1   **Evaluating the fitness of PA/I38T-substituted influenza A viruses with reduced**

2   **baloxavir susceptibility in a competitive mixtures ferret model**

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26 **Abstract (150/150 words)**

27 Baloxavir is approved in several countries for the treatment of uncomplicated influenza in

28 otherwise-healthy and high-risk patients. Treatment-emergent viruses with reduced

29 susceptibility to baloxavir have been detected in clinical trials, but the likelihood of

30 widespread occurrence depends on replication capacity and onward transmission. We

31 evaluated the fitness of A/H3N2 and A/H1N1pdm09 viruses with the polymerase acidic I38T-

32 variant conferring reduced susceptibility to baloxavir relative to wild-type (WT) viruses, using

33 a competitive mixture ferret model, recombinant viruses and patient-derived virus isolates.

34 The A/H3N2 I38T virus showed a reduction in within-host fitness but comparable between-

35 host fitness to the WT virus, while the A/H1N1pdm09 I38T virus had broadly similar within-

36 host fitness but substantially lower between-host fitness. Although I38T viruses replicate and

37 transmit between ferrets, our data suggest that viruses with this amino acid substitution have

38 lower fitness relative to WT and this relative fitness cost was greater in A/H1N1pdm09

39 viruses than in A/H3N2 viruses.

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Introduction

Antivirals are important for the treatment of influenza infections, particularly in high-risk individuals such as the immunocompromised and the elderly. Neuraminidase inhibitors (NAIs) such as oseltamivir are the current standard of care for treating influenza-related hospitalisations during seasonal epidemics (Heneghan et al., 2016), and are stockpiled in some countries as pandemic contingency (Patel et al., 2017). However, the high frequency of mutations during influenza virus replication combined with the selective pressure of antiviral treatment can lead to the emergence of viral variants with reduced antiviral susceptibility or resistance. The World Health Organization Global Influenza Surveillance and Response System regularly monitors circulating influenza viruses for antiviral resistance, as the prevalence of resistant variants circulating in the community is a key factor when considering which influenza antiviral drugs should be prescribed for clinical use. For example, M2 ion channel blockers are no longer prescribed to treat influenza A viruses, as nearly 100% of circulating influenza viruses contain an S31N amino acid substitution that confers resistance to these antiviral drugs (Hussain, Galvin, Haw, Nutsford, & Husain, 2017). Similarly, H275Y substitutions in viral neuraminidase (NA) can lead to reduced susceptibility of influenza viruses to oseltamivir and another NAI, peramivir (Li, Chan, & Lee, 2015). While localised clusters of H275Y oseltamivir-resistant A/H1N1pdm09 viruses have been detected (Hurt et al., 2012; Takashita et al., 2014), these variants are currently only detected at a frequency of <1% among circulating viruses (Takashita et al., 2020). There is an ongoing need for the development of antivirals that utilise novel mechanisms of action, such that multiple effective treatment options are available in the case that resistance towards an existing class of antiviral drugs becomes widespread.

Baloxavir (pro-drug: baloxavir marboxil; active form: baloxavir acid) has been approved for the treatment of uncomplicated influenza in otherwise-healthy and high-risk patients in several countries around the world. The influenza polymerase complex is a heterotrimer

67   comprising the polymerase acidic (PA) and two polymerase basic (PB1 and PB2) proteins.

68   Baloxavir targets the highly conserved cap-dependent endonuclease region of the PA to

69   inhibit 'cap-snatching', a critical stage of influenza virus replication (Dias et al., 2009).

70   Baloxavir has activity against influenza A, B, C and D viruses (Mishin et al., 2019; Noshi et

71   al., 2018).

72   Human clinical trial data have shown that a single oral baloxavir dose alleviates influenza

73   symptoms at least as effectively as a 5-day oral course of twice-daily oseltamivir, as well as

74   suppressing viral shedding more rapidly in all patient populations examined (Baker et al.,

75   2020; Hayden et al., 2018; Hirotsu et al., 2019; Ison et al., 2020). In 9.7% of otherwise-

76   healthy patients treated with baloxavir in the phase 3 CAPSTONE-1 trial, viruses with amino

77   acid substitutions at residue I38 of the PA protein were identified. The most frequent variant

78   was I38T, that has subsequently been shown to exhibit reduced susceptibility to baloxavir in

79   vitro (Hayden et al., 2018). I38T variants have emerged following baloxavir treatment in

80   subsequent clinical trials, with the highest rate of 23.4% observed in paediatric patients (Dias

81   et al., 2009; Hirotsu et al., 2019; Noshi et al., 2018). Although I38X variants are associated

82   with up to 50-fold reduction in baloxavir susceptibility in vitro compared to wild type (WT)

83   viruses (Omoto et al., 2018), clinical efficacy (time to alleviation of symptoms) is still

84   observed in baloxavir-treated patients in whom these variants develop, presumably in part

85   because resistance does not emerge until several days after treatment has begun (Uehara

86   et al., 2019). Although based on small patient numbers, post-hoc analyses of the phase 3

87   CAPSTONE-1 trial suggested these variants can be associated with prolonged virus

88   detection and uncommonly with symptom rebound (Uehara et al., 2019). However, analysis

89   of the CAPSTONE-2 trial (patients at high risk of complications) showed the opposite effect,

90   whereby I38X variants were associated with numerically faster resolution of symptoms than

91   those without (Ison et al., 2020), suggesting further investigation into the clinical impact of

92   I38X variants is required.

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93   Variant viruses containing amino acid substitutions that reduce influenza antiviral

94   susceptibility can also lower the efficiency of viral replication and transmission (Duan et al.,

95   2010; Herlocher et al., 2002; Ives et al., 2002; Tu et al., 2017; Yen et al., 2005). The extent

96   of these fitness impacts can vary depending on the specific substitution and the viral genetic

97   background, and compensatory mutations can develop which restore viral fitness in resistant

98   variants (Dong et al., 2015; Lackenby et al., 2018; Li et al., 2015; Simonsen et al., 2007). A

99   notable example of this has been the oseltamivir- and peramivir-resistant A/H1N1 H275Y

100   variant which, while initially showing impaired replicative capacity *in vitro* (Abed, Goyette, &

101   Boivin, 2005; Ives et al., 2002), acquired several compensatory mutations resulting in the

102   capacity for a fit H275Y variant to arise and spread globally in 2008 (Dharan et al., 2009;

103   Hurt et al., 2009; Meijer et al., 2009). The resistant virus was outcompeted from circulation

104   by the newly emergent oseltamivir-sensitive pandemic A/H1N1pdm09 strain in 2009.

105   Although treatment-emergent viruses with reduced susceptibility to baloxavir have been

106   detected in clinical trials, the public health risk and likelihood of their widespread occurrence

107   depends on their capacity to replicate and transmit compared with WT viruses. To evaluate

108   the intrinsic fitness of I38T-variant A/H1N1pdm09 or A/H3N2 viruses with those that have

109   reduced baloxavir susceptibility, we used in vitro assays and in vivo ferret models to

110   compare their replication and transmission efficiency relative to baloxavir-sensitive (WT)

111   viruses, using both recombinant viruses and patient-derived clinical virus isolates from

112   human trials.

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Results

Baloxavir susceptibility of I38T-variants from post-treatment clinical specimens

Pre- and post-treatment clinical isolate viruses were tested by plaque reduction assay to assess the baloxavir susceptibility of treatment-emergent variant viruses harbouring the I38T substitution. The mean baloxavir EC_{50} of viruses from pre-treatment samples (WT) were 1.1–1.5 and 0.31–0.69 ng/mL in A/H1N1pdm09 and A/H3N2 viruses, respectively, whereas the corresponding baloxavir EC_{50} of the post-treatment viruses (I38T) were 82–87 and 36–53 ng/mL, respectively. Therefore, A/H1N1pdm09 and A/H3N2 I38T-variants displayed 58–75- and 77–155-fold reductions in baloxavir susceptibility compared with their respective WT viruses, similar to levels reported previously (Imai et al., 2019; Noshi et al., 2018; Uehara et al., 2019).

In vitro comparison of WT and I38T-variant replication kinetics

The in vitro replicative capacity of pre-treatment WT and matched post-treatment I38T-substituted clinical isolates was compared in MucilAir™ human nasal epithelial cells (*Figure 1A*). For the two A/H1N1pdm09 virus pairs, we observed transient reductions of viral titres in the I38T viruses compared to WT at 48 hours in sample 2PQ003 ($p < 0.05$) and at 72 hours ($p < 0.05$) in sample 2HB001. No significant differences were observed in virus titres for A/H3N2 clinical isolates, indicating that in vitro growth kinetics of the A/H3N2 I38T-variants were comparable to the WT viruses.

A competitive mixtures experiment design was used to further investigate the effect of I38T on in vitro replication fitness (*Figure 1B and C*). Following inoculation of virus mixtures, we evaluated variant viral fitness based on the change in the percentage of I38T viruses (%I38T) in the viral population over three successive passages harvested from the infected cell supernatants. For all virus pairs tested, the proportion of the I38T virus replicating in MucilAir™ cells decreased
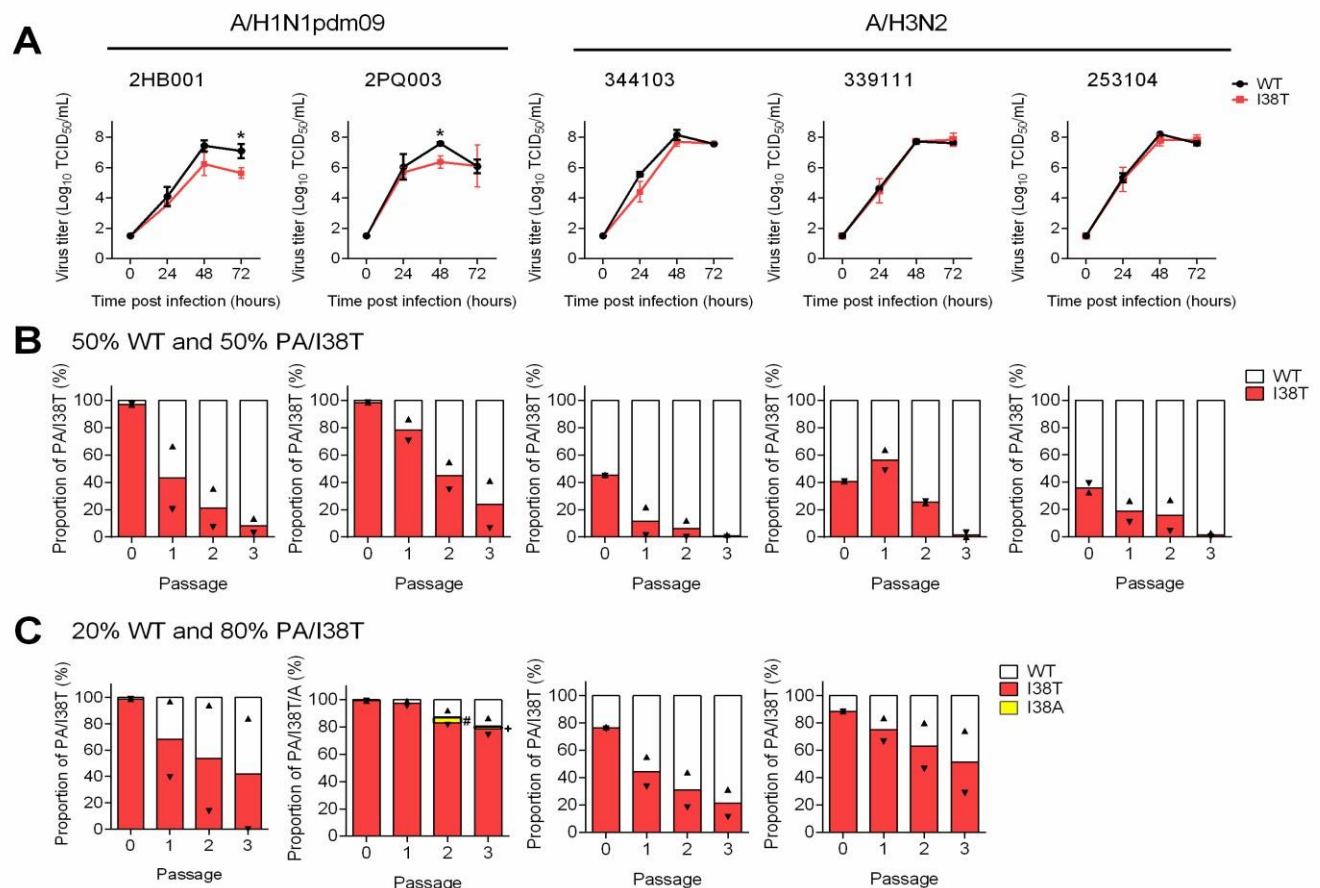
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Figure 1. Replicative capacity and competitive fitness of influenza PA/I38T substituted A/H1N1pdm09 and A/H3N2 viruses isolated from clinical samples. (A) MucilAir™ human nasal epithelial cells were infected with influenza clinical isolates at an MOI of 0.001 $TCID_{50}$/cell. Culture fluids were collected at 0, 24, 48 and 72 hours postinfection and viral titres were determined in MDCK-SIAT1 cells. The LLOQ for virus titre was 1.57 $\log_{10}$ $TCID_{50}$/mL and the viral titre lower than LLOQ was defined as 1.50 $\log_{10}$ $TCID_{50}$/mL. Statistical comparisons of virus titres between WT and viruses with I38T at each time-point were conducted using Welch's *t*-test at the 0.05 significance level (*p<0.05). Data represent mean ± SD of triplicate experiments. (B and C) MucilAir™ cells were co-infected with WT and I38T-substituted clinical isolates at (B) 50:50 or (C) 20:80 ratio based on viral titres ($TCID_{50}$) at an MOI of 0.002. At 48 hours post-infection, the culture fluids were collected and serially passaged three times at an MOI of 0.001. Each culture fluid was subjected to NGS to determine the population of amino acids at position 38 in the PA subunit. The lower detection limit for calling variant viruses was 1% and variant population less than the lower detection limit was set as 0 for the calculation. The bar and the triangles indicate the mean and the individual values of duplicate experiments, respectively. The symbols (# and +) indicate 3.8% and 1.5% of I38A proportion. LLOQ, lower limit of quantitation; MOI, multiplicity of infection; NGS, next generation sequencing; PA, polymerase acid; SD, standard deviation; $TCID_{50}$, 50% tissue culture infectious dose; WT, wild type.

