151   less markedly so than E26K (Figure 2C). Within the WF10 panel of viruses, the most striking

152   visual difference was caused by the introduction of K26E, which facilitated significant larger

153   plaque diameters (Figure 2B, C). D316G also gave a heterogenous but significantly larger

154   average plaque size than WT WF10. Mutations at position 26 were the only viruses to give

155   reciprocal plaque size phenotypes in both viral backgrounds, strongly suggesting that this

156   position is key to the phenotype.

157   **Residue 26 in PA modulates virus replication kinetics**

158   For influenza viruses, small plaque phenotypes are often used as a marker of poor virus

159   replication, thus we further investigated this phenotype by performing multiple cycle

160   replication kinetics experiments with the position 26 mutants.

161   In MDCK cells infected at a low MOI, by later time points (36 hours and after) UDL-01

162   WT clearly grew to higher titres than WF10 WT, consistent with the plaque assay

163   phenotypes (Figure 3A). UDL-01 E26K showed slightly attenuated growth compared to UDL-

164   01 WT while WF10 K26E showed slightly enhanced titres compared to WT WF10, which

165   were significantly different at the 48 and 72 hour time points (Figure 3A). Thus, PA residue

166   26 had significant reciprocal effects on the replication of UDL-01 and WF10 viruses in MDCK

167   cells, recapitulating the differences seen for the MDCK plaque size phenotype.

168   To test if the effect of PA residue 26 amino acid substitutions held true in more

169   biologically relevant avian systems, viral replication kinetics were assessed in primary

170   chicken kidney (CK) cells and embryonated chicken eggs (Figure 3B, C). In CK cells there

171   were consistent differences between the replication kinetics of the viruses similar to that

172   seen in MDCKs; viruses with PA 26E (UDL-01 WT and WF10 K26E) reached peak titres at 24

173   hours post-infection, while 26K-containing viruses replicated at a slower rate, achieving

174   maximum titres at 48 and 72 hour time points (Figure 3B). UDL-01 E26K trended towards

175   lower titres than UDL-01 WT, which was significant at 24 hours post-infection. Likewise,

176   WF10 K26E generally showed enhanced titres compared to WF10 WT, which was significant

177   at 8 hours post-infection.

178   In embryonated eggs, as in MDCK cells and CK cells, UDL-01 E26K showed attenuated

179   growth compared to UDL-01 WT, significantly so at 12 and 24 hours post-infection, while

180   WF10 K26E showed enhanced growth compared to WF10 WT, significantly at 12 hours post-

181   infection (Figure 3C). Considering these data together, we can conclude that amino acid

182   substitutions at position 26 of PA within WF10 and UDL-01 H9N2 AIVs significantly altered

183   the replication of the viruses in both mammalian cell lines, and avian systems indicating this

184   attenuation is not host-dependent.

185   **Impact of PA amino acid substitutions on polymerase activity**

186   As an integral part of the trimeric polymerase, influenza PA mutations have previously

187   been shown to impact polymerase activity due to its position within the heterotrimeric

188   polymerase complex (*e.g.* (29)). To investigate the role of K26E, as well as the other

189   mutations tested here on polymerase activity, minireplicon assays were performed in

190   chicken DF-1 cells. Cells were transfected with expression plasmids for either the UDL-01 or

191   WF10 polymerase components plus NP and a vRNA-reporter encoding luciferase under the

192   control of the avian RNA polymerase I promotor. No significant differences were seen

193   between the activities of polymerase complexes containing UDL-01 or WF10 WT

194   polymerases (Figure 3D). However, in contrast to the virus replication assays, UDL-01 E26K

195   showed a small (~ 2-fold) but significant increase in polymerase activity compared to UDL-01

196   WT. There were no further significant differences seen with any of the UDL-01 or WF10

197   mutants when compared to the activity of the relevant WT control, including the reciprocal

198   K26E change in WF10. Polymerase activity was also measured in mammalian 293T cells, but

199   no significant differences were seen (Figure 3E). These data suggest that the differences in

200   plaque phenotype observed between WF10 (progenitor) and UDL-01 (reassortant) H9N2

201   AIVs was not unambiguously related to the segment 3s ability to support polymerase

202   activity alone.

203   **PA-E26K attenuates virus replication and pathogenicity *in vivo***

204   The WF10-like K26E mutation appears to lead to an attenuated replication phenotype

205   for UDL-01 *in vitro*, *ex vivo* and *in ovo*, therefore we decided to assess the ability of UDL-01

206   E26K within the natural chicken host. Two groups of chickens were inoculated with either

207   UDL-01 WT or UDL-01 E26K virus and viral shedding, transmissibility, tissue tropism and

208   clinical signs were assessed. In both infected groups all directly inoculated birds shed virus

209   robustly into the buccal cavity, peaking early in infection (day 1 or 2) and then declining over

210   the subsequent days (Figure 4A). However, the UDL-01 E26K virus was shed in significantly

211   lower amounts and was cleared sooner; no swabs were found positive for infectious virus by

212   day 5 in the E26K group compared to day 7 for the WT group (Figure 4A). When the area

213   under the shedding curves (AUC) were calculated to assess the total virus shed throughout

214   the study period, birds directly infected with UDL-01 WT virus showed almost a ten-fold

215   increased AUC compared to birds infected with the mutant UDL-01 E26K virus (215,517

216   versus 23,886). Therefore, the mutant UDL-01 E26K virus showed a reduced total shedding

217   throughout the study by birds directly infected with virus.

218   Contact birds were introduced into each group 1 day post-inoculation. All contact birds

219   in both groups tested positive for infectious virus from the buccal cavity by 1 day post-

220   exposure indicating robust contact transmission for both viruses (Figure 4B). A significant

221   reduction in buccal shedding was seen in contact birds exposed to UDL-01 E26K compared

222   to UDL-01 WT from day 1 through to 4 post-exposure; however, the delayed clearance of

223   the mutant virus was not seen in the contact bird group, with both groups of birds clearing

224   virus by day 6 post-exposure and similar levels of virus being shed on day five post-exposure

225   (Figure 4B). When the AUC was calculated to assess the total virus shed throughout the

226   study period, contact birds infected with UDL-01 WT virus again showed around a ten-fold

227   greater AUC compared to birds infected with the mutant UDL-01 E26K virus (187,915 versus

228   17,367).

