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BRIEF REPORT

A novel E198K substitution in the PA gene of influenza A virus with reduced susceptibility to baloxavir acid

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Abstract

Baloxavir acid (BXA), the active compound in baloxavir marboxil (BXM), reduces the propagation of influenza A and B viruses by inhibiting the cap-dependent endonuclease activity of the polymerase acidic (PA) subunit. Although BXM has been used to treat influenza virus infections, recently, there has been general concern about the emergence of viruses with low susceptibility to BXA. Here, we identified a novel PA subunit substitution, PA E198K, that reduced susceptibility to BXA. The IC_{50} of BXA toward influenza A viruses containing PA E198K increased approximately 2- to 6-fold. These findings help to understand the mechanism by which PA substitutions reduce susceptibility to BXA.

Seasonal influenza, an acute respiratory infectious disease caused by influenza viruses, is a serious public health problem worldwide. The World Health Organization estimates that annual epidemics caused by influenza viruses are associated with 3 to 5 million cases of severe illness globally. Therefore, continuous efforts are needed to improve vaccines and develop antiviral drugs. Neuraminidase inhibitors (NAIs: oseltamivir, zanamivir, peramivir, and laninamivir octanoate), M2 ion-channel inhibitors (amantadine and rimantadine), and a viral RNA-dependent RNA polymerase inhibitor (baloxavir marboxil [BXM]) have been approved for clinical use [1]. Favipiravir (T-705) was approved for restricted use in Japan in 2014, but because of its teratogenicity and embryotoxicity, it has been strictly reserved for outbreaks of infections with novel or re-emerging influenza virus against which other influenza antiviral medications are either ineffective or insufficiently effective. However, current influenza virus strains are resistant to M2 inhibitors [2], and the antiviral potency of NAIs is relatively moderate [3, 4]. The use of NAIs may also lead to the emergence of resistant viruses [5–7].

The influenza A and B virus genomes consist of eight single-stranded negative-sense RNAs, and the viral genomic RNA (vRNA) forms a viral ribonucleoprotein (vRNP) by complexing with a heterotrimeric viral polymerase complex and nucleoprotein (NP). The viral polymerase complex consists of three subunits: the polymerase basic (PB) 2, PB1, and polymerase acidic (PA) subunits. At the initiation of viral mRNA transcription, the influenza virus polymerase uses a unique "cap-snatching" mechanism to steal the cap structure from host mRNAs because it cannot synthesize the cap structure *de novo*. The PB2 subunit binds the nascent capped host mRNA by recognizing its 5′ cap structure. Afterwards, cleavages at nucleotides 10-13 occur due to endonuclease activity of the PA subunit. Finally, the PB1 subunit synthesizes viral mRNA from the vRNP using the short capped RNA fragment as a primer [8–10]. Baloxavir acid (BXA), the active form of BXM, targets the endonuclease activity of the PA subunit of influenza A and B viruses [11]. BXM treatment has been shown to reduce the virus titer and mortality in a mouse model and the frequency of transmission in a ferret model [12, 13]. Moreover, a reduction in viral shedding from BXM-treated patients was observed in a clinical trial [14].

It has been shown that serial passages of viruses in the presence of BXA in cultured cells results in the emergence of a variants with an amino acid change at position 38 of the PA protein from isoleucine to threonine (PA I38T) [11]. In phase II and III clinical trials, PA I38F and PA I38M variants

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were detected in A/H1N1pdm and A/H3N2 influenza viruses from BXM-treated patients, in addition to the PA I38T variant [14, 15]. Moreover, surveillance studies have shown that PA I38T variants of A/H1N1pdm and A/H3N2 strains have been isolated from BXM-treated patients [16]. The variants were found to have similar replicative ability, pathogenicity, and transmission efficiency when compared with the wild-type isolates in an animal model [17, 18]. Additionally, in a clinical study, other amino acid substitutions in the PA protein, including A36V, E23K/G, L28P, V63I, A37T, and E199G substitutions, which were also associated with reduced susceptibility to BXM, were identified at a low frequency [15, 19, 20]. Therefore, the isolation, characterization, and monitoring of variants with reduced susceptibility to BXM are necessary for using this compound as an effective anti-influenza-virus drug. In this study, we identified a novel PA E198K substitution that reduced susceptibility to BXA *in vitro*.