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160   with each passage, regardless of virus subtype or initial ratio. Interestingly, a new I38A

161   variant virus was detected by next generation sequencing (NGS) in a #2PQ003 co-infection

162   group at P2 and P3 (*Figure 1C*), however this subpopulation was transient and remained at

163   low abundance. Compared to the A/H3N2 clinical isolates, the ratio of WT%:I38T%

164   determined by NGS for A/H1N1pdm09 clinical isolate pairs did not correlate well with the

165   intended mixture proportions based on infectious virus titre (used for preparing the P0

166   material), which may be due to the presence of non-replicating virions present in the viral

167   samples (*Figure 1B and C*). Despite these mixtures consisting of ~99% I38T viral copies

168   based on NGS, mixed virus populations similar to the expected proportions were established

169   at 48 hours following inoculation (P1, *Figure 1B and C*), and we identified that the

170   A/H1N1pdm09 I38T-variant population declined compared to WT with each successive

171   passage. In addition to these human cell experiments, we performed a competitive mixtures

172   experiment in MDCK cells using recombinant WT and I38X-variant virus pairs. As with

173   previous experiments, WT viruses to exhibit a replication advantage over I38X-variants

174   (*Figure 1 – figure supplement 1*).

175   The A/H3N2 clinical isolate pair #344103 (WT and I38T) and A/H1N1pdm09 clinical isolate

176   pair #2HB001 (WT and I38T) were selected to investigate further for viral fitness in the ferret

177   infection model (*Figure 1 – figure supplement 2*).

178   **Replication and direct contact transmission of WT/I38T clinical isolate virus pairs in a**

179   **competitive fitness ferret model (Melbourne)**

180   *A/H3N2 I38T fitness*

181   In the Melbourne laboratory, the in vivo replication and transmission capacity of the post-

182   treatment A/H3N2 I38T isolate was compared to the corresponding pre-treatment WT isolate

183   in ferret transmission chains. Infectious virus was detected in nasal washes of each recipient

184   ferret (RF) in transmission chains initiated with pure populations of either the WT or I38T

185   virus, indicating that the treatment-emergent I38T-variant was capable of at least three

186 successive transmissions in a direct contact model (*Figure 2A*). Pyrosequencing analysis

187 showed that the I38T-variant population was maintained over three generations of

188 transmission without substantial reversion to WT (*Figure 2 – figure supplement 1*). To

189 compare replication kinetics of A/H3N2 I38T virus with WT virus in vivo, the area under the

190 curve (AUC) of infectious viral titres in the nasal washes of RF1-RF3 (n = 3) was determined

191 over the course of infection (*Figure 2A*). Similar to the in vitro experiments (*Figure 1A*),

192 overall RF virus replication kinetics (AUC of the 50% tissue culture infectious dose [$TCID_{50}$] ±

193 standard deviation [SD]) for the A/H3N2 WT virus (18.42 ± 7.29) were comparable to the

194 I38T virus (17.58 ± 2.75, non-significant).

195 In parallel, transmission chains were initiated with competitive virus mixtures in donor ferrets

196 (DFs) inoculated with different ratios of A/H3N2 WT and I38T viruses based on infectious

197 titres. Our aim was to establish infections in DFs with WT and I38T-variant viruses present at

198 a range of different ratios, allowing us to evaluate changes over time for the proportion of

199 I38T-variant population in infected ferrets and the differences in variant population following

200 a transmission event to a recipient ferret. Pyrosequencing of viral RNA shedding in nasal

201 wash samples showed that the mixed infections established in DFs at 1 day post inoculation

202 (DPI) were similar to the intended infectious virus ratios in the inoculum mixture (*Figure 3*).

203 To evaluate the relative within-host fitness of the I38T-variant, in each individual ferret the

204 %I38T population on the final day of nasal washing was compared to the population present

205 on the first day of detection by pyrosequencing. We observed that the proportion of A/H3N2

206 I38T either remained stable (in DFs, with some decreases observed in [WT:I38T] 50:50 and

207 20:80 chains) or decreased over time (in RF1s) (*Figure 3*). The study of within host fitness of

208 A/H3N2 and I38T viruses was limited when the viral populations established in RF2-RF3

209 were nearly pure (*Figure 3*), and therefore this resulted in limited competition of WT and I38T

210 virus. The relative changes in A/H3N2 I38T proportion that occurred in each animal is

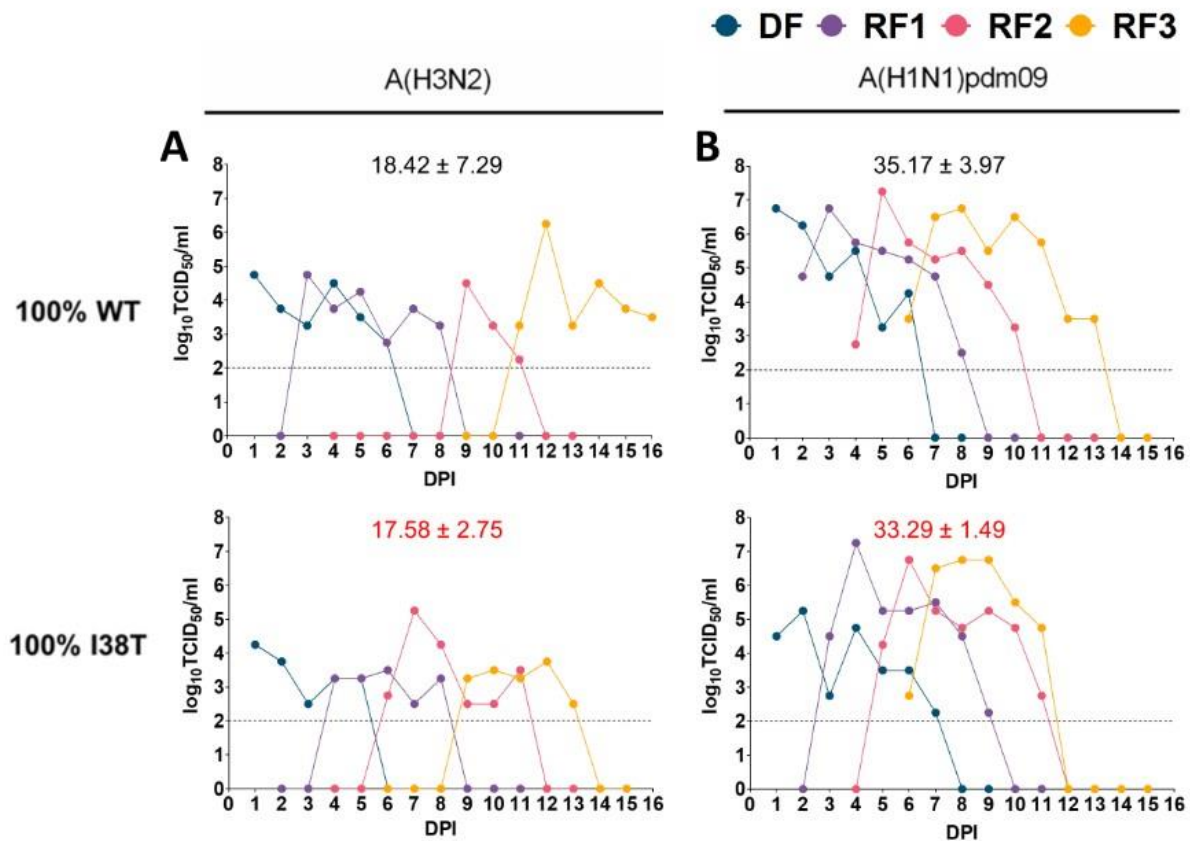211 summarised in *Figure 4A*; overall there was a modest reduction in the within-host fitness of

212 the

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Figure 2. Infectious viral titres (TCID$_{50}$) in nasal washes of direct contact recipient ferrets 1-3 (RF1-RF3) from pure (100%) WT and pure PA/I38T infection groups. (A) A/H3N2 clinical isolates; (B) A/H1N1pdm09 clinical isolates. Virus replication kinetics (TCID$_{50}$) in ferrets infected by direct contact transmission (DF and RF1-RF3) using pure populations of WT or I38T-variant virus for (A) A/H3N2 or (B) A/H1N1pdm09 subtypes. Points represent infectious titres (log$_{10}$ TCID$_{50}$/mL) in individual ferret nasal washes from each DPI of DF. The mean AUC of infectious virus shedding (AUC TCID$_{50}$ ± SD) for RF1-RF3 in each group is labelled (black text, WT; red text, I38T). AUC, area under the curve; DF, donor ferret; DPI, days post inoculation; RF, recipient ferret; SD, standard deviation; TCID$_{50}$, 50% tissue culture infectious dose; WT, wild type.
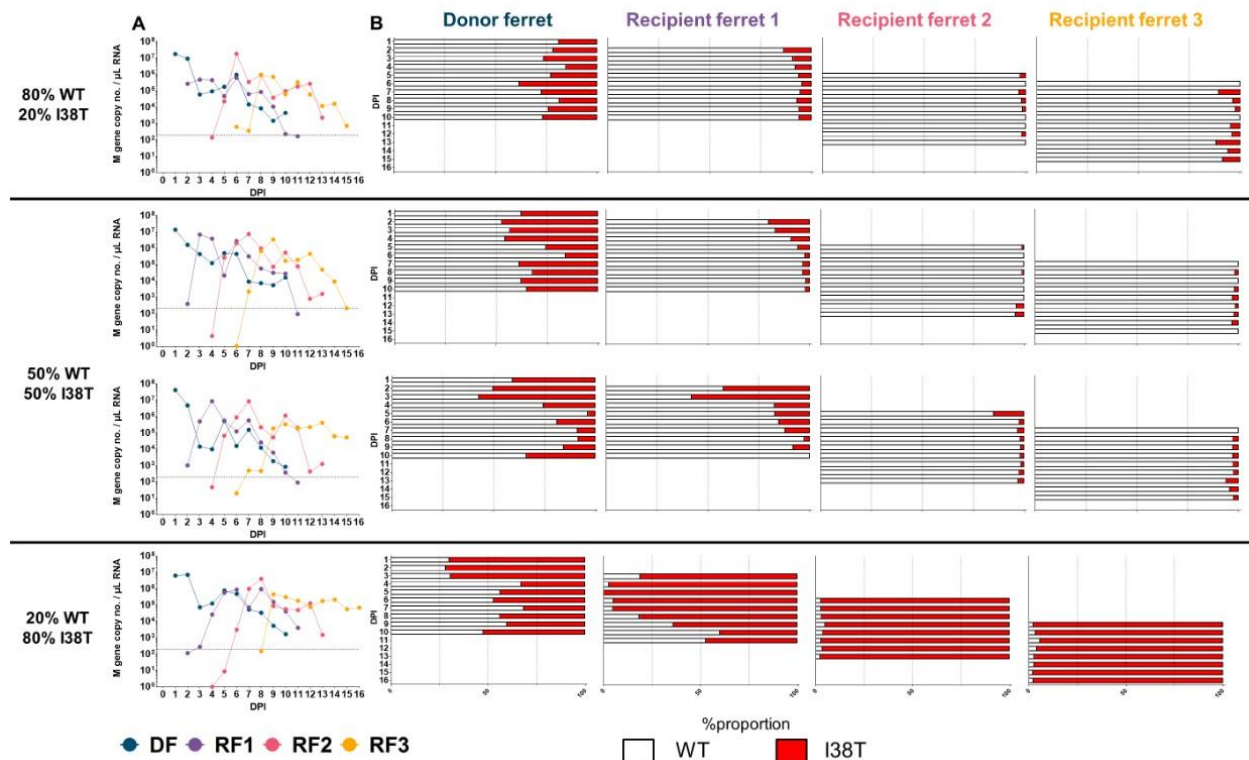
Clinical A/H3N2