229   Cloacal swabs from directly infected and contact birds were also analysed, shedding

230   was sporadic with not all birds yielding detectable infectious virus (Figure 4C). In total, six

231   UDL-01 WT and five E26K directly infected birds shed detectable virus along with a single

232   contact bird from each group. More birds shed virus on consecutive days in the WT group

233   than the E26K group (4 birds versus 1). This sporadic and low level virus shedding is

234   commonly seen for some AIV subtypes including the UDL-01 virus (30-33).

235   Clinical signs throughout the study were generally mild and the majority of birds

236   showed diarrhoea with listlessness as expected from previous reports of H9N2 infection,

237   including UDL-01 H9N2 (30, 33). However, between days three to six post-inoculation 33%

238   of birds (30% directly infected and 37.5% of contact birds) within the UDL-01 WT infected

239   group died (either spontaneously or due to reaching humane end points and being culled)

240   despite UDL-01 being classified as a LPAIV (Figure 4D); analysis of these survival curves

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241   showed a statistically significant difference (p <0.0001). UDL-01 has previously shown to

242   cause high levels of morbidity as well as occasionally low levels of mortality in

243   experimentally infected animals of certain chicken lines (30, 33). Birds infected with mutant

244   UDL-01 E26K showed no mortality, indicating this single mutation clearly attenuates the

245   virus for pathogenicity and mortality.

246

247   **Tropism of virus in infected chickens**

248   To determine whether the UDL-01 E26K mutation lead to any alteration in tropism,

249   tissues were taken from directly infected birds on days 1 and 3 post-inoculation. RNA

250   extracted from tissue samples was used for qRT-PCR reactions to detect the viral M gene

251   vRNA as a marker for presence of virus within tissues; which we have previously shown

252   correlates well with tissue infectious virus titres (30). At both time points, M gene copy

253   number was highest in the nasal and tracheal tissues but was also readily detectable within

254   the lung, colon, kidney, and spleen and intermittently detected in the liver (at least within

255   UDL-01 WT infected birds; Figure 4E, F). Overall, RNA copies were variable between days,

256   and between different birds, but levels were highest on day 3 post-inoculation, particularly

257   within the visceral organs. Within these animals, UDL-01 WT virus was consistently present

258   at higher levels in a number of tissues compared to birds infected with the mutant UDL-01

259   E26K virus. On day 1 post-inoculation there was significantly higher levels of RNA within the

260   lung, kidney, spleen and liver of the UDL-01 WT, compared to UDL-01 E26K infected birds

261   (Figure 4E). On day 3, UDL-01 E26K mutant virus was mostly undetectable in the visceral

262   organs, but detectable within the nasal tissue where levels remained high (10^4 to 10^5 copies

263   of viral M gene). The lung, colon and kidneys also showed significantly higher levels of UDL-

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264 01 WT RNA compared to the mutant UDL-01 E26K (Figure 4F). This suggested that although

265 the UDL-01 E26K virus was able replicate efficiently in the upper respiratory tract, it was less

266 able to disseminate through the bird and was more rapidly cleared. The expanded tissue

267 tropism of the UDL-01 WT virus likely explains the production of some mortality in this

268 experimental study.

269 **Polymorphisms at position 26 affect the host shutoff activity of the accessory protein**

270 **PA-X**

271 As we observed little or no difference in polymerase activity with the reciprocal

272 mutants at position 26 we hypothesized that the difference in replication could be due to

273 WF10 having poor PA-X activity. To test this, a previously described β-galactosidase (β-gal)

274 reporter assay was used to test the ability of the PA-X proteins from these viruses to cause

275 host cell shutoff (19, 34). Briefly, cells were co-transfected with expression plasmids

276 containing the different segment 3s along with a β-gal reporter plasmid, followed by

277 enzymatic readout of β-gal activity to give a measure of host gene expression in the

278 transfected cells and thus the ability of the different segment 3 plasmids to cause host

279 shutoff. Previous work has suggested that the majority of influenza host cell shutoff comes

280 from expression of PA-X rather than PA (19, 34, 35).

281 Mammalian 293T cells were transfected with plasmids with or without mutations in the

282 shared PA/PA-X endo domain or PA-X X-ORF and β-gal activity was measured. All data were

283 normalized to a control where segment 3 was substituted for an empty vector. UDL-01 WT

284 segment 3 significantly reduced levels of β-gal compared to the empty vector control

285 indicating robust shutoff activity (Figure 5A), as shown previously (34). In contrast WF10 WT

286 segment 3 displayed no detectable shutoff, giving equivalent β-gal signal to the empty

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287   vector control. When the reciprocal mutants were tested, only mutations at position 26 had

288   a reciprocal effect, significantly removing shutoff activity in a UDL-01 background and

289   causing shutoff activity in the WF10 background. In the UDL-01 background I118T

290   additionally showed significantly reduced shutoff activity but a reciprocal effect was not

291   seen in WF10. In the WF10 background the X-ORF mutation X-L221R also significantly

292   increased host shutoff activity. All other mutations showed the same phenotypes as their

293   respective WT segment 3s.

294   The host shutoff assay was also performed in avian DF-1 cells for the mutants at

295   positions 26, 118 and X-221 (Figure 5B). Although not significant, the change at position 26

296   trended towards switching the two segment 3s phenotypes – removing shutoff activity from

297   UDL-01 and introducing the activity into WF10 segment 3. Again UDL-01 I118T removed

298   shutoff activity and WF10 X-L221R partially introduced shutoff activity, but as in 293Ts,

299   these effects were not seen reciprocally.