BXM is a precursor to BXA and is administered as a prodrug. An esterase that is widely present in cells converts BXM to BXA. To isolate viruses with reduced susceptibility to BXA, we first performed cell culture passaging of influenza virus in the presence of BXM. We then tested the BXA sensitivity of the passaged viruses. Both BXM and BXA were purchased from Shionogi (Osaka, Japan). MDCK cells were infected with influenza virus (A/Puerto Rico/8/34; PR8) at a multiplicity of infection (MOI) of 0.01 and were incubated with 1 nM BXM. After 48 hpi, the supernatant was collected, and 0.5% of the supernatant was used as inoculum for the subsequent infection. After nine passages, a reduction in susceptibility to BXM was confirmed by the observation of an increased cytopathic effect in the presence of 1 nM BXM. Viral RNA was extracted from the supernatant by the phenol-chloroform method to determine the sequence of the PA gene. cDNA was synthesized using the primer Uni12 [21] and ReverTra Ace reverse transcriptase (Toyobo, Osaka, Japan). The PA coding region was amplified by PCR, and the sequence was determined by the Sanger method. Unexpectedly, we found an amino acid change at position 198 of the PA protein from glutamic acid to lysine (PA E198K), which had not been reported to reduce susceptibility to BXA. The virus with the PA E198K substitution became dominant after six passages. To confirm that PA E198K reduces susceptibility to BXM, recombinant PR8 viruses containing PA E198K and PA I38T were generated (PA E198K virus and PA I38T virus, respectively) by a reverse genetics approach [22, 23], and the virus was propagated in the presence of BXM. MDCK cells were infected with each virus at an MOI of 0.01 and then incubated with or without BXM until 24 h postinfection. The virus titers in the supernatants were determined using a plaque assay. Three independent experiments were performed. The titer of the PA E198K and PA I38T viruses, when grown in the absence of BXM, was tenfold lower than that of the wild-type PR8 virus (Fig. 1A). However, the virus titer of the supernatant of cells infected with the PA E198K and PA I38T viruses did not decrease in the presence of 5 nM BXM (Fig. 1A).

To confirm that the PA E198K mutation reduces susceptibility to BXA, virus propagation in the presence of BXA was analyzed. MDCK cells were infected with each virus at an MOI of 0.01 or 0.001 and incubated with or without BXA until 24 h postinfection. The virus titers of the supernatants were determined using a plaque assay. Three independent experiments were performed. After infection at an MOI of 0.001, the titer of the wild-type virus was reduced by 1 and 3 log PFU/mL in the presence of 1 and 5 nM BXA, respectively (Fig. 1B), whereas the titers of the PA E198K and PA I38T mutants did not decrease (Fig. 1B). After infection at an MOI of 0.01, the titer of the PA E198K virus decreased by 0.7 log PUF/mL, but that of wild-type virus decreased by 3 log PFU/mL in the presence of 5 nM BXA (Fig. 1B). These results suggest that the PA E198K substitution reduces the susceptibility to BXA.

Amino acid position 198 of the PA protein is located in the linker region between the endonuclease and PA C-terminal domains and is conserved among influenza A, B, and C viruses (Fig. 1C), indicating that this glutamic acid residue is likely to be essential for PA subunit functions. Based on previously reported structures, we deduced the likely three-dimensional positions of the E198K residue and BXA. The structure of PA E198K was computed using the SWISS-MODEL server homology modeling pipeline [24] based on the structure of the H3N2 viral polymerase complex (PDB ID: 6QNW) [25]. BXA was added to the structural model of PA E198K using coordinates obtained from the previously reported PA(1-193)-BXA complex structure [15]. PA E198 is located near the active center of the endonuclease, which is the target site of BXA (Fig. 1D). The substitutions resulting in reduced susceptibility to BXM that were identified in a clinical study, with the exception of V63I, are located near the active center [15, 19, 20]. PA E198 is not directly involved in the binding of BXA to the active center. The amino acid substitutions for Ile at position 38 do not alter the structure of the endonuclease active center, but these changes slightly reduce the van der Waals interaction between BXA and the amino acid at position 38. Therefore, the binding of BXA to the PA T38 variant requires local induced-fit rearrangements [15]. The binding of BXA to the PA K198 variant may also require structural alterations because of the electrostatic change from a negative to a positive side chain at amino acid position 198.