Figure 3. A) qPCR curves for A/H3N2 viral RNA and B) WT%:I38T% of viruses in ferret nasal washes. A/H3N2 DFs were infected (intranasal inoculation) with virus mixtures of WT and I38T at a total infectious dose of 10^5 TCID$_{50}$/ 500 µL. Daily nasal washes were collected from all animals for 10 consecutive days following inoculation/exposure, or until endpoint. RF1 was exposed to the DF by cohousing at 1 DPI. Naïve recipients were cohoused sequentially with the previous recipient (1:1) 1 day following detection of influenza A in RF nasal wash (Ct <35). (A) Viral RNA was quantified by number of M gene copies (influenza A)/µL of total RNA. (B) The relative proportion of virus encoding WT PA (white bars) and I38T (red bars) in each nasal wash sample was determined by pyrosequencing. DF, donor ferret; DPI, days post inoculation; RF, recipient ferret; TCID$_{50}$, 50% tissue culture infectious dose; WT, wild type.
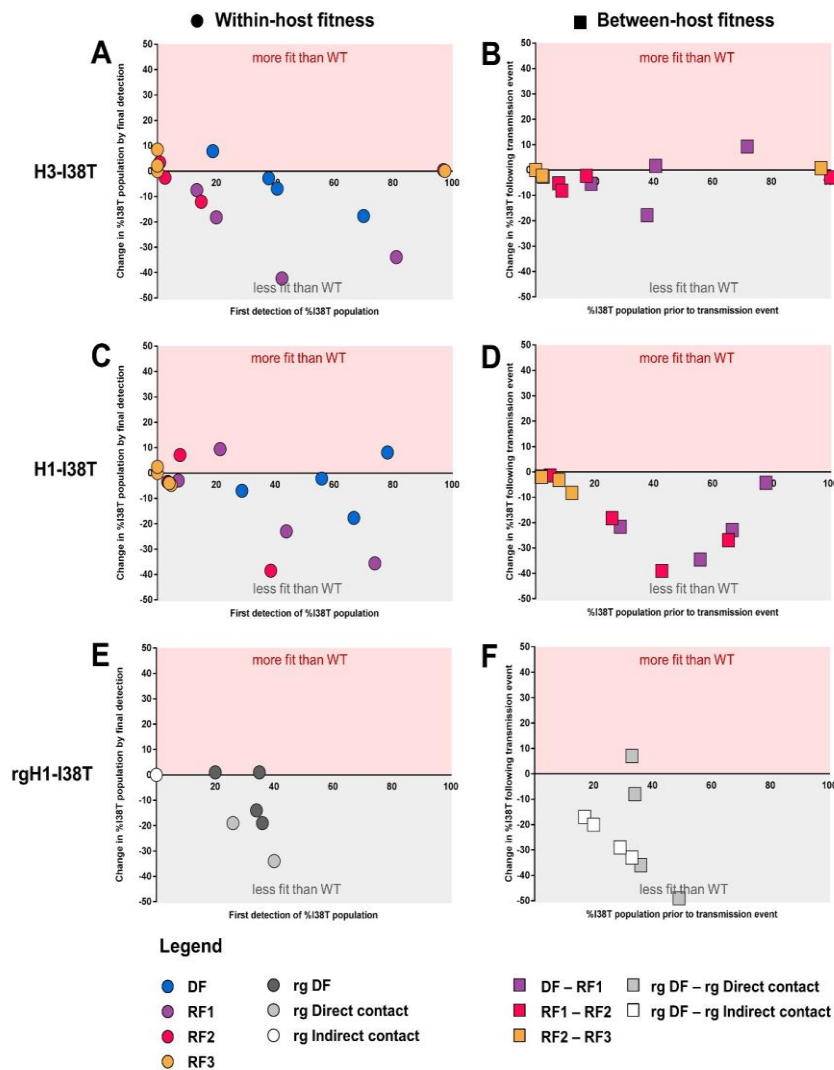
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Figure 4. Visualisations of estimated within-host fitness and between-host fitness for PA/I38T-variant viruses. Within-host, and between-host transmission fitness visualisation for I38T-substituted clinical isolates (A–D) and recombinant viruses (E and F). (A, C and E) Within-host fitness graph Y-axis plots the change in %I38T population from first valid pyrosequencing/NGS detection (on X-axis) compared to last detection in each individual ferret (coloured circles represent DF: blue; RF1: purple; RF2: pink; RF3; yellow; rg DF: charcoal; rg direct contact: grey; rg indirect contact: white). Points in red shaded area indicate %I38T increased over time, grey shaded area indicates %I38T reduced over time. (B, D and F) Between-host fitness graph Y-axis plots the change in %I38T population for each transmission event measured using the first valid pyrosequencing/NGS measurement of %I38T in the recipient compared to the %I38T in the respective donor on the previous day (donor value is the X-axis). Coloured squares represent generations of transmission (1st, DF-RF1: purple; 2nd, RF1-RF2: pink; 3rd, RF2-RF3: yellow) or route of transmission (grey: direct contact; white: indirect contact). Points in red shaded area indicate %I38T increased following transmission, grey shaded area indicates %I38T reduced following transmission. DF, donor ferret, NGS, next generation sequencing; RF, recipient ferret.

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261   A/H3N2 I38T-variant. Transmission or between-host fitness was evaluated according to the

262   change in variant virus population in the first pyrosequencing detection (post infection) in a

263   RF nasal wash, compared to the population detected in the cognate DF on the previous day.

264   Between-host fitness was similar in the I38T-variant and the WT A/H3N2 virus at each direct

265   contact transmission event (*Figure 4B*). Some shifts (both increase and decrease) in %I38T

266   were observed in the first generation of transmission (purple points), but apparent

267   transmission bottlenecks of near pure WT (left extreme of x-axis) or I38T (right extreme of x-

268   axis) had developed in the second (pink points) and third (yellow points) generations such

269   that competition between I38T and WT virus populations was effectively absent in RF2-RF3,

270   and minimal changes in proportion were observed (*Figure 4B*).

271   Overall, the populations of A/H3N2 I38T-variant diminished such that they were essentially

272   lost by RF2 in the three transmission chains that were initiated with either 20% or 50% of the

273   variant. However, in the transmission chain initiated with 80% A/H3N2 I38T, the WT virus

274   was outcompeted, likely to be due to the impact of a small transmission bottleneck in RF1

275   when the variant virus was still dominant in the viral mixture at 5 DPI (*Figure 3*). Therefore,

276   across the two measured components of viral fitness, we identified that the A/H3N2 I38T

277   virus had a modest reduction in within-host fitness, but comparable between-host fitness to

278   that of the WT virus.

279   ***A/H1N1pdm09 I38T fitness***

280   As observed in the A/H3N2 clinical isolate pair, infection with a pure population of the

281   A/H1N1pmd09 I38T virus was capable of three successive direct contact transmissions in

282   the ferret model (*Figure 2B*), and that the I38T substitution was maintained without reversion

283   to WT (*Figure 2 – figure supplement 2*). Similar to the in vitro data for 2HB001 (*Figure 1A*),

284   we measured a transient reduction in mean viral titre of A/H1N1pdm09 I38T recipients

285   compared to WT at 24 hours post-exposure ($p \leq 0.05$). However, the overall infectious virus

286   kinetics in ferrets that were infected with the A/H1N1pdm09 I38T virus (n = 3) (AUC 33.29 ±

13

287   1.49) was not significantly different to those infected with the WT virus (35.17 ± 3.97, non-

288   significant) (*Figure 2B*).

289   In transmission chains initiated with mixtures of A/H1N1pdm09 WT and I38T-variant viruses,

290   competitive co-infections were established at 1 DPI in DFs at similar proportions to the

291   infectious virus ratio in the inoculum (*Figure 5*). In terms of within-host fitness, the proportion

292   of A/H1N1pdm09 I38T remained relatively stable over time, except in the WT:I38T 20:80

293   chain, where some reductions were observed (*Figure 5*). This is further reflected in the

294   summary figure where the majority of within-host measurements show little or no change in

295   viral proportion, but in a small number of ferrets we observed a loss of the I38T-variant over

296   time (*Figure 4C*). The I38T-variant population decreased following every direct contact

297   transmission event, indicating the between-host fitness of A/H1N1pdm09 I38T was clearly

298   compromised compared to WT (*Figure 4D*). The largest reductions in %I38T were observed

299   in the first (purple points) and second (pink points) generations (*Figure 4D*), and by the

300   endpoint, the I38T-variant proportion had become a minority proportion in all transmission

301   chains (*Figure 5B*). Therefore, across the two measured components of viral fitness, we

302   identified that the A/H1N1 I38T virus had broadly similar within-host fitness, but substantially

303   lower transmission fitness compared to the WT virus.

304   **Replication and direct/indirect contact transmission of WT/I38T reverse genetics**

305   **A/H1N1pdm09 viruses in competitive fitness ferret model (London)**

306   In the London laboratory, we tested the fitness impact of the I38T amino acid substitution in

307   a reverse genetics-derived A/H1N1pdm09 virus using both direct and indirect transmission

308   routes. DFs were inoculated with a 50:50 mixture before exposure (1:1:1) to both a direct

309   contact recipient and an indirect contact recipient. NGS was used to determine the ratio of

310   WT:I38T virus populations in ferret nasal washes, and showed that mixed infections were

311   established in DFs at 1 DPI (*Figure 6*).

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Clinical A/H1N1pdm09
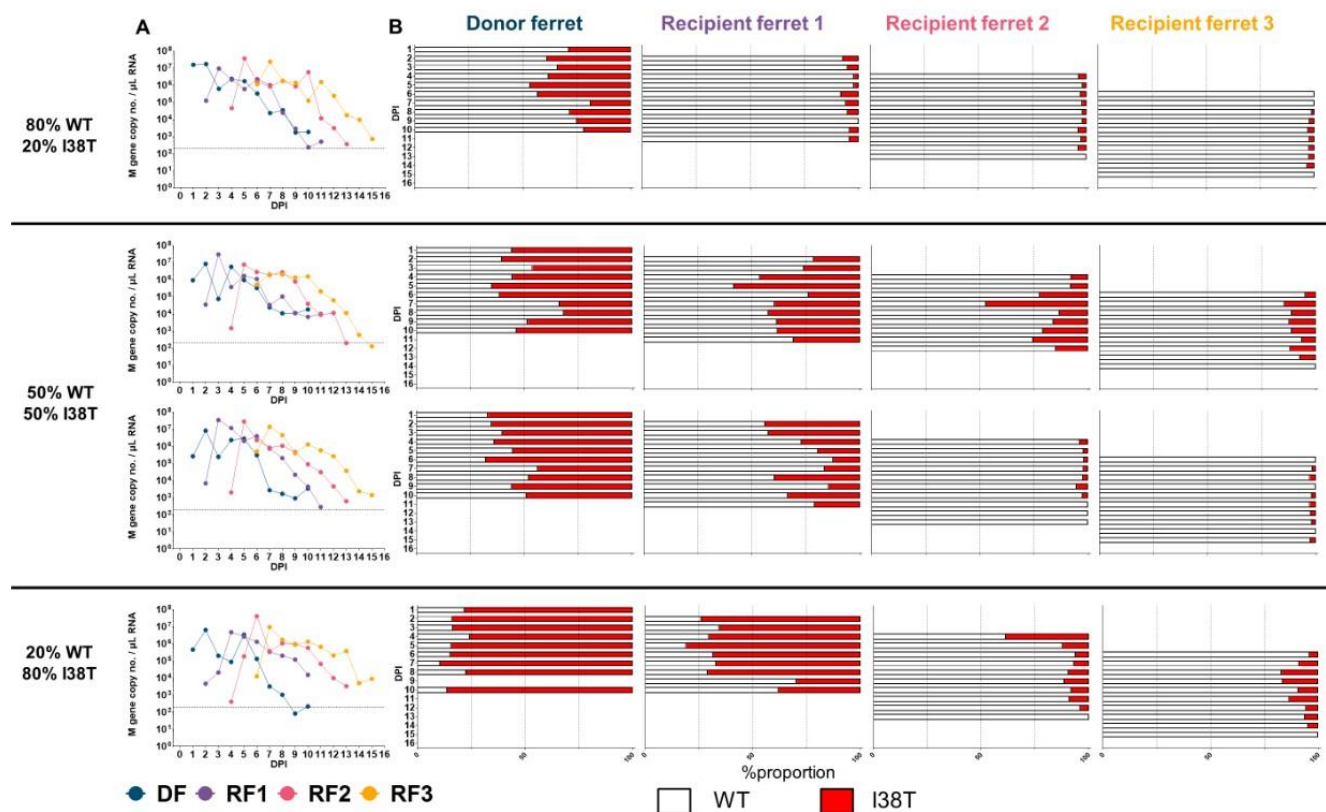
Figure 5. qPCR curves for A/H1N1pdm09 viral RNA and B) WT%:I38T% of viruses in ferret nasal washes. A/H1N1pdm09 DFs were infected (intranasal inoculation) with virus mixtures of WT and I38T at a total infectious dose of 10^4 $TCID_{50}$ / 500 µL. Daily nasal washes were collected from all animals for 10 consecutive days following inoculation/exposure, or until endpoint. RF1 was exposed to the DF by cohousing at 1 DPI. Naïve recipients were cohoused sequentially with the previous recipient (1:1) 1 day following detection of influenza A in RF nasal wash (Ct <35). (A) Viral RNA was quantified by number of M gene copies (influenza A) per µL of total RNA. (B) The relative proportion of virus encoding WT PA (white bars) and I38T (red bars) in each nasal wash sample was determined by pyrosequencing. DF, donor ferret; DPI, days post inoculation; PA, polymerase acid; RF, recipient ferret; $TCID_{50}$, 50% tissue culture infectious dose; WT, wild type.