300   To assess shutoff activity further, in the context of viral infection rather than

301   transfection and overexpression, we performed both radioactive and non-radioactive

302   metabolic labelling experiments. To test the shutoff activity in avian cells, primary chicken

303   embryonic fibroblast (CEF) cells were infected with a high MOI of virus containing mutations

304   in segment 3 and were subsequently pulsed with ^{35}S methionine then lysed and run on SDS-

305   PAGE. Autoradiography was performed and densitometry used to measure the abundance

306   of the highly abundant host protein, actin. In accordance with the reporter assays, UDL-01

307   WT virus showed efficient host shutoff with <10% of the levels of actin expressed in the

308   mock infected cells, whereas WF10 WT virus resulted in poor shutoff activity (>50% actin

309   expressed versus mock; Figure 5C, D). Reciprocal mutants at position 26 were the only

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310   mutants tested that showed any significant effect; UDL-01 E26K showed significantly poorer

311   host shutoff while WF10 K26E showed significantly more robust shutoff. Finally, a similar

312   experiment was performed in MDCK cells using the non-radioactive method of puromycin

313   pulsing and looking for levels of puromycinylated proteins in cell lysates (36). MDCKs were

314   either infected with WT viruses or the position 26 mutants. Levels of puromycinylated

315   products were then detected by western blot and quantified in the ~ 50 – 80 kDa range,

316   where no novel products likely corresponding to viral polypeptides were visible. As with the

317   previous assays, UDL-01 WT gave robust shutoff while WF10 WT gave poor shutoff which

318   could be switched, significantly, upon the introduction of the reciprocal mutations at

319   position 26 (Figure 5E, F).

320   Overall, these results show that UDL-01 has a PA-X capable of causing robust host

321   shutoff in avian and mammalian cells while WF10 does not, and the reason for this

322   difference maps to the identity of the amino acid at position 26.

323   **Differences in PA-X alone are not responsible for the attenuation of WF10 compared**

324   **to UDL-01**

325   The changes at PA position 26 are responsible for attenuation of WF10 *in vitro* and *in*

326   *vivo*; these correlated better with the *in vitro* host cell shutoff activity of PA-X than with

327   polymerase activity. To investigate whether the E26K polymorphism exerted its *in vivo*

328   phenotypic effect via PA-X rather than through PA, we introduced a well characterized set of

329   nucleotide substitutions into the frameshift site (FS mutant) of PA which has previously

330   been shown to inhibit expression of PA-X (19, 34, 35). We further combined this FS mutation

331   with the reciprocal mutants at position 26.

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332   We initially investigated the combined effect of position 26 and FS mutations on host

333   shutoff in mammalian and avian cells (Figure 6A,B). For UDL-01 WT introduction of either

334   the E26K or FS mutation individually or together ablated shutoff activity, indicating as we

335   and others have shown, that segment 3 shutoff activity maps to PA-X (19, 34, 35, 37, 38).

336   Conversely, as WF10 WT segment 3 had no shutoff activity the FS mutant alone had no

337   additional effect but ablated the increased shutoff activity seen when combined with WF10

338   K26E. An identical outcome of the mutations was observed in avian cells (Figure 6B). Finally,

339   using whole viruses with both mutations at position 26 and FS combined we showed that

340   when PA-X expression was ablated in both viruses, shutoff activity (as assayed by puromycin

341   incorporation) was lost (Figure 6C). Overall, these data indicate the shutoff activity of

342   segment 3s that contain PA 26E is entirely dependent on PA-X expression.

343   We next tested whether the difference in plaque and replication phenotype also

344   mapped to PA-X and whether the differences seen previously in this study were sensitive to

345   removal of PA-X expression. Looking at the plaque phenotypes of these combined mutants

346   we saw the frameshift mutant had little or no effect on the reciprocal plaque sizes seen in

347   the viruses; although the UDL-01 E26K + FS mutant had a significantly smaller plaque size

348   than UDL-01 WT, the WF10 K26E + FS virus retained its large plaque size , despite a lack of

349   PA-X expression and shutoff activity (Figure 6D, E). Furthermore, the replication kinetics of

350   the combined mutant viruses exhibited a similar phenotype – WF10 K26E + FS grew to

351   higher titres than WF10 WT, indicating again that the enhanced replication conferred by the

352   PA K26E mutation was independent of PA-X expression and shutoff activity (Figure 6F).

353   Overall this implies that the difference in virus replication, and potentially pathogenicity,

354   seen in viruses with differences at segment 3 position 26, may partially or fully map to PA,

355   rather than PA-X alone.

Discussion

357   In this study we investigated how differences in the PA gene of a progenitor (WF10) and

358   a contemporary reassortant (UDL-01) H9N2 viruses led to differences in replicative fitness.

359   We mapped these differences to a single amino acid change of K to E at PA residue 26,

360   which is within the endonuclease domain. Although changes at this residue did not affect

361   virus polymerase activity, they did cause reciprocal differences in replicative fitness in both

362   mammalian and avian systems, in cell lines, primary cells, embryonated eggs, as well as *in*

363   *vivo*, in chickens. We found that although these mutations strongly affected the shutoff

364   activity of the accessory protein PA-X, this did not explain the differences in *in vitro* virus

365   replication phenotype, indicating that it is likely that differences in PA function are partially,

366   or fully, responsible for this.

367   The influenza virus accessory protein, PA-X, has been described in multiple studies as a

368   virulence factor in avian influenza viruses (including H9N2 viruses) that can affect disease

369   outcome in mammals or birds (22, 24, 26, 34). Although other studies have found PA-X

370   expression can lead to an attenuated phenotype, particularly in highly pathogenic H5N1

371   viruses (23, 25). We found that differences between UDL-01 and WF10 at position 26 do

372   modulate PA-X shutoff activity; however, PA-X activity alone was not responsible for the

373   different replication phenotypes seen in these viruses, it is possible PA-X activity may still be

374   contributing *in vivo* but that this effect is overshadowed by a dominant PA-specific

375   replication effect. Although several further gene products are described as being generated

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376   from influenza A virus segment 3 (for example PA-N155 and PA-N182), these products do

377   not share the PA endo domain and therefore are unlikely to explain the difference in

378   phenotype between UDL-01 and WF10 (39).

