Impact of the Baloxavir-Resistant Polymerase Acid I38T Substitution on the Fitness of Contemporary Influenza A(H1N1)pdm09 and A(H3N2) Strains

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Background: Baloxavir is a cap-dependent inhibitor of the polymerase acid (PA) protein of influenza viruses. While appearing virologically superior to oseltamivir, baloxavir exhibits a low barrier of resistance. We sought to assess the impact of the common baloxavir-resistant I38T PA substitution on in vitro properties and virulence.

Methods: Influenza A/Quebec/144147/2009 (H1N1)pdm09 and A/Switzerland/9715293/2013 (H3N2) recombinant viruses and their I38T PA mutants were compared in single and competitive infection experiments in ST6GalI-MDCK cells and C57/BL6 mice. Virus titers in cell culture supernatants and lung homogenates were determined by virus yield assays. Ratios of wild-type (WT) and I38T mutant were assessed by digital RT-PCR.

Results: I38T substitution did not alter the replication kinetics of A(H1N1)pdm09 and A(H3N2) viruses. In competition experiments, a 50%:50% mixture evolved to 70%:30% (WT/mutant) for A(H1N1) and 88%:12% for A(H3N2) viruses after a single cell passage. The I38T substitution remained stable after 4 passages in vitro. In mice, the WT and its I38T mutant induced similar weight loss with comparable lung titers in both viral subtypes. The mutant virus tended to predominate over the WT in mouse competition experiments.

Conclusion: The fitness of baloxavir-resistant I38T PA mutants appears relatively unaltered in seasonal subtypes warranting surveillance for its dissemination.

Keywords. influenza; baloxavir; resistance; I38T; PA; A(H1N1)pdm09; A(H3N2).

Influenza infections constitute a major public health priority. Despite causing relatively benign symptoms in healthy adults, seasonal influenza viruses are associated with significant morbidity and mortality in high-risk groups, such as young children, elderly individuals, pregnant women, and immunocompromised patients [1]. Immunization with inactivated or live-attenuated influenza vaccines remains the first line of defense against influenza epidemics, but vaccine efficacy can be significantly reduced when antigenic drifts occur [2].

Antiviral agents constitute another option that can be used to prevent and treat influenza virus infection. Besides the use of neuraminidase inhibitors, including oseltamivir, zanamivir, and peramivir for more than a decade [3, 4], a new orally available drug (baloxavir marboxil [BXM]; Xofluza) has been approved in the United States since 2018 [5]. Baloxavir acid, the

active compound of BXM, is a cap-dependent endonuclease inhibitor of the influenza polymerase acid (PA) protein. This antiviral blocks the initiation of mRNA synthesis through its binding with divalent cations to the active site of the endonuclease enzyme [6]. Baloxavir demonstrated potent in vitro activity against influenza A and B viruses, including variants that were resistant to neuraminidase inhibitors and adamantanes [6]. In mice experimentally infected with influenza A/Puerto Rico/8/1934 (H1N1) virus, BXM provided a significant reduction in viral titers and proinflammatory cytokine production as well as prevented mortality [7]. A phase 3 clinical trial showed that oral administration of a single dose (40 or 80 mg) of BXM reduced the duration of influenza symptoms to an extent similar to that seen with oseltamivir (53.5 hours vs 53.8 hours) [8]. Furthermore, BXM was associated with a significantly greater reduction in the median duration of viral shedding than oseltamivir (24 hours vs 72 hours) [8].

On the other hand, BXM appears to have a low genetic barrier to the emergence of resistance. Indeed, a recent clinical trial showed that drug-resistant influenza A/B variants emerged in approximately 10% of baloxavir-treated patients [8]. The mechanism of resistance in most of these cases involved substitutions of the conserved isoleucine 38 residue within the endonuclease domain of the PA protein [8, 9]. When characterized in vitro

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using old laboratory strains of the A(H1N1) (A/WSN/33 strain) [10] and A(H3N2) (A/Victoria/3/75 strain) [6] subtypes, the I38T substitution greatly reduced susceptibility to baloxavir and the mutant had decreased replication kinetics at early time points compared to the wild-type (WT) virus.