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Recombinant A/H1N1pdm09
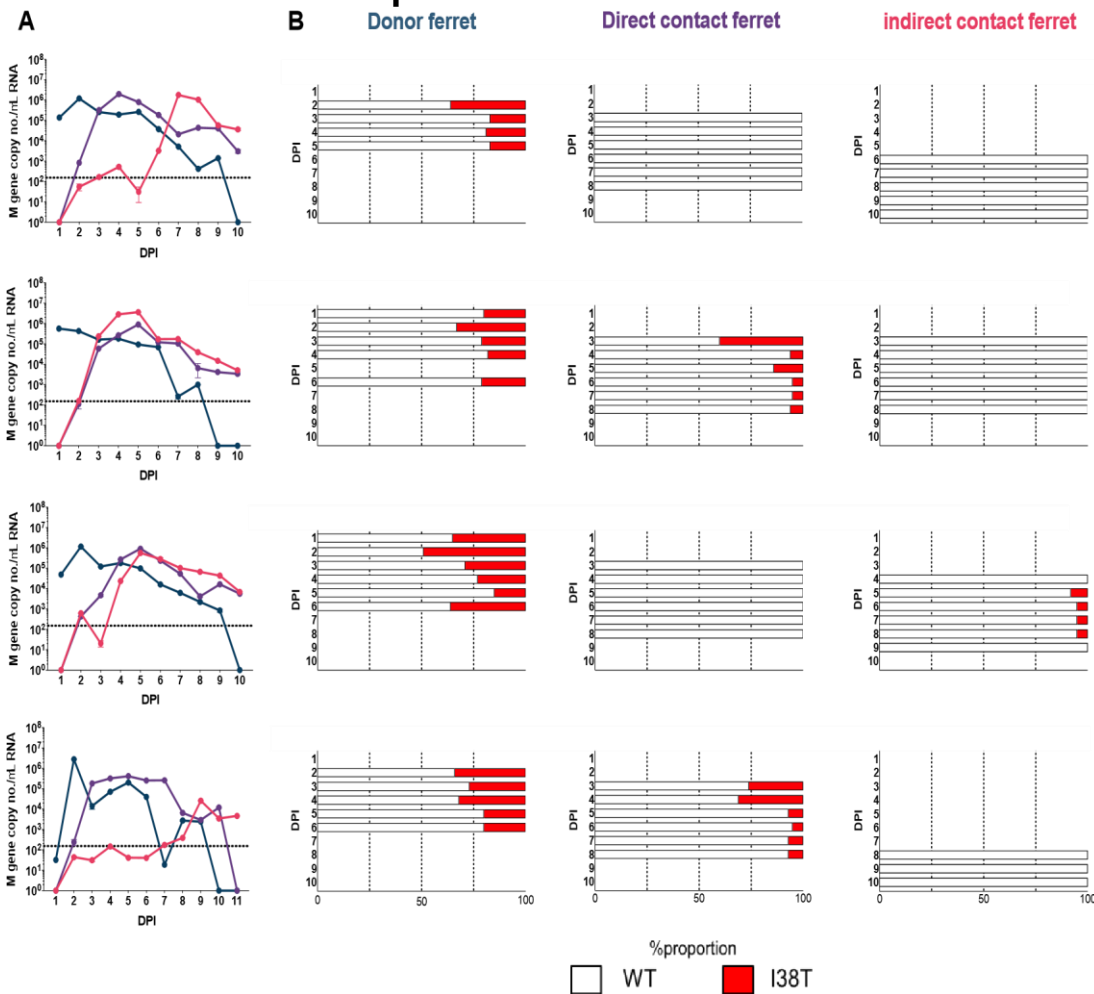
Figure 6. Change in WT/I38T virus proportion in nasal washes of donors compared to DC or IC recipients. Donor ferrets were infected (intranasal inoculation) with a recombinant A/H1N1pdm09 virus pair using 50:50 WT%: I38T% mixture based on infectious virus titre, at a total infectious dose of 10^4 PFU. Naïve recipient ferrets were exposed to infected donors at 1 DPI by cohousing in the same cage (direct contact), or by housing in an adjacent cage separated by a respiratory droplet-permeable interface (indirect contact) (1:1:1 ratio). The relative proportion of virus encoding WT PA (white bars) and I38T (red bars) in each nasal wash sample was determined by next generation sequencing. DPI, days post inoculation; inoc, inoculation; PA, polymerase acid; WT, wild type.

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341   In terms of within-host fitness, we observed that the I38T-variant population was maintained

342   at a similar proportion in DF throughout the course of infection, but diminished rapidly in the

343   direct contact ferrets where a mixed infection had been established by transmission (*Figure*

344   *6* and *4E*). In terms of between-host fitness the I38T-variant virus did not transmit by direct

345   contact in two of four occasions (*Figure 6*), but the proportion remained relatively stable

346   where transmission did occur (*Figure 4F*). Among indirect contact recipients, the I38T-variant

347   virus failed to transmit in three of four occasions, and in the one of four recipients where it

348   was detected it was at a very low abundance (<10%, near limit of detection) (*Figure 6*).

349   These data support the findings from the A/H1N1pdm09 clinical isolates derived from treated

350   patients, indicating that between-host fitness is substantially compromised by the I38T

351   substitution. Furthermore, the results suggest that the transmission fitness of the I38T virus

352   is lower in the indirect transmission model compared to the direct contact model (*Figure 4F*).

353

Discussion

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355 The detection of variant influenza viruses carrying the PA/I38T amino acid substitution in

356 baloxavir-treated patients raises the concern that such viruses with reduced drug

357 susceptibility could achieve sustainable transmission and circulate in the community, thereby

358 potentially compromising the effectiveness of baloxavir usage. The present study provides

359 evidence that the I38T substitution has some impact on aspects of the intrinsic viral fitness of

360 both A/H3N2 and A/H1N1pdm09 viruses, but that these fitness costs are modest and are not

361 completely deleterious to viral replication and transmission.

362 In existing studies investigating the fitness of PA/I38T-substituted influenza viruses in animal

363 models, variant influenza viruses of recombinant and patient-derived origins have been

364 capable of robust viral replication in mice, hamsters and ferrets (Checkmahomed et al.,

365 2019; Chesnokov et al., 2019; Imai et al., 2019; Jones et al., 2020). Ferrets represent the

366 gold standard transmission model for evaluating the fitness of influenza viruses (Belser,

367 Eckert, Tumpey, & Maines, 2016); of the available reports, patient-derived and recombinant

368 I38T viruses have previously been shown to transmit from experimentally inoculated DFs to

369 naïve recipients by direct (Jones et al., 2020) and airborne contact (Imai et al., 2019; Jones

370 et al., 2020) routes. In our study, patient-derived A/H3N2 and A/H1N1pdm09 I38T-variants,

371 when evaluated as pure viral populations, could sustain a chain of three sequential

372 generations of direct contact transmission. However, competition experiments allowed us to

373 determine more subtle differences in viral fitness, indicating that the treatment-emergent

374 I38T substitution compromised direct contact transmissibility of the A/H1N1pdm09 isolate,

375 whereas the same substitution appeared to more strongly attenuate within-host replication in

376 the A/H3N2 isolate. Furthermore, a recombinant I38T-variant A/H1N1pdm09 virus

377 demonstrated similar fitness characteristics in ferrets to the patient-derived A/H1N1pdm09

378 isolate, suggesting that transmission of the variant by respiratory droplets/aerosols was

379 restricted to a greater extent than transmission by direct contact. Our study revealed that

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380   populations of I38T-variants are outcompeted by WT viruses in both A/H1N1pdm09 and

381   A/H3N2; however, pure populations of I38T-variant viruses were still able to be transmitted

382   in ferrets in accordance with previous studies (Imai et al., 2019; Jones et al., 2020).

383   Recent reports using in vivo competitive mixture models suggest that the fitness impact of

384   I38T can vary based on the virus background. Checkmahomed and colleagues

385   (Checkmahomed et al., 2019) reported that I38T-variants of recombinant viruses from 2009

386   (pandemic A/H1N1pdm09) and 2013 (seasonal A/H3N2) outcompeted cognate WT viruses

387   within-host in mouse co-infections, whereas a separate study found a I38T-substituted

388   A/H3N2 clinical isolate from 2017 to be mildly attenuated compared with WT in ferrets

389   (Chesnokov et al., 2019). Furthermore, fitness observations from competition experiments in

390   vitro have not always accurately predicted in vivo fitness characteristics (Checkmahomed et

391   al., 2019). Such factors contribute to the difficulties associated with predicting the likely

392   widespread emergence of a strain, even if the virus in question has equivalent or greater

393   fitness than the respective WT virus being tested. A previous example of this has been

394   observed following a competitive fitness ferret study to evaluate the fitness of an oseltamivir-

395   resistant A/H1N1pdm09 virus with the H275Y NA substitution that caused a localised cluster

396   of cases among untreated patients in Australia and Japan in 2011/12 (Butler et al., 2014;

397   Hurt et al., 2012; Takashita et al., 2014). This study estimated that the oseltamivir-resistant

398   variant virus had equivalent or even greater fitness relative to the cognate WT virus, thereby

399   concluding that there was a significant risk that this virus would emerge and spread globally.

400   Despite this finding in ferrets, no such dissemination of A/H1N1pdm09 H275Y oseltamivir-

401   resistant viruses in the community has occurred in the intervening years (Lackenby et al.,

402   2018).

403   Permissive mutations in the NA and haemagglutinin (HA) proteins were thought to have

404   compensated for the intrinsic fitness costs of the H275Y mutation in former seasonal

405   A/H1N1 viruses, thereby enabling their capacity to spread globally in 2008 (Baranovich et

406   al., 2010; Bloom, Gong, & Baltimore, 2010; Takashita et al., 2015). This study utilised

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407   clinically derived and recombinant virus pairs, where the PA genes differed by the I38T

408   substitution alone, demonstrating that this baloxavir-resistance substitution is detrimental to

409   intrinsic viral fitness relative to WT. However, as I38T does not completely prohibit viral

410   replication and transmission, the potential exists for additional permissive mutations to

411   develop that recover any viral fitness loss associated with I38T, which may enable

412   increasing prevalence in community circulation. While specific compensatory mutations in

413   I38T-variants have not yet been identified, a previous report has highlighted an example of

414   this process developing for influenza viruses with reduced susceptibility to favipiravir,

415   another polymerase inhibitor drug. It was found that variant viruses harbouring a PB1

416   substitution conferring resistance to favipiravir caused a loss of viral fitness and polymerase

417   activity, which was rescued by a compensatory substitution in another part of the

418   polymerase complex to restore the fitness and polymerase function of the variant (Goldhill et

419   al., 2018).

420   The error-prone properties of influenza virus replication mean that substitutions leading to

421   resistance will inevitably occur. Amino acid substitutions that confer reduced susceptibility to

422   both the adamantanes and NAIs are well described, and likewise treatment with baloxavir

423   has been observed to select for variant viruses that have reduced baloxavir susceptibility in

424   clinical trials. Resistant viruses may retain sufficient fitness to transmit from person to

425   person, typically within households (Buchholz et al., 2010; Hayden et al., 1989). However,

426   from a public health perspective it is those resistant viruses that demonstrate the potential to

427   outcompete and displace circulating antiviral sensitive strains, as happened in 2007–2009

428   for the oseltamivir-resistant A/H1N1 virus, that are of significant concern. Our results in the

429   ferret model provide evidence that the intrinsic fitness of I38T-substituted viruses with

430   reduced baloxavir susceptibility is attenuated compared to baloxavir-sensitive WT strains.

431   Indeed, despite an estimated 5.5 million doses of baloxavir supplied to Japanese medical

432   institutions between October 2018 and January 2019 (Emi Takashita et al., 2019), there

433   have been very few reports of potential human-to-human spread of a I38T-variant in

434   Japanese households (E. Takashita et al., 2019; Emi Takashita et al., 2019). No clusters of

435   sustained transmission indicating epidemiological spread of I38T-variants have been

436   identified in any population or region to date and the number of baloxavir-resistant viruses

437   detected in circulating viruses between September 2019 and January 2020 demonstrated

438   that only a single E23K variant in 1355 A/H1N1pdm09 viruses, and a single I38M variant in

439   1012 A/H3N2 viruses, were detected in patients with no prior treatment (WHO, 2020). Thus,

440   it appears that baloxavir usage has not resulted in a significant number of circulating viruses

441   with reduced susceptibility to the drug. Nevertheless, our study suggests that the fitness

442   impact of the I38T substitution in A/H1N1pdm09 and A/H3N2 influenza viruses is relatively

443   modest, and the potential remains for the acquisition of permissive mutations to recover the

444   fitness cost of baloxavir resistance.

445   Materials and methods

446   Cells and viruses

447   Canine kidney MDCK and MDCK-SIAT1 cells were cultured as previously described

448   (*Supplementary File 1*) (Omoto et al., 2018; Uehara et al., 2019). The human nasal epithelial

449   cells (MucilAir™) were obtained from Epithelix Sarl (Geneva, Switzerland) and the cells were

450   maintained in MucilAir™ culture medium at 37 °C in a humidified 5% CO_2 incubator.

451   Three A/H3N2 clinical isolate pairs were derived from human nasopharyngeal/pharyngeal

452   swab samples from patients #344103, #253104, and #339111 from the CAPSTONE-1 phase

453   3 trial (otherwise-healthy patients with influenza; ClinicalTrials.gov identifier: NCT02954354)

454   as previously described (Uehara et al., 2019). Two A/H1N1pdm09 clinical isolate pairs were

455   derived from swabs from patients #2HB001 and #2PQ003 involved in a double-blind,

456   multicentre, placebo-controlled phase 2 study in Japan (Japic CTI-153090) (*Figure 1 – figure*

457   *supplement 3*). Pre-treatment samples contained viruses without an I38T substitution (WT)

458   and post-treatment samples contained viruses with an I38T substitution; full-length HA, NA

459   and PA sequences of clinical isolates from pre- and post-treatment samples were verified by

460   Sanger sequencing (*Supplementary Table*). Clinical isolates were propagated and titrated in

461   MDCK-SIAT1 cells. The selection criteria for clinical isolates used in the ferret study is

462   summarised in *Figure 1 – figure supplement 2.*

463   For in vitro experiments, recombinant viruses based on A/WSN/33 (H1N1) and

464   A/Victoria/3/75 (H3N2) were generated as previously described (Omoto et al., 2018). For in

465   vivo experiments, a recombinant A/H1N1pdm09 virus pair of WT and I38T viruses were

466   generated by reverse genetics using plasmids containing the gene segments from

467   A/England/195/2009 as previously described (Frise et al., 2016). Recombinant viruses were

468   propagated and titrated in MDCK cells.

469   **In vitro characterisation of clinical isolate pairs**

470   The baloxavir sensitivity of clinical isolate pairs (WT and I38T) was assessed by plaque

471   reduction assay in MDCK-SIAT1 cells as previously described (Uehara et al., 2019).

472   Baloxavir acid was synthesised by Shionogi & Co., Ltd. (Osaka, Japan). The effective

473   concentration of baloxavir achieving 50% inhibition of plaque formation (EC_{50}) was

474   calculated for each virus based on plaque numbers. The mean ($\pm$ SD) EC_{50} was determined

475   from three independent experiments performed in duplicate. Data for A/H3N2 viruses

476   (#344103, #253104, and #339111) were previously reported by Uehara et al. (Uehara et al.,

477   2019).