379   The poor replication and small plaque phenotype of WF10 has been previously

380   described by Wan and colleagues; the authors showed that the small plaque phenotype of

381   WF10 could be overcome by supplying the virus with the internal genes of a human H3N2

382   virus (40). In our study we further map these phenotypes to a single polymorphism in the PA

383   gene at position 26. In a separate study by Obadan and colleagues, a WF10 mutant virus

384   library with heterogeneity in the haemagglutinin receptor binding site was used to infect

385   quails. It was found that PA-K26E, the UDL-01-like mutation, was consistently found to

386   spontaneously arise – further suggesting that PA-K26 is responsible for the attenuated

387   phenotype seen in WF10, both *in vitro* and *in vivo* (41).

388   Throughout this study it has been shown that PA residue 26 is responsible for the

389   attenuated phenotype seen in WF10. When the relative distribution of polymorphisms at

390   position 26 is looked at in the population of H9N2 viruses, or throughout avian influenza

391   viruses in general, it becomes clear that the WF10-like K26 is very rare, with only a handful

392   of viruses sharing any kind of polymorphism at this position. Over 99% of avian influenza

393   viruses, including strains of the H5, H7 or H9 subtype contain the UDL-01-like E26 at this

394   position, with a very few viruses containing lysine, glycine, aspartic acid or glutamine (Table

395   2). Ultimately, the disease outcome of H9N2 infections in chickens is dependent on several

396   factors. The presence of co-infecting bacteria or viruses can have an overriding impact on

397   pathogenic outcome that exaggerates morbidity and mortality associated with H9N2

398   infection (42, 43). Likewise, the composition of the remaining influenza genes of H9N2

18

399   viruses can have a major influence on diseases outcome, outside of the effect of PA 26

400   studied here (44). The observation that H9N2 experimentally infected SPF chickens suffer

401   less mortality and morbidity compared to naturally infected farmed chickens can perhaps

402   partly be explained by these cofactors. Based on the data presented here, the apparent

403   abundance of PA 26E in sequenced H9N2 viruses, and the propensity for the PA K26E

404   substitution to occur in infected birds (41), detection of PA 26E in circulating closely related

405   G1 lineage viruses should be associated with adaptation of H9N2 to gallinaceous poultry.

406   Understanding the molecular basis of increased fitness of avian influenza viruses, both

407   in avian and mammalian cells, as they continue to circulate and adapt to avian hosts is key

408   to assessing the threat these viruses pose to food systems and to the human population. In

409   this study we describe a single naturally occurring polymorphism in the endo domain of PA

410   that leads to an attenuated replication and virulence phenotype. This work will help guide

411   future surveillance efforts and may help us better understand the molecular basis of viral

412   fitness and virulence in the avian host.

413   **Materials and Methods**

414   **Ethics Statement**

415   All animal experiments were carried out in strict accordance with the European and

416   United Kingdom Home Office Regulations and the Animal (Scientific Procedures) Act 1986

417   Amendment regulation 2012, under the authority of a United Kingdom Home Office Licence

418   (Project License Numbers: P68D44CF4 X and PPL3002952).

419   **Cell lines**

420   Madin_Darby canine kidney (MDCK) cells, Human embryonic kidney (HEK) 293T cells

421   and chicken DF-1 cells were maintained in Dulbecco's Modified Eagle Medium (DMEM;

422   Sigma) supplemented with 10% (v/v) FBS and 100U/ml Penicillin-Streptomycin (complete

423   DMEM). All cells were grown at 37°C, 5% CO_2.

424   Primary chicken kidney (CK) cells were generated as previously described (45). Briefly,

425   kidneys from three-week-old specific pathogen free (SPF) Rhode Island Red breed birds

426   were shredded, washed in PBS, trypsinised, then filtered. Cells were resuspended in CK

427   growth media (EMEM + 0.6% w/v BSA, 10% v/v tryptose phosphate broth, 300U/ml

428   penicillin/streptomycin), plated and grown at 37°C, 5% CO2.

429   Primary chicken embryo fibroblasts (CEFs) were generated from 10-day-old chicken

430   embryos. Embryos were homogenised and treated with trypsin/EDTA solution.

431   Supernatants were passed through a metal mesh filter and centrifuged to pellet cells. Cells

432   were resuspended in CEF media (M199, 4% (v/v) FBS and 100U/ml Pen/Strep), plated and

433   grown at 37°C, 5% CO2.

434   **Viruses and reverse genetics**

435   A pair of H9N2 viruses were used through this study, A/chicken/Pakistan/UDL-01/2008

436   (UDL-01) and A/Guinea Fowl/Hong Kong/WF10/1999 (WF10). Both virus reverse genetics

437   systems were created using the bi-directional pHW2000 plasmids (46, 47). Mutant PA

438   segments were generated by site directed mutagenesis or Gibson assembly (NEB).

439   Reverse genetics viruses were generated as previously described (46). Briefly, 250ng of

440   each plasmid for either UDL-01 and WF-10 viruses were co-transfected into 6 well plates of

441   293Ts using lipofectamine 2000. 16h post transfection, media was changed to reverse

442   genetics media (DMEM + 2mM glutamine, 100U/ml penicillin, 100U/ml streptomycin, 0.14%

443   (w/v) BSA, 5μg/ml TPCK-treated trypsin). Following a 48h incubation at 37°C, 5%CO_2

444   supernatants were collected and inoculated into embryonated hens' eggs to grow virus

445   stocks. All viruses were Sanger sequenced to confirm no further mutation had occurred

446   upon growth of egg stocks. The generation of LPAI RG viruses was conducted in accordance

447   with the Pirbright Institute's risk assessments approved by the Pirbright Institute's Health

448   and Safety Biosafety department. All LPAI viruses were handled in class II microbiology

449   safety cabinets.