In this study, we sought to assess the impact of the I38T substitution on replication kinetics of contemporary recombinant influenza A(H1N1)pdm09 and A(H3N2) influenza strains, both in vitro and in animal models, using a competition approach.

MATERIALS AND METHODS

Cells and Viruses

Madin-Darby canine kidney cells overexpressing the α 2,6 sialic acid receptor (ST6-GalI-MDCK cells; kindly provided by Y. Kawaoka from the University of Wisconsin, Madison, WI [11]) and human embryonic kidney 293T cells (ATCC, CRL3216) were maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (Invitrogen, Carlsbad, CA) and antibiotics.

The WT influenza A/Quebec/144147/09 (H1N1)pdm09 virus (GenBank No. [FN434457-FN434464](#)), the mouse-adapted A/Switzerland/9715293/13 (H3N2) virus (GenBank No. [KY116075-KY116082](#)) and their respective I38T PA variants were generated by reverse genetics and polymerase chain reaction (PCR)-mediated mutagenesis as previously described [12]. Viral stocks were prepared in ST6-GalI-MDCK cells and PA genes were reverse transcription-PCR (RT-PCR) amplified and sequenced using the automated ABI Prism 377 DNA sequencer (Applied Biosystems, Foster City, CA).

Determination of Drug Susceptibility Phenotypes

The phenotype of susceptibility to baloxavir acid was determined for recombinant A(H1N1)pdm09 and A(H3N2) WT viruses and their respective I38T PA mutants using viral yield assays. Briefly, confluent ST6GalI-MDCK cells in 12-well plates were infected with 50 plaque-forming units (PFU)/well in the presence of baloxavir acid at concentrations ranging between 0 and 250 nM, using 2-fold change serial dilutions. After 3 days of incubation at 37°C, supernatants were collected and titrated as 50% tissue culture infectious dose (TCID₅₀)/mL.

Minigenome Assay for Polymerase Activity

To investigate the effect of the I38T PA substitution on viral RNA polymerase activity, a reconstituted minigenome assay was performed. As a reporter signal, the coding sequence of the *Gaussia* luciferase gene, flanked by noncoding regions of the influenza A/Quebec/144147/09 nonstructural (NS) gene, was amplified by PCR and inserted into the pHH21 plasmid [13]. The resulting reporter plasmid (pHH21-vNS-Luc) was cotransfected with pLLBG plasmids containing NP, PB1, PB2, and PA genes of A(H1N1)pdm09 or A(H3N2) viruses in 293T cells using Lipofectamine 2000 reagent (Invitrogen, Carlsbad,

CA). At 18 hours posttransfection, cells were harvested and luminescence was measured with a multilabel plate reader (Victor; PerkinElmer, Waltham, MA), using an acquisition period of 1 second. Luciferase activity values were average of 3 independent experiments.

Droplet Digital Reverse-Transcription PCR Studies

The droplet digital reverse-transcription PCR (ddRT-PCR) tests were designed as previously reported [14] to determine WT and I38T ratios in samples collected from in vitro and mouse competition experiments. Viral RNA was first extracted from cell culture supernatants or lung homogenates using the QIAmp Viral RNA Mini Kit (QIAGEN, Mississauga, Ontario). The workflow and data analyses were performed with the One-Step ddRT-PCR supermix according to the manufacturer's instructions. The primers and probes targeting the I38 (WT) and T38 (mutant) variants for both A(H1N1)pdm09 and A(H3N2) strains are available upon request. The cycling and postcycling steps were done as previously reported [14]. The cycled plate was then transferred and read in the FAM and HEX channels of the QX200 reader (Bio-Rad, Montréal, Canada).