478   The replication of clinical isolate virus pairs was compared in vitro using primary human

479   airway epithelial MucilAir™ cells. Confluent monolayers of MucilAir™ cells in transwell 24-

480   well plates (5.0×10^5 cells/well) were infected with either WT or I38T virus at a multiplicity of

481   infection (MOI) of 0.001 for both A/H1N1pdm09 and A/H3N2 viruses. The culture

482   supernatants were collected at 0, 24, 48 and 72 hours after inoculation and the viral titre was

483   determined by $TCID_{50}$ measured in MDCK-SIAT1 cells.

484   Competitive fitness experiments were conducted in MucilAir™ cells using A/H3N2 and

485   A/H1N1pdm09 clinical isolate pairs. The WT virus and the virus harbouring the I38T

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486   substitution were diluted to equivalent infectious viral titres calculated by $TCID_{50}$ in MDCK-

487   SIAT1 cells and mixed at 50:50 or 20:80 volumetric ratios. Confluent monolayers of

488   MucilAir™ cells in transwell-24 plates were infected at an MOI of 0.002. Cell culture

489   supernatant was collected 48 hours post infection and viral titres determined by $TCID_{50}$. The

490   virus supernatant were serially passaged three times in duplicate cultures at an MOI of

491   0.001, and the proportion of viruses harbouring I38X substitutions were evaluated by deep

492   sequencing as previously described (Uehara et al., 2019). The PA coding region

493   (nucleotides 1–466; amino acids 1–155 for A/H3N2 viruses and nucleotides 1–419; amino

494   acids 1–139 for A/H1N1pdm09 viruses) were amplified from total RNA extracted from each

495   culture supernatant (MagNA Pure 96 system; Roche Life Science, Superscript III reverse

496   transcriptase; Invitrogen, HotStar Taq DNA polymerase; Qiagen, Venlo, The Netherlands).

497   Fragmented and barcoded DNA libraries were generated (Nextera XT Library Prep Kit;

498   Illumina) and sequenced using one flow cell on an Illumina MiSeq instrument. All reads were

499   mapped to the PA gene of the reference strain A/Texas/50/2012 (H3N2) or

500   A/California/07/2009 (H1N1) using the CLC Genomics Workbench Version 10.0.1. The

501   average coverage was more than 10,000 and the percentage of reads with a quality score

502   greater than 30 was more than 69%. A threshold frequency of >1% was adopted for calling

503   variants, and the calculated risk of false positive variants was 0.21% for A/H3N2 and 0% for

504   A/H1N1pdm09 strains, respectively, which were determined by sequencing duplicate

505   influenza A virus stock samples.

506   **Ferret studies**

507   *Melbourne experiments*

508   The replication and transmission of clinical isolate virus pairs was evaluated in vivo using a

509   competitive fitness experiment design in ferrets (*Figure 3 – figure supplement 1*). Outbred

510   male and female ferrets (*Mustela putorius furo*) >12 weeks old (independent vendors),

511 weighing 600–1800 g and seronegative against recently circulating influenza viruses were

512 used.

513 Virus stocks for ferret inoculation were diluted to equivalent viral titres in PBS (10^5

514 $TCID_{50}$/500 µL for the A/H3N2 viruses and 10^4 $TCID_{50}$/500 µL for the A/H1N1pdm09 viruses)

515 and the WT and I38T-variant pairs were then mixed at various volumetric ratios: 100% WT :

516 0% I38T, 80% WT : 20% I38T, 50% WT : 50% I38T (×2 groups), 20% WT : 80% I38T, and

517 0% WT : 100% I38T; n = 4 ferrets per transmission chain, total n = 24 ferrets per

518 experiment). Within each transmission chain, DFs were anaesthetised by intramuscular

519 injection of a 1:1 (v/v) xylazine/ketamine (10 mg/kg, Troy Laboratories) mixture, and

520 inoculated with pure or mixed virus suspension (250 µL per nostril) by the intranasal route.

521 The first naïve recipient (RF1) was exposed to the DF 1 DPI of virus. Ferrets were nasal

522 washed daily under xylazine sedation (5 mg/kg, Troy Laboratories) and confirmed to be

523 influenza positive based on a real-time reverse-transcriptase polymerase chain reaction (RT-

524 PCR) cycle threshold (C_t) of <35. Following detection of virus in RF1, it was cohoused 1:1

525 with a subsequent naïve recipient (RF2) the next day. Similarly, once influenza was detected

526 in RF2 it was cohoused with RF3, and the transmission chain was terminated.

527 Daily nasal washes were collected under xylazine sedation (0.5 mg/kg) by instilling 1 mL

528 PBS (1% w/v BSA) into the nostrils for up to 10 consecutive days following

529 inoculation/exposure. Nasal wash samples were stored at –80 ℃ until determination of

530 infectious virus titres by in MDCK-SIAT1 cells as previously described (Oh, Barr, & Hurt,

531 2015). The lower limit of quantitation (LLOQ) was 2.0 $\log_{10}$ $TCID_{50}$/mL. The experiment was

532 terminated at 16 DPI, regardless of transmission status.

533 ***London experiments***

534 Female ferrets (20–24 weeks old) weighing 750–1000 g were used (independent vendors).

535 After acclimatisation, sera were obtained and tested by HI assay for antibodies against

536 A/England/195/2009/H1N1pdm09. All ferrets were confirmed to be seronegative against the

537 NP protein of influenza A virus by using an ID Screen Influenza virus A antibody competition

538 enzyme-linked immunosorbent assay kit (ID.vet) at the start of the experiments.

539 DFs (n = 4) were anaesthetised with ketamine (22 mg/kg) and xylazine (0.9 mg/kg) for

540 intranasal inoculation with 10^4 PFU of the 50% WT : 50% I38T mixture of the reverse

541 genetics A/England/195/1009 virus pair diluted in PBS (100 μL per nostril). The volumetric

542 ratio of virus inoculation was determined based on infectious virus titre (PFU/mL). At 24

543 hours post inoculation, a direct contact recipient was exposed by cohousing with the donor in

544 the same cage, while simultaneously an indirect contact recipient was placed in an adjacent

545 cage (minimum 6 cm apart) which only allowed respiratory droplet/aerosol exposure from the

546 donor cage (*Figure 6 – figure supplement 1*). Exposure of recipient animals to donor animals

547 (1:1:1 ratio) was continued until 3 DPI (48-hour exposure period).

548 All animals were nasal washed daily, while conscious, by instilling 2 mL PBS into the nostrils.

549 The nasal wash sample was used for virus titration by plaque assay in MDCK cells. The

550 LLOQ in the plaque assays was 10 PFU/mL.

551 **Quantitative real-time RT-PCR (qRT-PCR)**

552 For the Melbourne experiments, viral RNA was extracted from 200 μL nasal wash samples

553 using the NucleoMag VET isolation kit (Macherey Nagel) on the KingFisher Flex

554 (ThermoFisher Scientific) platform according to manufacturer's instructions. Primer/probe

555 sets were obtained from CDC Influenza Division: Influenza virus M gene copy number per

556 μL RNA was determined by qRT-PCR using the SensiFAST Probe Lo-ROX One-Step qRT-

557 PCR System Kit (Bioline) on the ABI 7500 Real Time PCR System (Applied Biosystems)

558 under the following conditions: 45 °C for 10 min, one cycle; 95 °C for 2 min, one cycle; 95 °C

559 for 5 s then 60 °C for 30 s, 40 cycles. Influenza A genomic copies were quantitated by the

560 standard curve method using influenza A RNA samples of known copy number generated in-

561 house. The M gene-targeted primers/TaqMan probe for the detection of influenza A viruses

562 are sourced from the CDC Influenza Division (Atlanta, USA): forward primer (5'-

25

563   ACCRATCCTGTCACCTCTGAC-3'), reverse primer (5'-

564   GGGCATTYTGGACAAAKCGTCTACG-3') and TaqMan probe (6FAM-

565   TGCAGTCCTCGCTCACTGGGCACG-BHQ1). Results were analysed by 7500 Fast System

566   SDS software v1.5.1. The LLOQ was 100 M gene copies/µL RNA, based on the first RNA

567   standard to yield C_t <35.

568   For the London experiments, viral RNA was extracted from 140 µL nasal wash samples

569   using the QIAamp viral RNA mini kit (Qiagen) according to manufacturer's instructions. Real-

570   time RT-PCR was performed using 7500 Real Time PCR system (ABI) in 20 µL reactions

571   using AgPath-ID™ One-Step RT-PCR Reagents 10 µl RT-PCR buffer (2X) (Thermo Fisher),

572   4 µL of RNA, 0.8 µl forward (5'-GACCRATCCTGTCACCTCTGA-3') and reverse primers (5'-

573   AGGGCATTYTGGACAAAKCGTCTA-3') and 0.4 µl probe (5'-FAM-

574   TCGAGTCCTCGCTCACTGGGCACG-BHQ1-3'). The following conditions were used: 45 °C

575   for 10 min, one cycle; 95 °C for 10 min, one cycle; 95 °C for 15 s then 60 °C for 1 min, 40

576   cycles. For each sample, the C_t value for the target M gene was determined, and absolute M

577   gene copy numbers were calculated based on the standard curve method. The LLOQ was

578   153 M gene copies/µL RNA, based on the results from the samples of uninfected ferrets

579   (mean + 2*SD).

Quantitation of I38T-variant population for ferret studies

581   For the Melbourne experiments, detection of the I38T-variant in a virus sample was

582   performed by pyrosequencing as previously described (Koszalka, Farrukee, Mifsud,

583   Vijaykrishna, & Hurt, 2020). The MyTaq One-Step RT-PCR Kit (Bioline) was used to

584   generate and amplify cDNA from viral RNA isolated from nasal wash samples (as above).

585   Samples containing fewer than 200 M gene copies/µL RNA were excluded from analysis.

586   RT-PCR product was processed in the PyroMark Vacuum Workstation according to

587   manufacturer's protocol, and pyrosequencing was performed using the PyroMark ID System

588   (Biotage). Primers were designed to investigate SNPs at position 38 of the PA gene of

589   influenza A/H3N2 and A/H1N1pdm09 (*Supplementary File 1*). Analysis was performed using

590   PyroMarkQ96 ID Software to quantify the percentage of WT and variant in a mixed virus

591   population. The error associated with estimating pure populations of WT or I38T virus was

592   such that values <5% or >95% could not be accurately quantified – therefore, values <5% or

593   >95% were considered to be pure WT or I38T, respectively (Koszalka et al., 2020).

594   For the London experiments, whole-genome NGS was performed using a pipeline at Public

595   Health England (PHE). Viral RNA was extracted from viral lysate using easyMAG

596   (bioMérieux). One-step RT-PCR was performed using SuperScript III (Invitrogen), Platinum

597   Taq HiFi polymerase (Thermo Fisher), and influenza-specific primers (Zhou et al., 2009).

598   The entire ORF of the PA segment was amplified in two overlapping fragments using two

599   pairs of primers (*Supplementary File 1*). Samples were prepared using the Nextera XT

600   library preparation kit (Illumina) and sequenced on an Illumina MiSeq generating a 150-bp

601   paired-end reads. Reads were mapped with BWA (version 0.7.5) and converted to BAM files

602   using SAMTools (version 1.1.2). Variants were called using QuasiBAM, an in-house script at

603   PHE. Analysis of prepared minority variant mixture controls has shown that a cut-off set at

604   5% coupled with a minimum depth of 2000 were reliable parameters to quantify the I38T-

605   variant with 99% confidence (data not shown).

606   We considered the I38T-variant proportion measured at experiment endpoint as dependent

607   on both within-host and between-host components of viral fitness. Within-host fitness was

608   regarded as the observed trend in the proportion of %I38T changing in each individual ferret

609   over time. Between-host fitness was regarded as the observed differences in the measured

610   proportion of I38T-variant in the RF compared to the proportion measured in the DF on the

611   previous day for each transmission event.

612   **Statistical analysis**

613   For in vitro experiments, comparisons of virus titres between WT and I38T viruses at each

614   time-point were conducted using Welch's *t*-test by using the statistical analysis software,

615   SAS version 9.2 at the 0.05 significance level using two-sided tests. No adjustment for

616   multiple testing was performed for exploratory analysis.

617   For the Melbourne in vivo experiments, 2-way ANOVA followed by Bonferroni's post-tests

618   (Prism 5) were performed on daily virus titre data from pure WT- and I38T-group ferrets

619   which were infected by direct contact transmission (n = 3 per group). Data from DF were not

620   included in this analysis as the replication kinetics of experimental infection by direct

621   inoculation of virus differs from the surrogate for 'natural' infection that is achieved by direct

622   contact. AUC analysis was performed, and AUC values for WT and I38T groups were

623   compared by two-tailed Mann-Whitney U test (Prism 5). $p \leq 0.05$ was considered statistically

624   significant.