450   **Virus plaque assays**

451   All plaque assays were performed in MDCK cells using 0.6% agarose overlay. Cells were

452   stained with either 0.1% crystal violet solution (20% methanol). When plaques needed to be

453   visualised by immunofluorescence, cells were fixed with 10% neutral buffered formalin

454   followed by permeabilisation with PBS (0.2 %Triton X-100) then incubated with mouse

455   monoclonal α-NP (Iqbal laboratory, 1:2000). Primary antibody was detected with goat anti-

456   mouse IgG 568 conjugated secondary antibody (LICOR; 1:10000) Plates were imaged using

457   an Odyssey Clx Near-Infrared Fluorescence Imaging System (LICOR). ImageJ was used to

458   measure and analyse plaques.

459   **Minireplicon assays**

460   DF-1 cells were seeded into 24 well plates were co-transfected with PB2, PB1, PA and

461   NP, along with a firefly luciferase reporter construct under an avian polI promoter (CKpPol I

462   Luc) at the following concentrations: PB2- 160ng, PB1- 160ng, PA- 40ng, NP- 320ng, pPol I

463   Luc- 160ng. 48 hours post-transfection cells were lysed in Passive Lysis Buffer (Promega) and

464   lysates were read on a Promega GloMax Multi Detection unit using Luciferase Assay

465   Reagent II (Promega) following the manufacturer's instructions.

466   **Virus replication *in vitro* and *in ovo***

467   MDCK and CK cells were inoculated with virus diluted in serum free DMEM for 1 h at 37°C

468   at an MOI of 0.01. Cell supernatants were taken at 4-, 8-, 12-, 24-, 48- and 72-hours post-

469   infection. After 1 hour incubation with virus, cells were then washed twice to remove unbound

470   virus, and media was replaced with virus growth medium - DMEM plus 2 μg/ml tosyl

471   phenylalanyl chloromethyl ketone (TPCK)-treated trypsin for MDCK cells or Eagle's minimum

472   essential medium (EMEM), 7% bovine serum albumin and 10% tryptose phosphate broth for

473   CKCs.. Viruses were titred by plaque assay on MDCK cells.

474   10-day-old embryonated hens' eggs (VALO breed) were inoculated with 100 pfu of virus

475   into the allantoic cavity. Eggs were incubated for 4-72h and culled via the schedule one

476   method of refrigeration at 4°C for a minimum of 6h. Harvested allantoic fluid from each egg

477   was collected and clarified by centrifugation, virus titres were assessed by plaque assay on

478   MDCKs.

479   **Virus infection, transmission and clinical outcome *in vivo***

480   Rhode Island Red (RIR) embryos were purchased from the National Avian Research

481   Facility (University of Edinburgh; http://www.narf.ac.uk/) and housed within the Pirbright

482   Institute BSU. Embryonated eggs were incubated for 21 days and hatched at the Pirbright

483   Institute. Prior to the commencement of the study, all birds were swabbed (in both

484   oropharyngeal and cloacal cavities) and bled via wing prick to confirm they were naïve to

485   the virus. All infection experiments were performed in self-contained BioFlex B50 Rigid Body

22

486   Poultry isolators (Bell Isolation Systems) at negative pressure. 20 birds per group were

487   directly inoculated with 10^4 pfu of virus intranasally. Mock infected birds were instead

488   inoculated with sterile PBS. One day post-inoculation 8 naïve contact birds were introduced

489   into each isolator to determine virus transmission.

490   Throughout the experiment, birds were swabbed in the buccal and cloacal cavities

491   (days 1-8, 10 and 14 post-infection). Swabs were collected into 1ml of virus transport media

492   (WHO standard). Swabs were soaked in media and vortexed for 10 seconds before

493   centrifugation. Viral titres in swabs were determined by plaque assay on MDCKs.

494   At day 1 and 3 post-inoculation, directly infected birds were euthanized and a panel of

495   tissues were collected and stored in RNA later at -80°C until further processing. On day 14

496   post-infection, all remaining birds were culled via overdose of pentobarbital or cervical

497   dislocation.

498   Birds were observed twice daily and whilst procedures were carried out. Birds were

499   monitored for the presence of clinical signs of infection. Mild clinical signs expected during

500   the study included ruffled feathers, pale comb/wattles, eye and nasal discharge, reddened

501   eyes, snicking and listlessness. Additional moderate clinical signs that may be expected

502   included drooping wings, swollen heads and sporadic diarrhoea. If any signs of severe

503   disease were identified, including laboured breathing, persistent diarrhoea, sitting alone,

504   not attempting to evade capture, or paralysis and unconsciousness then birds were

505   euthanised via a schedule one method and post mortem examination carried out.

506   **RNA extraction and RT-PCR from chicken tissues**

507   30mg of tissue collected in RNA later was mixed with 750µl of Trizol. One sterile 5mm

508   stainless steel bead was added per tube and tissues were homogenised using the Retsch

509   MM 300 Bead Mill system (20Hz, 4 min). 200µl of chloroform was added per tube and tubes

510   were shaken vigorously and incubated for 5 min at room temperature. Samples were

511   centrifuged (9,200xg, 30 min, 4°C) and the top aqueous phase containing total RNA was

512   added to a new microcentrifuge tube and the remaining fluid discarded. RNA extraction was

513   then carried out using the QIAGEN RNeasy mini kit following manufacturers' instructions.

514   100ng of RNA extracted from tissue samples was used for qRT-PCR. All qRT-PCR was

515   completed using the Superscript III platinum One step qRT-PCR kit (Life Technologies)

516   following manufacturer's instructions for reaction set up. Cycling conditions were as follows:

517   i) 5 min hold step at 50°C, ii) a 2 min hold step at 95°C, and 40 cycles of iii) 3 sec at 95 °C and

518   iv) 30 sec annealing and extension at 60 °C. Cycle threshold (CT) values were obtained using

519   7500 softwarev2.3. Mean CT values were calculated from triplicate data. Within viral M

520   segment qRT-PCR an M segment RNA standard curve was completed alongside the samples

521   to quantify the amount of M gene RNA within the sample from the CT value. T7 RNA

522   polymerase-derived transcripts from UDL-01 segment 7 were used for the preparation of

523   the standard curve.