To analyze the susceptibility of virus strains containing PA E198K to BXA, we generated H1N1 (A/California/7/2009; California) and H3N2 (A/Aichi/2/68; Aichi) influenza virus strains containing PA E198K and PA I38T substitutions. The viral genomes of A/California/7/2009 and

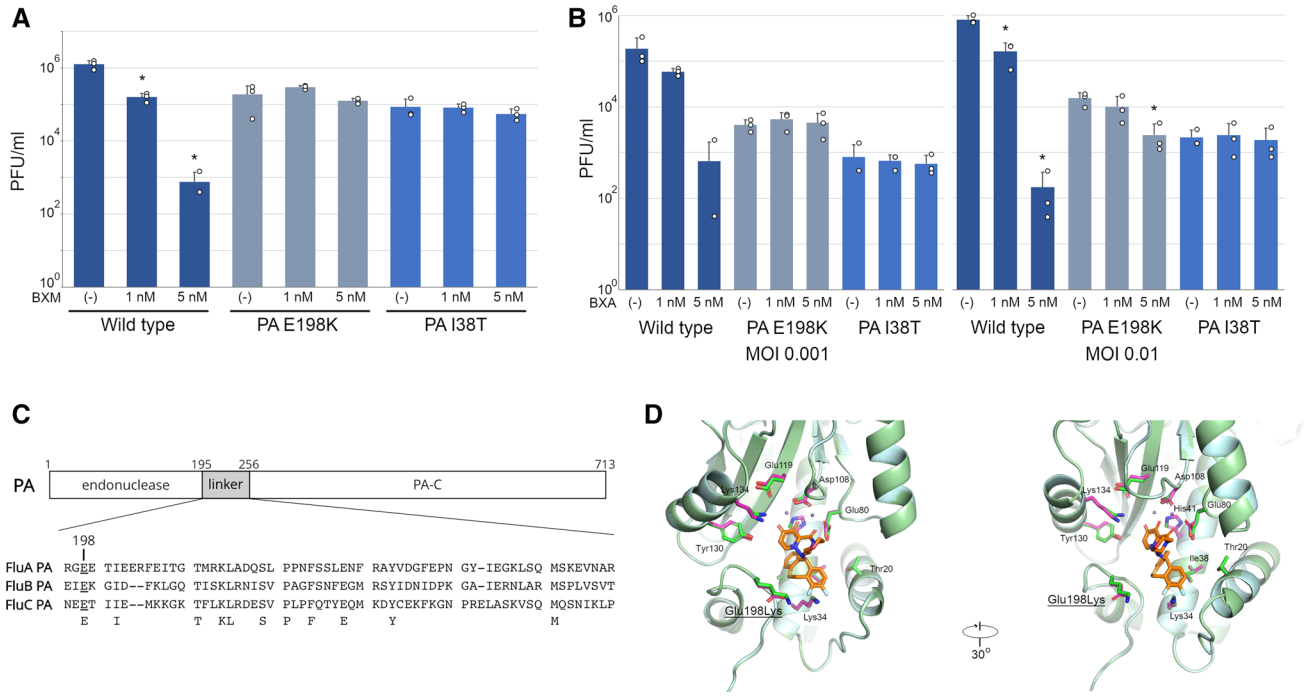

Fig. 1 Reduced BXM susceptibility of viruses containing PA E198K. (A) Propagation of wild-type (A/Puerto Rico/8/34), PA E198K, and PA I38T viruses in the presence of BXM. MDCK cells were infected with each virus at an MOI of 0.01 and incubated in MEM containing trypsin and the indicated concentration of BXM. The graph shows average values with standard deviations from three independent experiments. The circles show the values from each experiment. *P*-values were calculated using Student's *t*-test and were adjusted using Holm's method for multiple comparisons. An asterisk indicates a *p*-value < 0.05. (B) Propagation of wild-type, PA E198K, and PA I38T viruses in the presence of BXA. MDCK cells were infected with each virus at an MOI of 0.01 or 0.001 and incubated in MEM containing trypsin and the indicated concentration of BXA. The graph shows average values with standard deviations from three independent experiments. The circles show the values from each experiment. *P*-values were calculated using Student's *t*-test and were adjusted using Holm's method for multiple comparisons. An asterisk indicates a *p*-value < 0.05. (C) Amino acid sequence alignment of the PA linker domains of influenza A, B, and C viruses. Conserved residues are indicated under the aligned sequence, and E198 is underlined. (D) The position of residue E198 in a three-dimensional model of PA. The modeled structure of PA E198K was merged with the structure of wild-type PA from the H3N2 viral polymerase complex (PDB ID: 6QNW). BXA was also merged based on the structure of the PA(1-193)-BXA complex (PDB ID: 6FS6). The side chains interacting with BXA and those of amino acid 198 (in magenta for wild-type PA and in green for PA E198K) are represented as sticks.