625   **Ethics**

626   The Melbourne ferret experiments were conducted with approval (AEC#1714278) from the

627   University of Melbourne Biochemistry & Molecular Biology, Dental Science, Medicine,

628   Microbiology & Immunology, and Surgery Animal Ethics Committee, in accordance with the

629   NHMRC Australian code of practice for the care and use of animals for scientific purposes

630   (8th edition). For the London ferret experiments, all work performed was approved by the

631   local genetic manipulation (GM) safety committee of Imperial College London, St. Mary's

632   Campus (centre number GM77), and the Health and Safety Executive of the United

633   Kingdom.

Acknowledgements

639   **Competing interests**

646   **Additional files**

647   **Supplementary files**

648   • Supplementary file 1. Supplemental methods and figures

649

References

Abed, Y., Goyette, N., & Boivin, G. (2005). Generation and Characterization of Recombinant Influenza A (H1N1) Viruses Harboring Amantadine Resistance Mutations. *Antimicrobial Agents and Chemotherapy, 49*(2), 556-559. doi:10.1128/aac.49.2.556-559.2005

Baker, J., Block, S. L., Matharu, B., Burleigh Macutkiewicz, L., Wildum, S., Dimonaco, S., . . . Piedra, P. A. (2020). Baloxavir Marboxil Single-dose Treatment in Influenza-infected Children: A Randomized, Double-blind, Active Controlled Phase 3 Safety and Efficacy Trial (miniSTONE-2). *Pediatr Infect Dis J.* doi:10.1097/INF.0000000000002747

Baranovich, T., Saito, R., Suzuki, Y., Zaraket, H., Dapat, C., Caperig-Dapat, I., . . . Suzuki, H. (2010). Emergence of H274Y oseltamivir-resistant A(H1N1) influenza viruses in Japan during the 2008-2009 season. *J Clin Virol, 47*(1), 23-28. doi:10.1016/j.jcv.2009.11.003

Belser, J. A., Eckert, A. M., Tumpey, T. M., & Maines, T. R. (2016). Complexities in Ferret Influenza Virus Pathogenesis and Transmission Models. *Microbiology and molecular biology reviews : MMBR, 80*(3), 733-744. doi:10.1128/MMBR.00022-16

Bloom, J. D., Gong, L. I., & Baltimore, D. (2010). Permissive secondary mutations enable the evolution of influenza oseltamivir resistance. *Science (New York, N.Y.), 328*(5983), 1272-1275. doi:10.1126/science.1187816

Buchholz, U., Brockmann, S., Duwe, S., Schweiger, B., an der Heiden, M., Reinhardt, B., & Buda, S. (2010). Household transmissibility and other characteristics of seasonal oseltamivir-resistant influenza A(H1N1) viruses, Germany, 2007-8. *Euro Surveill, 15*(6).

Butler, J., Hooper, K. A., Petrie, S., Lee, R., Maurer-Stroh, S., Reh, L., . . . Hurt, A. C. (2014). Estimating the Fitness Advantage Conferred by Permissive Neuraminidase Mutations

676   in Recent Oseltamivir-Resistant A(H1N1)pdm09 Influenza Viruses. *PLOS Pathogens,*

677   *10*(4), e1004065. doi:10.1371/journal.ppat.1004065

678 Checkmahomed, L., M'hamdi, Z., Carbonneau, J., Venable, M.-C., Baz, M., Abed, Y., &

679   Boivin, G. (2019). Impact of the baloxavir-resistant polymerase acid (PA) I38T

680   substitution on the fitness of contemporary influenza A(H1N1)pdm09 and A(H3N2)

681   strains. *The Journal of Infectious Diseases.* doi:10.1093/infdis/jiz418

682 Chesnokov, A., Patel, M. C., Mishin, V. P., De La Cruz, J. A., Lollis, L., Nguyen, H. T., . . .

683   Gubareva, L. V. (2019). Replicative Fitness of Seasonal Influenza A Viruses With

684   Decreased Susceptibility to Baloxavir. *The Journal of Infectious Diseases, 221*(3),

685   367-371. doi:10.1093/infdis/jiz472

686 Dharan, N. J., Gubareva, L. V., Meyer, J. J., Okomo-Adhiambo, M., McClinton, R. C.,

687   Marshall, S. A., . . . Oseltamivir-Resistance Working Group, f. t. (2009). Infections

688   With Oseltamivir-Resistant Influenza A(H1N1) Virus in the United States. *JAMA,*

689   *301*(10), 1034-1041. doi:10.1001/jama.2009.294

690 Dias, A., Bouvier, D., Crepin, T., McCarthy, A. A., Hart, D. J., Baudin, F., . . . Ruigrok, R. W.

691   (2009). The cap-snatching endonuclease of influenza virus polymerase resides in the

692   PA subunit. *Nature, 458*(7240), 914-918. doi:10.1038/nature07745

693 Dong, G., Peng, C., Luo, J., Wang, C., Han, L., Wu, B., . . . He, H. (2015). Adamantane-

694   Resistant Influenza A Viruses in the World (1902–2013): Frequency and Distribution

695   of M2 Gene Mutations. *PLOS ONE, 10*(3), e0119115.

696   doi:10.1371/journal.pone.0119115

697 Duan, S., Boltz, D. A., Seiler, P., Li, J., Bragstad, K., Nielsen, L. P., . . . Govorkova, E. A.

698   (2010). Oseltamivir–Resistant Pandemic H1N1/2009 Influenza Virus Possesses

699   Lower Transmissibility and Fitness in Ferrets. *PLOS Pathogens, 6*(7), e1001022.

700   doi:10.1371/journal.ppat.1001022

701 Frise, R., Bradley, K., van Doremalen, N., Galiano, M., Elderfield, R. A., Stilwell, P., . . .

702   Barclay, W. S. (2016). Contact transmission of influenza virus between ferrets

703   imposes a looser bottleneck than respiratory droplet transmission allowing

704   propagation of antiviral resistance. *Sci Rep, 6*, 29793. doi:10.1038/srep29793

705   Goldhill, D. H., te Velthuis, A. J. W., Fletcher, R. A., Langat, P., Zambon, M., Lackenby, A., &

706   Barclay, W. S. (2018). The mechanism of resistance to favipiravir in influenza.

707   *Proceedings of the National Academy of Sciences, 115*(45), 11613-11618.

708   doi:10.1073/pnas.1811345115

709   Hayden, F. G., Belshe, R. B., Clover, R. D., Hay, A. J., Oakes, M. G., & Soo, W. (1989).

710   Emergence and apparent transmission of rimantadine-resistant influenza A virus in

711   families. *N Engl J Med, 321*(25), 1696-1702. doi:10.1056/nejm198912213212502

712   Hayden, F. G., Sugaya, N., Hirotsu, N., Lee, N., de Jong, M. D., Hurt, A. C., . . . Watanabe,

713   A. (2018). Baloxavir Marboxil for Uncomplicated Influenza in Adults and Adolescents.

714   *New England Journal of Medicine, 379*(10), 913-923. doi:10.1056/NEJMoa1716197

715   Heneghan, C. J., Onakpoya, I., Jones, M. A., Doshi, P., Del Mar, C. B., Hama, R., . . .

716   Jefferson, T. (2016). Neuraminidase inhibitors for influenza: a systematic review and

717   meta-analysis of regulatory and mortality data. *Health Technol Assess, 20*(42), 1-

718   242. doi:10.3310/hta20420

719   Herlocher, M. L., Carr, J., Ives, J., Elias, S., Truscon, R., Roberts, N., & Monto, A. S. (2002).

720   Influenza virus carrying an R292K mutation in the neuraminidase gene is not

721   transmitted in ferrets. *Antiviral research, 54*(2), 99-111.

722   doi:https://doi.org/10.1016/S0166-3542(01)00214-5

723   Hirotsu, N., Sakaguchi, H., Sato, C., Ishibashi, T., Baba, K., Omoto, S., . . . Watanabe, A.

724   (2019). Baloxavir marboxil in Japanese pediatric patients with influenza: safety and

725   clinical and virologic outcomes. *Clin Infect Dis*. doi:10.1093/cid/ciz908

726   Hurt, A. C., Ernest, J., Deng, Y. M., Iannello, P., Besselaar, T. G., Birch, C., . . . Barr, I. G.

727   (2009). Emergence and spread of oseltamivir-resistant A(H1N1) influenza viruses in

728   Oceania, South East Asia and South Africa. *Antiviral research, 83*(1), 90-93.

729   doi:10.1016/j.antiviral.2009.03.003

730 Hurt, A. C., Hardie, K., Wilson, N. J., Deng, Y. M., Osbourn, M., Leang, S. K., . . . Barr, I. G.

731 (2012). Characteristics of a widespread community cluster of H275Y oseltamivir-

732 resistant A(H1N1)pdm09 influenza in Australia. *J Infect Dis, 206*(2), 148-157.

733 doi:10.1093/infdis/jis337

734 Hussain, M., Galvin, H. D., Haw, T. Y., Nutsford, A. N., & Husain, M. (2017). Drug resistance

735 in influenza A virus: the epidemiology and management. *Infection and drug*

736 *resistance, 10*, 121-134. doi:10.2147/IDR.S105473

737 Imai, M., Yamashita, M., Sakai-Tagawa, Y., Iwatsuki-Horimoto, K., Kiso, M., Murakami, J., . .

738 . Kawaoka, Y. (2019). Influenza A variants with reduced susceptibility to baloxavir

739 isolated from Japanese patients are fit and transmit through respiratory droplets.

740 *Nature Microbiology*. doi:10.1038/s41564-019-0609-0

741 Ison, M. G., Portsmouth, S., Yoshida, Y., Shishido, T., Mitchener, M., Tsuchiya, K., . . .

742 Hayden, F. G. (2020). Early treatment with baloxavir marboxil in high-risk adolescent

743 and adult outpatients with uncomplicated influenza (CAPSTONE-2): a randomised,

744 placebo-controlled, phase 3 trial. *Lancet Infect Dis*. doi:10.1016/S1473-

745 3099(20)30004-9

746 Ives, J. A., Carr, J. A., Mendel, D. B., Tai, C. Y., Lambkin, R., Kelly, L., . . . Roberts, N. A.

747 (2002). The H274Y mutation in the influenza A/H1N1 neuraminidase active site

748 following oseltamivir phosphate treatment leave virus severely compromised both in

749 vitro and in vivo. *Antiviral research, 55*(2), 307-317. doi:10.1016/s0166-

750 3542(02)00053-0

751 Jones, J. C., Pascua, P. N. Q., Fabrizio, T. P., Marathe, B. M., Seiler, P., Barman, S., . . .

752 Govorkova, E. A. (2020). Influenza A and B viruses with reduced baloxavir

753 susceptibility display attenuated in vitro fitness but retain ferret transmissibility.

754 *Proceedings of the National Academy of Sciences*, 201916825.

755 doi:10.1073/pnas.1916825117

756 Koszalka, P., Farrukee, R., Mifsud, E., Vijaykrishna, D., & Hurt, A. C. (2020). A rapid

757 pyrosequencing assay for the molecular detection of influenza viruses with reduced

758   baloxavir susceptibility due to PA/I38X amino acid substitutions. *Influenza Other*

759   *Respir Viruses*. doi:10.1111/irv.12725

760 Lackenby, A., Besselaar, T. G., Daniels, R. S., Fry, A., Gregory, V., Gubareva, L. V., . . .

761   Meijer, A. (2018). Global update on the susceptibility of human influenza viruses to

762   neuraminidase inhibitors and status of novel antivirals, 2016-2017. *Antiviral research,*

763   *157*, 38-46. doi:10.1016/j.antiviral.2018.07.001

764 Li, T. C., Chan, M. C., & Lee, N. (2015). Clinical Implications of Antiviral Resistance in

765   Influenza. *Viruses, 7*(9), 4929-4944. doi:10.3390/v7092850

766 Meijer, A., Lackenby, A., Hungnes, O., Lina, B., van-der-Werf, S., Schweiger, B., . . .

767   Zambon, M. (2009). Oseltamivir-resistant influenza virus A (H1N1), Europe, 2007-08

768   season. *Emerg Infect Dis, 15*(4), 552-560. doi:10.3201/eid1504.181280

769 Mishin, V. P., Patel, M. C., Chesnokov, A., De La Cruz, J., Nguyen, H. T., Lollis, L., . . .

770   Gubareva, L. V. (2019). Susceptibility of Influenza A, B, C, and D Viruses to

771   Baloxavir. *Emerg Infect Dis, 25*(10), 1969-1972. doi:10.3201/eid2510.190607

772 Noshi, T., Kitano, M., Taniguchi, K., Yamamoto, A., Omoto, S., Baba, K., . . . Naito, A.

773   (2018). In vitro characterization of baloxavir acid, a first-in-class cap-dependent

774   endonuclease inhibitor of the influenza virus polymerase PA subunit. *Antiviral*

775   *research*. doi:10.1016/j.antiviral.2018.10.008

776 Oh, D. Y., Barr, I. G., & Hurt, A. C. (2015). A Novel Video Tracking Method to Evaluate the

777   Effect of Influenza Infection and Antiviral Treatment on Ferret Activity. *PLOS ONE,*

778   *10*(3), e0118780. doi:10.1371/journal.pone.0118780

779 Omoto, S., Speranzini, V., Hashimoto, T., Noshi, T., Yamaguchi, H., Kawai, M., . . . Cusack,

780   S. (2018). Characterization of influenza virus variants induced by treatment with the

781   endonuclease inhibitor baloxavir marboxil. *Sci Rep, 8*(1), 9633. doi:10.1038/s41598-

782   018-27890-4

783 Patel, T. S., Cinti, S., Sun, D., Li, S., Luo, R., Wen, B., . . . Stevenson, J. G. (2017).

784   Oseltamivir for pandemic influenza preparation: Maximizing the use of an existing

785   stockpile. *Am J Infect Control, 45*(3), 303-305. doi:10.1016/j.ajic.2016.09.024

786   Simonsen, L., Viboud, C., Grenfell, B. T., Dushoff, J., Jennings, L., Smit, M., . . . Holmes, E.

787   C. (2007). The genesis and spread of reassortment human influenza A/H3N2 viruses

788   conferring adamantane resistance. *Mol Biol Evol, 24*(8), 1811-1820.