524   **Host shutoff assays**

525   β-galactosidase (β-gal) shutoff reporter assays were performed as previously described

526   (19). Briefly, 293T or DF-1 cells were co-transfected with expression plasmids for the

527   influenza segment 3 and β-gal reporter. 48 hours later, cells were lysed with Reporter lysis

528   buffer (Promega). β-gal expression was measured using the β-galactosidase enzyme assay

529   system (Promega). A Promega GloMax Multi Detection unit was employed to read

530   absorbance at 420nm.

531   For the radio-labelling shutoff activity assays using live virus, chicken embryonic

532   fibroblast (CEFs) were infected with 7:1 (PR8: H9N2) reassortant viruses containing the

533   described PAs at an MOI of 3. At 6 hours post-infection, cells were washed and overlaid with

534   1ml of methionine-and cysteine- free DMEM supplemented with 5% dialysed FCS and 2mM

535   L-Glutamine to starve the cells of methionine and cysteine. At 8 hours post-infection, cells

536   were washed and overlaid with methionine- and cysteine- free DMEM (supplemented as

537   above) including ^{35}S-methionine/cysteine protein labelling mix (Perkin/Elmer) at 0.8mBq/ml.

538   Cells were incubated at 37°C in a vented box containing activated charcoal (Fisher) for 1

539   hour. Cells were washed once with ice-cold PBS and then cells lysed in protein loading

540   buffer for SDS-PAGE and processed via autoradiography. Gels were fixed in gel fix solution

541   (50% methanol, 10% acetic acid) for 5-15 minutes. Fix solution was replaced for 2 more

542   rounds of fixing. Gels were dried in a gel dryer (Bio-Rad) by heating up to 80°C for 2-4h

543   under vacuum pressure. Dried gels were placed in a sealed cassette with an X-ray film

544   (Thermo Fisher) overnight, at a minimum or until the desired signal strength was achieved.

545   X-ray films were developed using a Konica SRX-101A X-ograph film processor using

546   manufacturers' instructions.

547   For the non-radioactive shutoff activity assays using live virus, MDCKs were infected

548   with whole H9N2 virus at an MOI of 5. At 7.5 hours post-infection cells were washed and the

549   medium changed to complete DMEM containing 10μg/ml of Puromycin dihydrochloride

550   from *Streptomyces alboniger* for 30 minutes. Cells were washed then lysed in protein

551   loading buffer for SDS-PAGE and western blotted, probing for puromycin. Puromycylated

552   protein synthesis was quantified in the region of the gel between 45kDa and 80kDa.

553   Protein quantification following autoradiography or anti-puromycin western blot was

554   determined by densitometry using ImageJ analysis software.

555   **Bioinformatics analysis**

556   To assess the prevalence of different polymorphisms at position 26 of PA, every amino

557   acid sequence of full length PA isolates from avian hosts, excluding duplicate sequences,

558   was   downloaded   from   the   NCBI   Influenza   Virus   Database

559   (https://www.ncbi.nlm.nih.gov/genomes/FLU/Database/nph-select.cgi), as of the 23rd May,

560   2020. Sequences were aligned using Geneious R11.1.5 and the distribution of different

561   amino acids was recorded.

562

563   **Statistical analysis**

564   All statistical analysis was carried out using GraphPad Prism 6/7 software. Distribution

565   of data was assessed prior to deciding on the statistical test to use. For statistical analysis of

566   plaque diameter, Kruskal-Wallis with Dunn's multiple comparisons was conducted. For

567   comparison of *in vitro* and *in vivo* replication and polymerase activity, unpaired T-tests or

568   Mann-Whitney Tests were conducted. Log rank Mantel-Cox tests were conducted for

569   survival curves. One-way ANOVA with multiple comparisons or Kruskal-Wallis with multiple

570   comparisons were conducted for β-galactosidase reporter assays

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751   **Table 1: Amino acid differences between the N-terminus of progenitor (WF10) and**

752   **reassortant (UDL-01) H9N2 segment 3 products.**

Amino acid position	WF10	UDL-01	Domain
3	D	N	Endo
20	T	A	Endo
26	K	E	Endo
85	A	T	Endo
86	M	L	Endo
100	V	I	Endo
101	D	E	Endo
118	T	I	Endo
160	D	E	Endo
184	S	N	Endo
213	R	K	Linker
237	K	E	Linker
316	D	G	PA-C
318	R	K	PA-C
319	E	D	PA-C
323	I	V	PA-C

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327	E	K	PA-C
335	I	L	PA-C
352	D	E	PA-C
367	M	K	PA-C
X-221	L	R	X-ORF (PA-X)
X-250	R	Q	X-ORF (PA-X)

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765 **Table 2. Prevalence of PA residue 26 polymorphisms in avian influenza viruses.**

Viruses	Glutamic acid (%)	Lysine (%)	Other (%)
All avian influenza viruses	99.92	0.02	0.04 (glycine), 0.01 (aspartic acid), 0.01 (glutamine)
H5Nx	99.73	0.03	0.07 (glycine), 0.11 (aspartic acid), 0.03 (glutamine)
H7Nx	99.92	0.00	0.08 (glycine)
H9Nx	99.87	0.13	0.00

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776   **Figure legends**

777   **Figure 1. The small plaque phenotype of WF10 maps to the N-terminus of segment 3.**

778   Phylogenetic trees of haemagglutinin (A) and PA (B) were constructed to highlight the

779   evolutionary relationship between H9N2 viruses UDL-01 and WF10. Sequences included for

780   comparison are from lineage-defining viruses of the H9N2 subtype; sequences for H7N3 (red

781   branch;   A/chicken/Pakistan/NARC-100/2004)   and   H7N9   (green   branch;

782   A/pigeon/Wuxi/0405007/2013)   were   included   to   highlight   past   reassortment.   The

783   maximum likelihood method was used in tree generation with 1000 bootstrap replicates in

784   MEGA7. All nucleotide sequences were downloaded from the NCBI database. The indicated

785   viruses were titrated in MDCK cells via plaque assay under a 0.6% agarose overlay and 72

786   h.p.i., fixed and stained for NP using immunofluorescence. (C) and (E) show representative

787   images of plaque sizes of UDL-01 and WF10 RG viruses. (D) shows diameter of 20

788   plaques/virus measured using Image J analysis software. The graphs represent the average

789   plaque diameter +/- SD. (D) Kruskal-Wallis with Dunn's multiple comparison test was used to

790   determine the statistical differences between the plaque sizes.   **** P value < 0.0001 (F)

791   Structure of the trimeric polymerase with vRNA (dark orange) with PB2 (green), PB1 (blue)

792   and PA (light orange).N-terminus half PA differences between WF10 and UDL-01 shown in

793   cyan, zoomed in PA endonuclease domain included in right panel, PA endonuclease active

794   set residues H41, E80, D108, E119 and K134 shown in red (PDB ID: 4WSB)(27).