Replication Kinetics Experiments

Replicative capacities of A(H1N1)pdm09 and A(H3N2) WT recombinant viruses and their respective I38T PA mutants were evaluated by infecting 12-well plates of confluent ST6GalI-MDCK cells at a multiplicity of infection of 0.0001 PFUs/cell (50 PFUs per well). Supernatants were collected at serial indicated time points for determination of viral titers (TCID₅₀/mL) in ST6GalI-MDCK cells.

In Vitro Genetic Stability

The WT A(H1N1)pdm09 and A(H3N2) recombinant viruses and their respective I38T PA mutants were submitted to 4 serial passages in ST6GalI-MDCK cells. Confluent cells in 6-well plates were washed with phosphate-buffered saline before viral inoculation using a multiplicity of infection (MOI) of 0.001 PFU/cell. After a 1-hour adsorption step at 37°C, the supernatant was removed and fresh DMEM medium containing 1 µg/mL tosyl phenylalanyl chloromethyl ketone-treated trypsin (Sigma, Oakville, ON, Canada) was added. After 3 days, supernatants were used to infect freshly prepared confluent cells. The PA, PB1, PB2, HA, and NA genes from supernatant samples were amplified by RT-PCR and sequenced.

In Vitro Competition Experiments

Viral stocks were titrated by plaque assays using ST6-GalI-MDCK cells and pure A(H1N1)pdm09 or A(H3N2) viral populations (100%) as well as mixtures between the WT and its respective I38T variant, at equal amounts (50%:50%) and at a lower quantity of the mutant versus the WT (90%:10%–70%:30%), based on PFU numbers, were used to infect ST6-GalI-MDCK cells at an MOI of 0.001. After 3 days of incubation,

viruses were collected from supernatants, titrated (TCID₅₀/mL) in ST6GalI-MDCK cells and quantified by RT-ddPCR for WT and I38T viral populations.

Experimental Infections of Mice

Groups of 12 female C57/BL6 6- to 8-week old mice (Charles River, St-Constant, Canada) were housed 4 per cage and kept under conditions that prevent cage-to-cage transmission. Mice were infected under isoflurane anesthesia by intranasal inoculation of pure (100%) as well as 50%:50% and 70%:30% or 90%:10% mixtures (based on the number of PFUs) of WT and I38T recombinants. Infection of animals was performed with 5×10^4 PFUs for A(H1N1)pdm09 and 10^4 PFUs for A(H3N2) viruses. Animals inoculated with pure viral populations were weighed daily for 14 days. Four mice per group were sacrificed on days 3 and 6 postinfection for determination of viral titers (TCID₅₀/mL) in ST6-GalI-MDCK cells. RNA samples were extracted from lung homogenates at both time points and tested by ddRT-PCR as described above. These samples also served for PCR amplification and Sanger sequencing of PA, PB1, PB2, HA, and NA genes.

Statistical Analyses

Viral titers from cell culture supernatants as well as from lungs of mice, viral RNA copy numbers, and polymerase activities were compared using the unpaired Student *t* test. Mouse body weight changes were compared with the 2-tailed Mann-Whitney test. All analyses were made using PRISM software (GraphPad Inc., San Diego, CA).

RESULTS

Impact of I38T Substitution on the Phenotype of Susceptibility to Baloxavir

Viral yield assays confirmed the role of the I38T PA substitution on susceptibility phenotype to baloxavir acid. The A(H1N1)pdm09 WT virus mean 50% inhibitory concentration (IC₅₀) value was 0.42 ± 0.37 nM; by contrast, the I38T variant had a mean IC₅₀ value of 41.96 ± 9.42 nM (100-fold increase). For the A(H3N2) virus, mean IC₅₀ values for the WT and its I38T mutant were 0.66 ± 0.17 nM and 139.73 ± 24.97 nM, respectively (211-fold increase).