789   doi:10.1093/molbev/msm103

790   Takashita, E., Daniels, R. S., Fujisaki, S., Gregory, V., Gubareva, L. V., Huang, W., . . .

791   Meijer, A. (2020). Global update on the susceptibilities of human influenza viruses to

792   neuraminidase inhibitors and the cap-dependent endonuclease inhibitor baloxavir,

793   2017-2018. *Antiviral research, 175*, 104718. doi:10.1016/j.antiviral.2020.104718

794   Takashita, E., Ejima, M., Itoh, R., Miura, M., Ohnishi, A., Nishimura, H., . . . Tashiro, M.

795   (2014). A community cluster of influenza A(H1N1)pdm09 virus exhibiting cross-

796   resistance to oseltamivir and peramivir in Japan, November to December 2013. *Euro*

797   *Surveill, 19*(1). doi:10.2807/1560-7917.es2014.19.1.20666

798   Takashita, E., Ichikawa, M., Morita, H., Ogawa, R., Fujisaki, S., Shirakura, M., . . . Odagiri, T.

799   (2019). Human-to-Human Transmission of Influenza A(H3N2) Virus with Reduced

800   Susceptibility to Baloxavir, Japan, February 2019. *Emerg Infect Dis, 25*(11), 2108-

801   2111. doi:10.3201/eid2511.190757

802   Takashita, E., Kawakami, C., Ogawa, R., Morita, H., Fujisaki, S., Shirakura, M., . . . Odagiri,

803   T. (2019). Influenza A(H3N2) virus exhibiting reduced susceptibility to baloxavir due

804   to a polymerase acidic subunit I38T substitution detected from a hospitalised child

805   without prior baloxavir treatment, Japan, January 2019. *Euro Surveill, 24*(12),

806   1900170. doi:10.2807/1560-7917.ES.2019.24.12.1900170

807   Takashita, E., Kiso, M., Fujisaki, S., Yokoyama, M., Nakamura, K., Shirakura, M., . . .

808   Tashiro, M. (2015). Characterization of a large cluster of influenza A(H1N1)pdm09

809   viruses cross-resistant to oseltamivir and peramivir during the 2013-2014 influenza

810   season in Japan. *Antimicrob Agents Chemother, 59*(5), 2607-2617.

811   doi:10.1128/aac.04836-14

812   Tu, V., Abed, Y., Barbeau, X., Carbonneau, J., Fage, C., Lagüe, P., & Boivin, G. (2017). The

813   I427T neuraminidase (NA) substitution, located outside the NA active site of an

35

814   influenza A(H1N1)pdm09 variant with reduced susceptibility to NA inhibitors, alters

815   NA properties and impairs viral fitness. *Antiviral research, 137*, 6-13.

816   doi:https://doi.org/10.1016/j.antiviral.2016.11.007

817 Uehara, T., Hayden, F. G., Kawaguchi, K., Omoto, S., Hurt, A. C., De Jong, M. D., . . . Kida,

818   H. (2019). Treatment-Emergent Influenza Variant Viruses With Reduced Baloxavir

819   Susceptibility: Impact on Clinical and Virologic Outcomes in Uncomplicated Influenza.

820   *The Journal of Infectious Diseases*. doi:10.1093/infdis/jiz244

821 WHO. (2020). Recommended composition of influenza virus vaccines for use in the 2020–

822   2021 Northern Hemisphere influenza season. Retrieved from

823   https://www.who.int/influenza/vaccines/virus/recommendations/202002_recommenda

824   tion.pdf

825 Yen, H.-L., Herlocher, L. M., Hoffmann, E., Matrosovich, M. N., Monto, A. S., Webster, R. G.,

826   & Govorkova, E. A. (2005). Neuraminidase Inhibitor-Resistant Influenza Viruses May

827   Differ Substantially in Fitness and Transmissibility. *Antimicrobial Agents and*

828   *Chemotherapy, 49*(10), 4075-4084. doi:10.1128/aac.49.10.4075-4084.2005

829 Zhou, B., Donnelly, M. E., Scholes, D. T., St George, K., Hatta, M., Kawaoka, Y., &

830   Wentworth, D. E. (2009). Single-reaction genomic amplification accelerates

831   sequencing and vaccine production for classical and Swine origin human influenza a

832   viruses. *J Virol, 83*(19), 10309-10313. doi:10.1128/JVI.01109-09

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835 **Supplementary file 1**

836 **Supplementary methods**

837 - Cell culture

838   o MDCK-SIAT1 cells were obtained from the European Collection of Cell

839   Cultures and were grown in Dulbecco's modified Eagle's medium (DMEM)

840   supplemented with 2 mM L-glutamine solution (Nacalai Tesque, Inc.), 1

841   μg/mL Geneticin^{TM} selective antibiotic (Thermo Fisher Scientific, Inc.), 10%

842   fetal bovine serum (FBS) (Sigma-Aldrich Co., Ltd.), and penicillin-

843   streptomycin (Thermo Fisher Scientific, Inc.).

844 - Virus titration

845   o The virus titre of clinical isolates was determined by the 50% tissue culture

846   infectious dose (TCID$_{50}$) method in MDCK-SIAT1 cells in DMEM containing

847   0.2% bovine serum albumin (BSA) (Sigma-Aldrich Co., Ltd.), 25 mM HEPES

848   (Thermo Fisher Scientific, Inc.), 2 mM L-glutamine solution, 1 μg/mL trypsin

849   from bovine pancreas-TPCK treated (TPCK trypsin) solution (Sigma-Aldrich

850   Co., Ltd.), and penicillin-streptomycin.

851 - In vitro competitive fitness of rg virus pairs:

852   o Competitive fitness experiments were conducted in MDCK cells using 50:50

853   mixtures of WT/I38X virus pairs prepared volumetrically based on infectious

854   viral titres (TCID$_{50}$). The presence of I38X variant in cell culture supernatant

855   was determined by Sanger sequencing.

856 Melbourne methods:

857 - Cell culture

858   o MDCK-SIAT1 cells were obtained from ATCC. Cultures were grown in

859   Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% (v/v)

860   foetal bovine serum, 2 mM GlutaMAX, 0.05% sodium bicarbonate, 100 μM

861   MEM non-essential amino acid, 20 mM HEPES, 500mg Geneticin and 50,000

862   U penicillin-streptomycin.

863   • Virus titration

864   o The virus titre of clinical isolates was determined by the $TCID_{50}$ method in

865   MDCK-SIAT1 cells in DMEM containing 2 mM GlutaMAX, 0.05% sodium

866   bicarbonate, 100 μM MEM non-essential amino acid, 20 mM HEPES,

867   100,000 U penicillin-streptomycin, 1000 μg Amphotericin B and 4 μg/mL of

868   TPCK-treated trypsin.

869   • Ferret handling procedures

870   o Identification/temperature-monitoring chips (LifeChip, Bio-Thermo) were

871   implanted subcutaneously on the dorsal region of each ferret. Animals were

872   monitored at least once daily for temperature and weight changes, had *ad*

873   *libitum* access to pellet feed (Eukanuba) and water and were provided with

874   supplementary wet food (Hill's Pet Nutrition, Australia) as ethically required

875   (below 90% baseline weight). Nasal wash samples were collected using 1 mL

876   of PBS containing 1% w/v BSA. At the end of each experiment, ferrets were

877   anaesthetised before sacrifice by intracardiac injection of sodium

878   pentobarbitone (≤1,000 mg/kg, Troy Laboratories).

879   • Pyrosequencing primers (Melbourne)

880   o A/H3N2: Forward (Biotin-5'-TTGTCGAACTTGCAGAAAAGGC-3'), reverse

881   (5'-GCCATTGTTCTGTCTCTCCCCT-3') and pyrosequencing (5'-

882   CATACCTCCAAGTGAGTGCA-3', reverse orientation)

883   o A/H1N1pmd09: Forward (Biotin-5'-CAATCCAATGATCGTCGAGC-3'),

884   reverse (5'-GGTGCTTCAATAGTGCATTTGG-3') and pyrosequencing (5'-

885   CAAACTTCCAAATGTGTGCA-3', reverse orientation)

886   London methods:

887   • Ferret handling

38

888   o   Body weight was measured daily, and strict procedures were followed to

889   prevent aberrant cross-contamination between animals. Sentinel animals

890   were handled before inoculated animals, and work surfaces and handlers'

891   gloves were decontaminated between animals.

892   •   Cell culture:

893   o   MDCK cells were maintained in Dulbecco's modified Eagle's medium (DMEM;

894   Gibco, Invitrogen) supplemented with 10% FBS and 1%

895   penicillin/streptomycin (Sigma-Aldrich) and 1% non-essential amino acid

896   (Gibco, Invitrogen).

897   •   Plaque assays:

898   o   100% confluent MDCK cell monolayers were inoculated with 100 µL of serially

899   diluted samples and overlaid with 0.6% agarose (Oxoid) in supplemented

900   DMEM with 1 µg trypsin (Worthington) mL−1 and incubated at 37°C for 3

901   days.

902   •   Next-generation sequencing primer sets:

903   o   Full genome sequencing as described previously (Zhou et al., 2009)

904   o   PA ORF amplified in two overlapping fragments PA-1 and PA-2. PA-1 forward

905   (5'-AGCAAAAGCAGGTACTGATCCAA-3'), PA-1 reverse (5'-

906   CTTGAATCAGTCAATTCACATGCC-3'), PA-2 forward (5'-

907   ATGCAATCAAATGCATGAAGA-3'), PA-2 reverse 5'-

908   ATATGGTCTCGTATTAGTAGAAACAAGGTACTT-3')

909

910 **Supplementary Table.** Amino acid and nucleotide difference in A/H1N1pdm09 and A/H3N2

911 viruses isolated from baloxavir-treated patients.

	Segment	A/H1N1pdm09		A/H3N2		
		2HB001	2PQ003	344103	339111	253104
Amino acid	HA	-*	-	-	-	-

	NA	-	-	-	-	-
	PA	I38T	I38T	I38T	I38T	I38T
Nucleotide	HA	-	-	t885c	-	-
	NA	g1245a	-	-	-	a732g
	PA	t113c	t113c	t113c	t113c g1698a	t113c

912   *No mutation was detected compared with the cognate wild type virus in amino acid or

913   nucleotide. HA, haemagglutinin NA, neuraminidase; PA, polymerase acidic
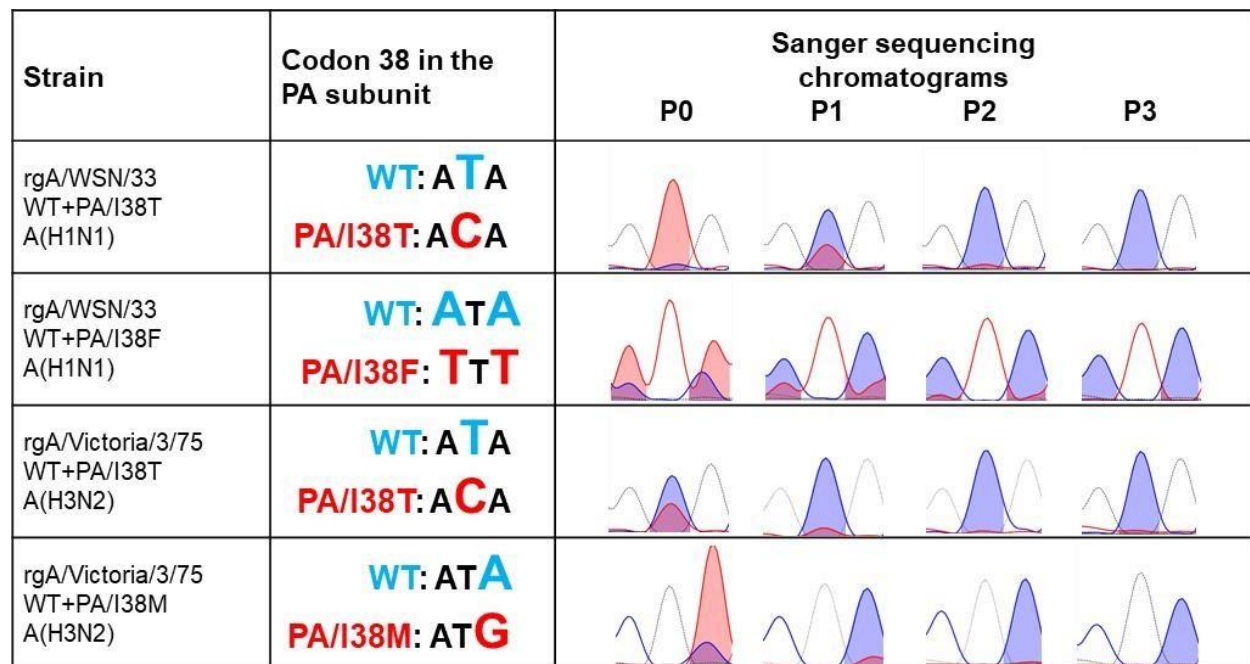
914