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796   **Figure 2. The small plaque phenotype of WF10 maps to PA position 26.** H9N2 AIVs

797   with either a UDL-01 or WF10 backbone were rescued via reverse genetics. The plaque

798   phenotypes of the rescues were assessed via plaque assay on MDCK cells under a 0.6%

799   agarose overlay. After 72 hours cells were fixed and stained with 0.1% crystal violet solution

800   and plaques imaged. (A) Visual representation of UDL-01 virus panel containing PA

801   mutations to make them WF10-like. (B) Visual representation of WF10 virus panel

802   containing PA mutations to make them UDL-01 like. (C) 20 plaque diameters per virus

803   performed on the same day were measured using ImageJ analysis software and the average

804   plaque diameter calculated. Graph represents the average +/- SD. P values= ****: <0.0001;

805   **: <0.0039 (Kruskal-Wallis with Dunn's multiple comparisons).

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808   **Figure 3. Variation at position 26 leads to differences in replication but not**

809   **polymerase activity.** (A) MDCK cells and (B) CK cells were infected with the specified virus

810   (UDL-01 WT, UDL-01 E26K, WF10 WT or WF10 K26E) at a low MOI (0.01). (C) 10-day-old

811   fertilised hens' eggs infected with 100pfu of virus. Samples were taken at the indicated time

812   points for titration via plaque assay. No virus was detected prior to 8 h.p.i. Data represents

813   the average +/- SD of 3 independent experiments (cells) or 5 eggs per timepoint. Significant

814   differences (unpaired T-tests (A; UDL-01 36 and 72 h.p.i. WF10 24, 36, 48 and 72 h.p.i., B:

815   UDL-01 8, 12, 24 and 48 h.p.i. WF10 8, 48 and h.p.i. C: UDL-01 12 h.p.i.) or Mann-Whitney

816   Test: (A; UDL-01 4, 8, 12, 24 and 48 h.p.i. WF10 4, 8 and 12 h.p.i. B; UDL-01 4 and 72 h.p.i.

817   and WF10 4 , 12 and 72 h.p.i. C: UDL-01 8, 24 and 48 h.p.i. WF10 all data points) between

818   WT and corresponding mutant at each time point depending on distribution of data are

819   represented with asterisks in orange (UDL-01 pair) or blue (WF10 pair). P values: *= <0.035;

820   ** = <0.008; **** = <0.0001. (D and E) Polymerase activity of the different mutants was

821   assessed using an influenza minireplicon assay. (D) DF-1 cells or (E) 293T were transfected

822   with the components of the polymerase complex (PB1, PB2, PA and NP) plus a vRNA mimic

823   encoding luciferase under the control of an avian RNA polymerase I promotor. For 293T cells

824   the PB1, PB2 and NP were from PR8 to overcome the restriction of avian polymerase in

825   these cells. 48 hours post transfection cells were lysed and luciferase levels measured. Data

826   represents the mean +/- SD of 3 independent experiments.

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828   **Figure 4. In the background of UDL-01, K26E leads to reduced shedding, mortality and**

829   **tissue tropism.** Groups of 20 chickens were infected with 10^4pfu of either UDL-01 WT or

830   mutant UDL-01 E26K viruses. One day post-infection 8 naïve contact birds were cohoused

831   with each group. Birds were swabbed in the buccal and cloacal cavities throughout the study

832   duration. (A, B) average buccal shedding profile of directly infected or contact birds, (C)

833   individual cloacal shedding profiles of birds through duration of experiment. (D) Survival

834   curve of birds exposed to each virus, graph includes both birds that died spontaneously or

835   which reached a humane end point (E, F) qRT-PCR for detection of M gene of viral RNA from

836   chicken tissues of birds culled on day 1 and 3 post-infection. Mann-Whitney Test was

837   conducted for A and B. For E and F, unpaired T-test (Day 1- Trachea, Kidney, Spleen and

838   Liver, Day 3- Nasal, Trachea, Colon, Kidney, Spleen) or Mann Whitney Tests (Day 1- Lung and

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839   Colon, Day 3- Lung and Liver) between UDL-01 WT and UDL-01 E26K infected birds was

840   conducted. P values = ****:<0.001, ***: <0.0005, **: <0.0082, *:<0.033. Error bars

841   represent +/- SD of all birds swabbed (at least 4 birds/group). For survival curves (D) P value

842   = <0.0001 (Log rank (Mantel-Cox) test).

843

844   **Figure 5. Lysine at position 26 correlates with a lack of host shutoff activity in PA-X.**

845   WT or mutant segment 3 plasmids were co-transfected into (A) 293T cells or (B) DF-1 cells

846   with a β-gal reporter plasmid. 48 hours post-transfection cells were lysed and levels of β-gal

847   assessed by colorimetric enzyme assay. Results were normalised to a sample where the PA

848   plasmid was replaced with the empty vector control. Graph represents the average of 3

849   independent experiments +/- SD. P values: ****= <0.0001; ***= 0.0003; * = <0.003 (A, B)

850   one-way ANOVA with multiple comparisons (all 293T and WF10 panel in DF-1) or Kruskal

851   Wallis with multiple comparisons (UDL-01 panel in DF-1). (C, D) CEF were infected with a

852   high MOI (3) of virus. 7 hours post-infection cells were pulsed with 35-S methionine for 1

853   hour then lysed and proteins separated by SDS-PAGE. Radiolabelled proteins were detected

854   by autoradiography. (C) Representative SDS-PAGE gel with specific proteins and the

855   positions of molecular mass (kDa) markers indicated. (D) Levels of radiolabelled actin were

856   quantified by densitometry using ImageJ analysis software. Graph represents the average of

857   3 independent experiments +/- SD P values = *: 0.02, ****: <0.0001 (one-way ANOVA with

858   multiple comparisons). (E, F) MDCK cells were infected with a high MOI (10) of each virus.