Impact of I38T Substitution on Polymerase Activity

In minigenome assays (Figure 1), no significant difference was observed between the pol activities of the WT and I38T mutant in the A(H1N1)pdm09 background while the I38T mutation was associated with a significant increase of pol activity in the A(H3N2) subtype (+45.7%; $P < .01$).

Impact of I38T Substitution on In Vitro Replication Kinetics

In replication kinetics experiments using ST6GalI-MDCK cells, the recombinant I38T mutant viruses reached titers comparable to their respective WT viruses based on mean TCID₅₀/mL and quantitative ddRT-PCR assays (data not shown). As shown in

Figure 1. Polymerase activity of contemporary wild-type (WT) and I38T A(H1N1)pdm09 and A(H3N2) viruses by minigenome assays. 293T cells were cotransfected with NP, PA, PB1, PB2, and the reporter luciferase plasmids. Mean luciferase values of 3 independent experiments were measured. The data were analyzed with 1-way ANOVA Dunnett multiple comparisons test. **, $P < .01$.

Figure 2, recombinant WT viruses and their respective mutants followed an exponential replication curve until 48 hours. The peak A(H1N1)pdm09 viral loads were $10^{7.8}$ and $10^{7.4}$ TCID₅₀/mL for the WT and the I38T mutant, respectively (Figure 2A). On the other hand, the peak A(H3N2) viral loads for the WT and its I38T mutant were $10^{7.8}$ and $10^{7.6}$ TCID₅₀/mL, respectively (Figure 2B).

After 4 passages in ST6GalI-MDCK cells, the I38T substitution was conserved in the 2 viral subtypes with no additional PA, PB2, HA, or NA substitutions. However, a R721K substitution was detected in the PB1 gene of the A(H1N1)pdm09 I38T mutant but not in that of the respective WT-passaged virus. Notably, this substitution was present in the rescued mutant before passaging.

In Vitro Competition Experiments

The results of competition experiments using pure viruses as well as 90%:10%; 70%:30%, and 50%:50% WT:I38T mixtures based on viral yield titers are summarized in Figure 3. For the A(H1N1)pdm09 subtype, pure WT and I38T viral populations reached mean viral titers of $10^{6.51}$ TCID₅₀/mL and $10^{6.39}$ TCID₅₀/mL, and mean ddRT-PCR copy numbers of 4.07×10^8 and 1.32×10^9 copies/mL ($P < .05$), respectively (Supplementary Table 1). There was a slight increase in favor of the WT strain over the mutant after a single cell passage of the different viral mixtures. Using initial viral WT:mutant ratios of 90%:10%, 70%:30%, and 50%:50%, the WT strain was subsequently detected at ratios of 99.3%, 85.6%, and 69.8%, respectively (Figure 3A).

For the A(H3N2) subtype, mean viral loads for the pure WT and I38T viral populations after a single passage were $10^{7.44}$

Figure 2. In vitro replicative capacity of recombinant (A) A(H1N1)pdm09 and (B) A(H3N2) viruses. Confluent ST6Gall-MDCK cells were infected with recombinant viruses at a multiplicity of infection of 0.0001. Supernatants were harvested at the indicated time points and viral titers (TCID₅₀/mL) were determined in ST6Gall-MDCK cells. Data were analyzed using the Mann-Whitney test. Abbreviation: WT, wild type.

TCID₅₀/mL and $10^{7.08}$ TCID₅₀/mL, respectively, while their respective mean ddRT-PCR copy numbers were $1.08 \pm 0.12 \times 10^{10}$ and $5.71 \pm 1.55 \times 10^8$, respectively ($P < .05$) (Supplementary Table 1). Here again, the superiority of the WT strain over the I38T mutant was maintained for the different viral mixtures. For instance, the initial 90%:10%, 70%:30%, and 50%:50% WT:mutant ratios became 98.5%:1.5%, 94.5%:5.5%, and 88.5%:11.5%, respectively, after a single cell passage (Figure 3B).