915 **Supplementary figures**

916

917 **Figure 1 – figure supplement 1. Competitive replicative capacity of influenza**
918 **reverse genetics-derived viruses with PA/I38 substitutions.** Competitive
919 replicative capacity of reverse genetics-derived influenza viruses with I38
920 substitutions. MDCK cells were co-infected with reverse genetics-derived WT and
921 I38-substitutited viruses at 50:50 ratio based on viral titres at an MOI of 0.001 each.
922 At 48 hours post infection, the culture supernatant was collected and serially
923 passaged three times. The virus passage experiments were conducted in duplicate
924 as lineage 1 and lineage 2. Each culture supernatant was subjected to Sanger
925 sequencing to analyse the change of amino acid at 38 in PA subunit. Sanger
926 sequence chromatograms of amino acid at position 38 in the PA subunit were
927 analysed using BioPython, and blue chromatograph represents WT and red shows
928 /I38-substituted viruses. Representative sequencing chromatographs of lineage 1
929 were shown because the similar results were obtained from both lineages. MOI,
930 multiplicity of infection; PA, polymerase acid; P0–P3, passage 0 to passage 3; WT,
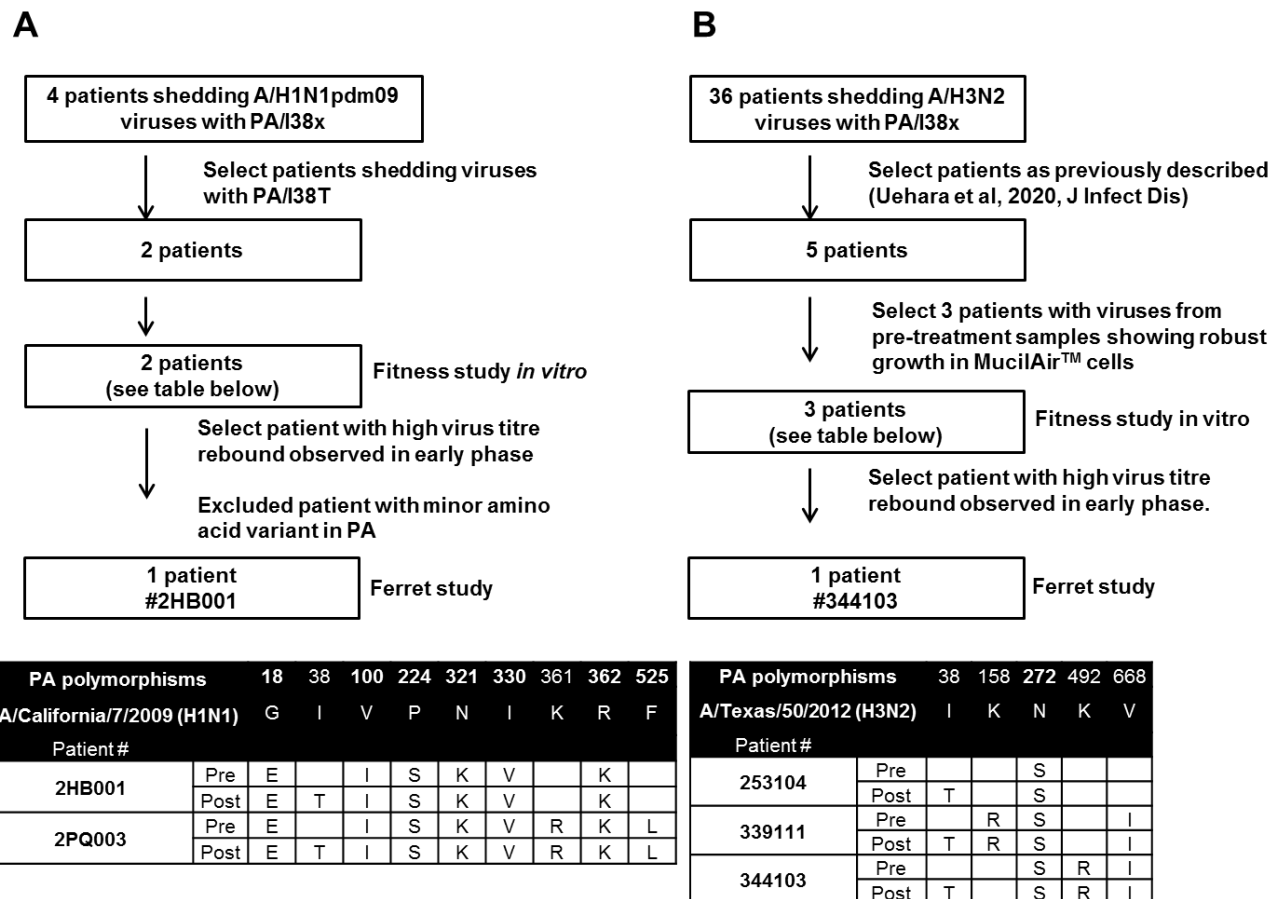931 wild type.

A

4 patients shedding A/H1N1pdm09 viruses with PA/I38x

↓ Select patients shedding viruses with PA/I38T

2 patients

↓

2 patients (see table below) — Fitness study *in vitro*

↓ Select patient with high virus titre rebound observed in early phase

↓ Excluded patient with minor amino acid variant in PA

1 patient #2HB001 — Ferret study

B

36 patients shedding A/H3N2 viruses with PA/I38x

↓ Select patients as previously described (Uehara et al, 2020, J Infect Dis)

5 patients

↓ Select 3 patients with viruses from pre-treatment samples showing robust growth in MucilAir™ cells

3 patients (see table below) — Fitness study in vitro

↓ Select patient with high virus titre rebound observed in early phase.

1 patient #344103 — Ferret study

PA polymorphisms		18	38	100	224	321	330	361	362	525
A/California/7/2009 (H1N1)		G	I	V	P	N	I	K	R	F
Patient #										
2HB001	Pre	E		I	S	K	V		K	
	Post	E	T	I	S	K	V		K	
2PQ003	Pre	E		I	S	K	V	R	K	L
	Post	E	T	I	S	K	V	R	K	L

PA polymorphisms		38	158	272	492	668
A/Texas/50/2012 (H3N2)		I	K	N	K	V
Patient #						
253104	Pre			S		
	Post	T		S		
339111	Pre		R	S		I
	Post	T	R	S		I
344103	Pre			S	R	I
	Post	T		S	R	I

932

933 **Figure 1 – figure supplement 2. Sample selection for fitness study in vitro and**
934 **ferret transmission study.** Sample selection process for in vitro fitness and ferret
935 transmission studies of I38-substituted viruses propagated from swab samples. Flow
936 diagram of sample selection for fitness study in vitro and ferret transmission study for
937 (A) influenza A/H1N1pdm09 and (B) influenza A/H3N2 viruses. Polymorphic amino
938 acid substitutions in PA protein aligned with (A) influenza A/California/7/2009
939 (A/H1N1) and (B) influenza A/Texas/50/2012 (A/H3N2) are also shown. PA,
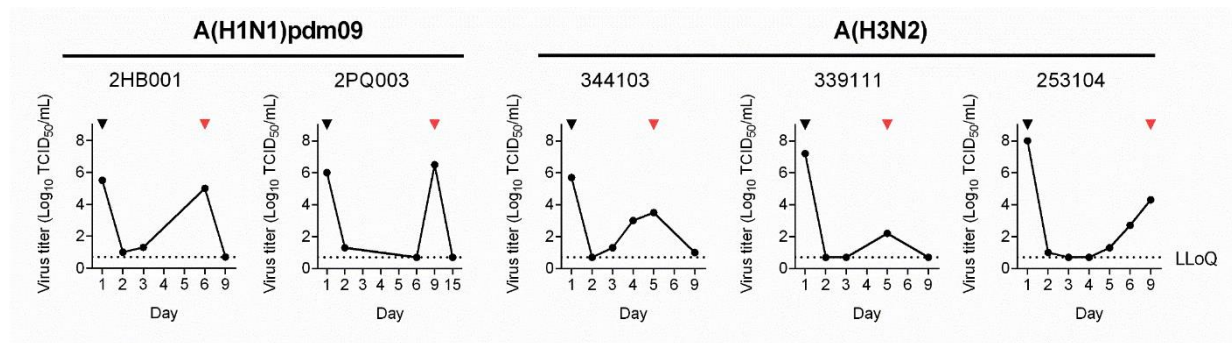940 polymerase acidic.
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942
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Figure 1 – figure supplement 3. Influenza virus titre (log10 TCID50/mL) in individual patients with PA/I38T-substituted viruses. Influenza virus titre ($\log_{10}$ $TCID_{50}$/mL) in individual patients with I38T-substituted viruses. Time course of influenza virus titre of individual baloxavir-treated patients with I38X-substituted viruses, determined by $TCID_{50}$ assay (values below LLOQ were set at 0.7 $\log_{10}$ $TCID_{50}$/mL). The black and red arrowheads indicate the sampling time-points for WT viruses (prebaloxavir treatment) and I38T-substituted viruses (post-baloxavir treatment), respectively. Black dotted line = LLOQ at 0.7 $\log_{10}$ $TCID_{50}$. LLOQ, lower limit of quantitation; $TCID_{50}$, 50% tissue culture infectious dose.
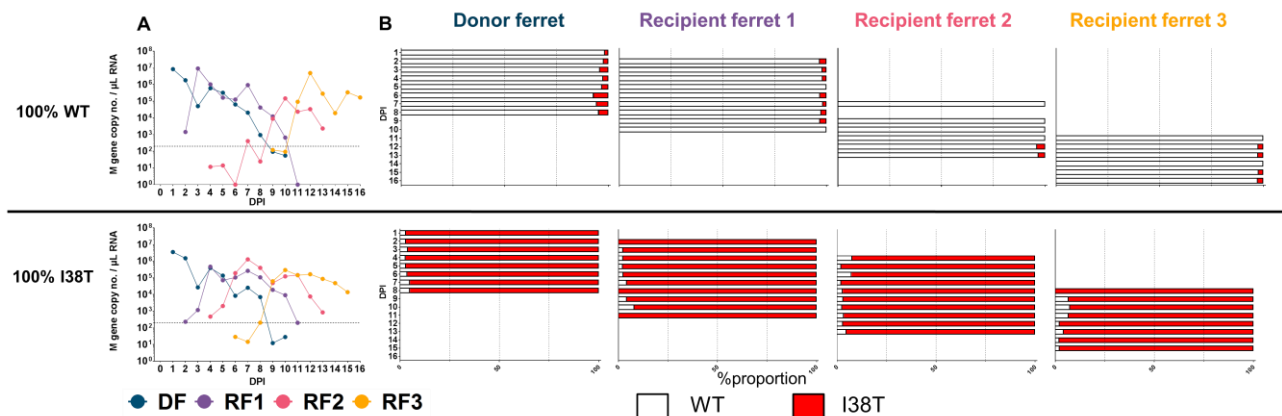
Figure 2 – figure supplement 1. Pyrosequencing of ferret nasal washes from WT or I38T-variant A/H3N2 pure population groups. (**A**) Viral RNA and (**B**) pyrosequencing of ferret nasal washes from A/H3N2 WT or I38T pure population groups.
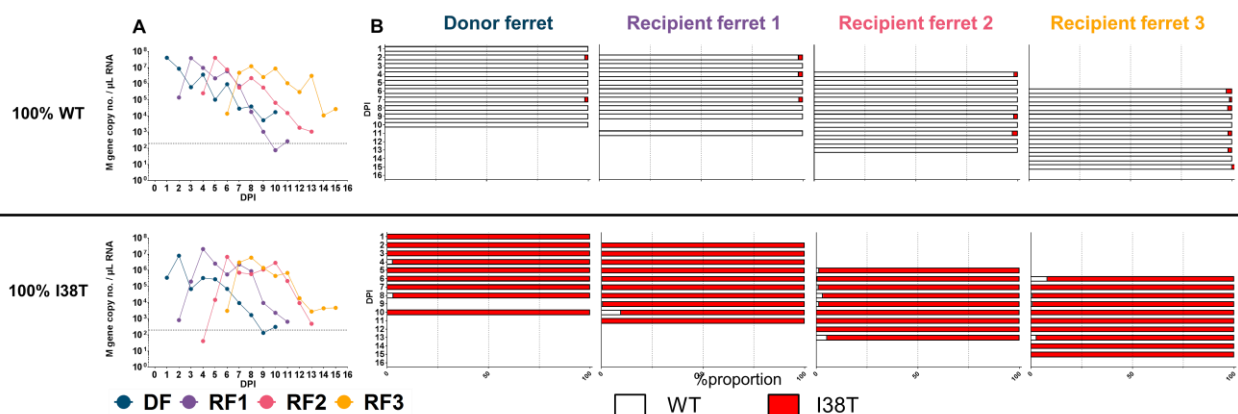DPI, days post inoculation; WT, wild type.

Figure 2 – figure supplement 2. Pyrosequencing of ferret nasal washes from WT or I38T-variant A/H1N1pdm09 pure population groups. (A) Viral RNA and (B) pyrosequencing of ferret nasal washes from A/H1N1pdm09 WT or I38T pure population groups. DPI, days post inoculation; WT, wild type.
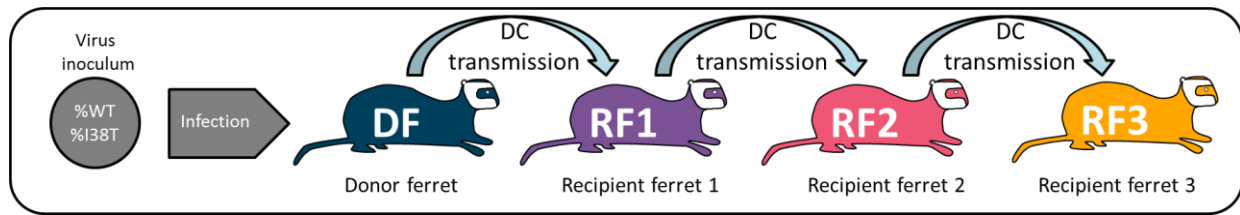
983

Figure 3 – figure supplement 1. Ferret experiment designs. Ferret transmission experiment design schematics for Melbourne clinical isolate studies. DC, direct contact; DF, donor ferret; RF, recipient ferret; WT, wild type.
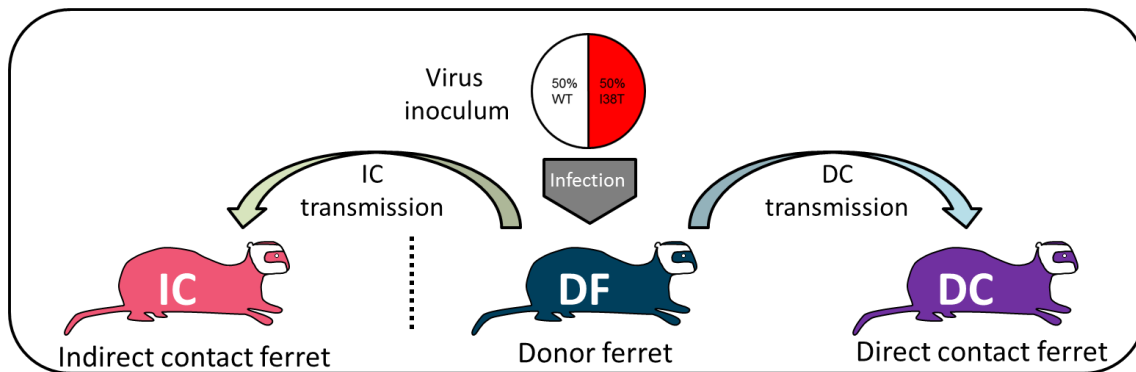
987

988

989

Figure 6 – figure supplement 1. Ferret experiment designs. Ferret transmission experiment design schematics the London recombinant virus study. DC, direct contact; DF, donor ferret; IC, indirect contact; WT, wild type.

993