859   7.5 hours post-infection cells were pulsed with puromycin for 30 minutes. Cells were lysed,

860   run on SDS-PAGE gels and western blotted for puromycin. (E) Representative western blot

861   gel probed for puromycin. (F) The bracket in (E) covering the areas above 45.7kDa indicates

862   the region quantified using ImageJ analysis software to measure the area under the curve

863   following densitometry of this region. Data were converted to a percentage of the value

864   seen in mock infected cells. Graph represents average +/- SD of 3 independent

865   experiments. P values = *: 0.0124, **: 0.007 (unpaired T-test).

866

867   **Figure 6. The attenuated replication of WF10 is independent of PA-X expression.**

868   H9N2 segment 3s with differing mutations were transfected into (A) 293T cells or (B) DF-1

869   cells along with a β-galactosidase (β-gal) reporter plasmid. 48 hours post transfection cells

870   were lysed and then levels of β-gal assessed by colorimetric enzyme assay. Results were

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871   normalised to an empty vector control where no shut off host gene expression was

872   expected. Graph represents the average of 3 independent experiments +/- SD., P values:

873   ***= 0.0001; **= <0.0097 * = <0.045 (one-way ANOVA – 293T all and DF-1 WF10 panel or

874   Kruskal Wallis – DF-1 UDL-01 panel with multiple comparisons). (C) Host cell shut off within

875   viral infection was then assessed, MDCK cells were infected with each virus at a high MOI

876   (5). 7 hours post-infection cells were pulsed with puromycin for 30 minutes. Cells were lysed

877   in SDS-PAGE buffer and western blotted for puromycin. The area under the curve for each

878   section was calculated and then compared to the mock infected sample. The Graph displays

879   average +/- SD of 3 independent experiments. P values = **: 0.0042 (unpaired T-test). (D,E)

880   The plaque phenotype of viruses containing both polymorphisms at position 26 and a PA-X

881   frameshift mutation was assessed. Viral plaque phenotypes were assessed in MDCK cells

882   under 0.6% agarose. 48 hours post-infection cells were fixed and stained with 0.1% crystal

883   violet solution and (D) plaques imaged. (E) ImageJ analysis software was used to measure

884   the diameters of 20 plaques per virus. Graph represents average +/- SD. P values =

885   **:0.0015, ****=<0.0001 (unpaired T-test). (F) MDCK cells were infected with a low MOI of

886   virus (0.01), cell supernatants were harvested at various time points post- infection and viral

887   titres determined via plaque assay. Graph represents the average of 3 independent

888   experiments +/- SD. P values =, ***= 0.006 (unpaired T-test – UDL-01 4 and 24 h.p.i. WF10 8,

889   12, 24, 48 h.p.i. or Mann Whitney – UDL-01 8, 12 and 48 h.p.i. WF10 4 h.p.i.).   *** =

890   between WF10 WT and WF10 K26E +FS.

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A

A/guinea fowl/Hong Kong/WF10/1999
A/chinese hwamei/Vietnam/38/2006   Eastern
A/quail/Hong Kong/G1/1997   G1 lineage
A/chicken/Pakistan/UDL-01/2008   Western   Eurasian branch
A/chicken/Beijing/1/1994   BJ94 lineage
A/duck/Hong Kong/Y439/1997
A/chicken/Korea/38349-p96323/1996 Y439/Korean lineage
A/turkey/Wisconsin/1/1966

0.02

B

A/duck/Hong Kong/Y439/1997
A/Chinese Hwamei/Vietnam/38/2006
A/Chicken/Korea/38349-p96323/96
A/chicken/Pakistan/UDL-01/2008
A/chicken/Karachi/NARC-100/2004
A/chicken/Wenzhou/606/2013
A/pigeon/Wuxi/0405007/2013
A/chicken/Beijing/1/1994
A/Guinea Fowl/Hong Kong/WF10/1999
A/quail/Hong Kong/G1/1997
A/turkey/Wisconsin/1/1966

0.02

C   WT PA   PA swap

UDL-01

WF10

D

Plaque diameter (mm)

PA segment

UDL-01 backbone   WF10 backbone

E   Chimeric PA
     U/W   W/U

UDL-01

WF10

F

vRNA
PA-endo
PA-C
PB1
PA-Linker
PB2
endo active site

A MDCKs

Virus titre (pfu/ml (log$_{10}$))

Time (hours)

- UDL-01 WT
- UDL-01 K26E
- WF10 WT
- WF10 E26K

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B Chicken Kidney cells

Virus titre (pfu/ml (log$_{10}$))

Time (hours)

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C Embryonated Eggs

Virus titre (pfu/ml (log$_{10}$))

Time (hours)

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D DF-1

Relative luminescene units

WT A20T E26K T85I I100V E101D I118T R221L E237K Q250R G316D K327E K367M -PA | WT T20A K26E I85T V100I/ D101E T118I L221R K237E R250Q D316G E327K M367K -PA

UDL-01   WF10

**

E 293T

Relative luminescene units

WT A20T E26K T85I V100I/ D101E I118T R221L E237K Q250R G316D K327E K367M | WT T20A K26E I85T V100I/ D101E T118I L221R K237E R250Q D316G E327K M367K -PA PR8 WT

UDL-01   WF10

A — Directly infected
B — Contacts
C
D
E — Day one post-infection
F — Day three post-infection