In Vivo Competition Experiments

As shown in Figure 4A, infection of C57/BL6 mice with recombinant WT and I38T A(H1N1)pdm09 viruses resulted in a significant body weight loss compared to uninfected animals. Major weight losses were recorded between days 3 and 8 postinfection with no significant differences between the 2 infected groups (WT and I38T) except on day 4 postinfection when the I38T-infected group lost more weight

Figure 3. In vitro quantification of wild-type (WT) and I38T populations in (A) A(H1N1)pdm09 and (B) A(H3N2) viruses. Proportion of WT viruses and their respective I38T mutants are indicated in competition experiments performed in ST6Gall-MDCK cells. Cells were infected using a multiplicity of infection of 0.001. Proportions of each viral population were determined by digital droplet reverse transcription polymerase chain reaction and are expressed as a percentage of the total viral population. Mean proportion values from quadruplicate experiments are shown.

than the WT (14% vs 9.6%, $P < .05$). Lung viral titers were similar between the WT and I38T groups at both day 3 ($10^{6.5}$ TCID₅₀/mL versus $10^{6.4}$ TCID₅₀/mL) and day 6 postinfection ($10^{6.3}$ TCID₅₀/mL versus $10^{6.1}$ TCID₅₀/mL; Figure 4B). The respective ddRT-PCR copy numbers were 4.57×10^7 versus 7.63×10^7 copies/mL ($P < .05$) at day 3 postinfection and 2.02×10^7 versus 1.03×10^7 copies/mL ($P < .05$) at day 6 postinfection (Supplementary Table 2).

Figure 4. Impact of the I38T PA mutation in mice experimentally infected with A(H1N1)pdm09 viruses. *A*, Mean body weight loss $\pm$ standard deviation of mice infected with recombinant A(H1N1)pdm09 wild-type (WT) and mutant viruses. Groups of 6 mice were infected with 5×10^4 plaque-forming units of the recombinant A(H1N1)pdm09 and its I38T variant. Percent body weight losses as compared to initial weights were recorded daily until day 14 postinoculation. * $P < .05$. *B*, Mean viral titers in lungs $\pm$ standard deviation were determined as TCID₅₀/mL in ST6Gall-MDCK cells for groups of 4 mice euthanized on days 3 and 6 postinoculation. *C*, Proportions of WT and I38T viral populations in lungs of mice as determined by digital droplet reverse transcription polymerase chain reaction.

Sequencing of viral genes from lung samples confirmed the presence of the I38T substitution in the mutant with no additional PA or PB2 changes. However, the 4 lung viral samples (day 6 postinfection) of the mutant contained the R721K PB1 substitution, which was present in the viral inoculum.

In mouse competition experiments, the proportion of the I38T mutant in lungs slightly decreased from 32.5% (in the

inoculum) to 22.8% and 21.7% at days 3 and 6 postinfection, respectively (Figure 4C). By contrast, when present at a higher proportion in the inoculum (56.1%), the I38T population increased to 73.1% and 72.7% at days 3 and 6 postinfection, respectively.

Experimental infections of mice with WT or I38T mutant A(H3N2) viruses also resulted in body weight losses that were

Figure 5. Impact of the I38T PA mutation in mice experimentally infected with A(H3N2) viruses. *A*, Mean body weight losses $\pm$ standard deviation of mice infected with recombinant A(H3N2) wild-type (WT) and I38T viruses. Groups of 6 mice were infected with 10^4 plaque-forming units of each virus. Percent body weight losses as compared to initial weights were recorded daily until day 14 postinoculation. *B*, Mean viral titers in lungs $\pm$ standard deviation were determined as TCID₅₀/mL in ST6Gall-MDCK cells for groups of 4 mice euthanized on days 3 and 6 postinoculation. *C*, Proportions of WT and I38T viral populations in lungs of mice as determined by digital droplet reverse transcription polymerase chain reaction.

less important than in A(H1N1)pdm09 experiments (Figure 5A). At day 4 postinfection, mean weight losses recorded in WT and I38T groups were 6% and 5%, respectively. WT and I38T infections were also associated with similar lung viral titers and ddRT-PCR copy numbers ($10^{7.01}$ vs $10^{6.7}$ TCID₅₀/mL and 8.66×10^7 vs 3.19×10^8 copies/mL at day 3 postinfection as well as $10^{5.2}$ TCID₅₀/mL vs $10^{4.7}$ TCID₅₀/mL and 1.73×10^7 copies/mL vs 1.27×10^7 copies/mL at day 6 postinfection; Figure 5B and Supplementary Table 2).

Sequencing of viral genes from lung samples confirmed the presence of the I38T substitution in the mutant with no additional PA changes. The PB2 sequence of 1 lung viral sample (day 6 postinfection) from the mutant group contained an R755G substitution that was absent in the WT group. No other changes could be detected in the PB1, HA, and NA genes.

In competition experiments, the proportion of the I38T mutant changed from 11% (in the inoculum) to 17% (day 3 postinfection) and 9.3% (day 6 postinfection) and from 42% (in the inoculum) to 55% (day 3 postinfection) and 54% (day 6 postinfection) (Figure 5C).

DISCUSSION

The clinical usefulness of antiviral drugs can be severely compromised by the emergence of resistant viruses with unaltered viral fitness. Therefore, the characterization of seasonal influenza variants with a high potential to emerge during BXM treatment is of significant clinical importance. Clinical trials revealed that a single dose of BXM could select for I38T/M/F substitutions with the largest effect on the resistance phenotype being observed for the I38T substitution, causing >30- to 50-fold increases in IC₅₀ values [10]. A recent study exploring factors associated with the development of resistance in baloxavir-treated individuals showed that the emergence of I38T/M substitutions was associated with a rebound in viral titers, initial delay in symptom alleviation, and prolonged viral shedding [15]. Noteworthy, among the 36 variants with PA mutations, including 35 from A(H3N2) infections and 1 from A(H3N2)/influenza B coinfection, the I38T substitution was detected in 30 variants, confirming that this change is the most common pathway leading to baloxavir resistance [15]. In the present study, we assessed the role of the I38T PA substitution on susceptibility profile, polymerase activity, and replicative properties both in vitro and in mice. Contrasting with previous studies using laboratory strains, we focused on contemporary influenza viruses belonging to the current seasonal subtypes, that is A(H1N1)pdm09 and A(H3N2). For better assessing viral fitness, we used competition experiments in addition to comparing the WT and its I38T variant in single (pure) infection experiments.

Despite the absence of a reference susceptibility method for determining the phenotype of resistance to baloxavir, evidence for reduced susceptibility to this PA inhibitor could be made by

cell culture-based experiments [16]. By performing viral yield assays in this study, we could confirm that the I38T PA substitution confers high levels of resistance to baloxavir acid in both A(H1N1) and A(H3N2) backgrounds (100- and 211-fold increases in IC₅₀ values vs the WT, respectively). However, a standardized technique is still required to allow interlaboratory comparisons of phenotypic results and for surveillance of baloxavir susceptibility purposes. Of interest, the I38T PA substitution was also selected in influenza A/California/04/2009 (H1N1)pdm09 and A/PR8/1934 (H1N1) strains during in vitro passages in presence of another investigational PA inhibitor (ie, RO-7) [9], highlighting the potential cross-resistance impact of this substitution. Such in vitro resistance could be attributed to a decreased affinity of PA to the inhibitor. Indeed, Jones et al [9] observed that I38T resulted in a 500-fold decrease in the affinity of the PA to RO-7, compared to the WT. In another crystal structure study, the I38T substitution resulted in a reduction of van der Waals contacts between baloxavir acid and the influenza endonuclease, lowering the binding stability [10]. Whether the I38T substitution could also impact the ability of the endonuclease to cleave host mRNA caps remains unclear.

When performing minigenome assays, we did not observe a significant impact of I38T on the polymerase activity of the A/Quebec/144147/2009 (H1N1)pdm09 virus. A similar observation was made when the I38T PA mutation was assessed in the A/California/04/2009 (H1N1)pdm09 background [9]. By contrast, our study showed that the pol activity increased by approximately 45% in the A/Switzerland/9715293/2013 (H3N2) mutant compared to its parental virus, suggesting that the impact of I38T could differ with regard to the viral strain/subtype [9].

Regarding the potential impact of I38T on viral fitness, our in vitro data demonstrated comparable viral titers in replication kinetics experiments between the WT and I38T mutant for the 2 viral subtypes. By contrast, viral titers collected at 24 hours postinfection for both influenza A/WSN/33 (H1N1) and A/Victoria/3/75 (H3N2) viruses were significantly reduced for the I38T PA mutants [10], highlighting again the contrasting effect of the I38T substitution on viral strain/subtype. Competition experiments in ST6GalI-MDCK cells showed a tendency for a WT predominance for both subtypes although pure WT and mutant populations reached the same peak viral titers. Of note, the I38T substitution was stable after 4 passages in cell culture in the 2 viral backgrounds.

In experimentally infected mice, the A(H1N1)pdm09 and A(H3N2) mutant viruses were found to be at least as virulent as their parental viruses, when assessing weight loss and viral titers in lung homogenates collected at 2 different time points (days 3 and 6 postinfection). Additionally, competition experiments using a 50%:50% ratio of WT and mutant viruses revealed a slight predominance of the I38T mutant in lungs of mice with no reversions or additional PA mutations being detected.

As stated above, the I38T PA substitution was selected under RO-7 pressure in A/California/04/2009 (H1N1)pdm09 and A/PR8/34 (H1N1) viruses. In that study, the I38T substitution was maintained after in vitro passages without drug; however, other PA (E199K and E349G) and PB1 (E636K) substitutions could be identified in the A/California/04/2009 (H1N1)pdm09 context while E199G and D347N PA substitutions together with PB1 (T228A), PB2 (I504V and K702R), HA (N73N and L345I), and NS1 (K78E) changes were detected in the genome of the A/PR8/34 (H1N1) mutant [9]. Contrasting with that report, no additional PA substitutions could be identified in our A(H1N1)pdm09 and A(H3N2) variants after in vitro passages or in lungs of mice; nevertheless, we detected PB1 (R721K) and PB2 (R755G) substitutions in the A(H1N1)pdm09 and A(H3N2) mutants, respectively, whose potential compensatory role still needs to be investigated.

Although our report clearly demonstrated a tendency for influenza A(H1N1)pdm09 and A(H3N2) I38T variants to retain a significant level of viral replication in vitro and in mice, we could not make a conclusion with regard to the potential for this variants to transmit efficiently. The potential human-to-human transmission of baloxavir-resistant influenza A variants is of a significant clinical importance warranting further investigations such as transmission studies in ferrets and a household-based clinical trial, which is underway in Japan (JapicCTI-184180) [15]. Nevertheless, our study is in line with a recent clinical report that suggested the possibility of an efficient transmission of A(H3N2) I38T variants among untreated children [17].

In conclusion, our experimental results combined with previously reported epidemiological findings suggest that it is pertinent to carefully monitor for the possible emergence and transmission of the BXM-resistant I38T PA mutants in clinic. Other therapeutic alternatives, including combined PA/NA inhibitors strategies, should be also assessed, particularly in immunocompromised individuals.

Supplementary Data

Supplementary materials are available at *The Journal of Infectious Diseases* online. Consisting of data provided by the authors to benefit the reader, the posted materials are not copyedited and are the sole responsibility of the authors, so questions or comments should be addressed to the corresponding author.

Notes

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editors consider relevant to the content of the manuscript have been disclosed.

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