A/Aichi/2/68 were cloned into pHH21 plasmids. A recombinant virus of the California strain could not be recovered in our systems. Therefore, we generated a chimeric California strain whose HA and NA segments were replaced with those of the PR8 strain. MDCK cells were infected with these recombinant viruses at an MOI of 0.01 and were incubated with or without BXA until 24 h postinfection. The virus titers of the supernatants were determined using a plaque assay. Three independent experiments were performed, and the IC_{50} of BXA was calculated from the virus titer. The IC_{50} of BXA for the wild-type PA was approximately 0.2 nM for all three viral strains, whereas those for PA E198K viruses were 3-5 times higher in the PR8 and Aichi strains (Table 1). The IC_{50} of BXA for PA I38T viruses was 25-50 times higher than those for the wild-type PA viruses of all three strains (Table 1). These results suggest that the BXA

Table 1 IC_{50} of BXA against wild-type and mutant influenza A virus

Strain	Wild type	PA I38T	PA E198K
Puerto Rico/8/34 (H1N1)	0.12 ± 0.01 nM	6.93 ± 3.73 nM*	0.30 ± 0.09 nM*
California/7/2009 (H1N1)[#]	0.23 ± 0.10 nM	5.32 ± 1.69 nM*	0.42 ± 0.36 nM
Aichi/2/68 (H3N2)	0.10 ± 0.07 nM	2.61 ± 1.00 nM*	0.62 ± 0.14 nM*

[#]Segment 4 and segment 6 of these recombinant viruses were derived from strain PR8

*$p < 0.05$ in comparison with wild type. *P*-values were calculated using Student's *t*-test and were adjusted by Holm's method for multiple comparisons

susceptibility of viruses containing PA E198K was reduced in the PR8 (H1N1) and Aichi (H3N2) strains, although PA E198K was less effective than PA I38T in reducing susceptibility to BXA.

To analyze whether the viral polymerase influenced the BXA-resistant and attenuated phenotype of PA E198K, a mini-replicon assay was performed using viral polymerases from H1N1 and H3N2 influenza A virus strains [26]. The viral polymerase and NP expression vectors of each strain, a viral RNA expression reporter vector that encodes luciferase, and a *Renilla* luciferase expression vector were introduced into HEK293T cells by transfection [26]. BXA was added to the medium immediately after transfection. After incubation for 24 h, the luciferase activity, which reflects the viral polymerase activity, was measured and normalized to the *Renilla* luciferase activity. Subsequently, three independent experiments were performed. The relative luciferase activity was calculated by setting the luciferase activity of cells transfected with wild-type PA vector and not treated with BXA to 1. The relative luciferase activity of cells transfected with wild-type or PA E198K vector was decreased by BXA treatment in a dose-dependent manner. In contrast, the cells transfected with the PA I38T vector showed similar relative luciferase levels with or without BXA treatment (Fig. 2A-C). To further compare the rates of reduction of luciferase activity, the relative luciferase activity was recalculated by setting the luciferase activity of BXA-untreated cells in each case to 1. Using the PR8 backbone, the relative luciferase activity of the wild-type polymerase decreased by 10^5-fold in the presence of 0.5 μM BXA, whereas that of the polymerase containing PA E198K decreased by 10^2-fold (Fig. 2D). The relative luciferase activity of the polymerase containing PA I38T was comparable with and without BXA. Similarly, the relative luciferase activity of the wild-type California and Aichi strain polymerases decreased 10^4- and 10^5-fold, respectively, in the presence of 5.0 μM BXA. In contrast, that of the polymerases containing PA E198K decreased 10^1-fold and 10^2-fold, respectively (Fig. 2E and F). These results suggest that the PA E198K substitution reduces the BXA susceptibility of the viral polymerases of H1N1 and H3N2 strains in the mini-replicon assay.

We identified a novel PA E198K substitution that reduced susceptibility to BXA by cell culture passaging of influenza virus in the presence of BXM. Our results indicated that the PA E198K virus was more sensitive to BXA than the PA I38T virus (Table 1). However, the PA substitutions identified in previous clinical studies were not isolated by our selection method, in which the virus was passaged in the presence of 1 nM BXM. At this concentration, the growth rate of the PA E198K virus is comparable with that of the PA I38T virus. Therefore, the PA I38T virus, which first acquired the mutation based on probability, would become dominant.

Amino acid position 198 of PA is located in a flexible loop region that is not necessary for the endonuclease activity of the PA subunit [8, 9]. However, PA E198 contributes to the viral polymerase activity, because this amino acid is conserved in influenza virus strains, and the PA E198K/P substitutions result in reduced virus growth and viral polymerase activity [27]. Since substitutions in the PA loop region induce temperature-sensitive phenotypes and negatively modulate PA-PB1 interactions or complex stability at nonpermissive temperatures [27], PA E198K probably affects the stability of the viral polymerase complex.

In the case of influenza B virus, the PA I38T/M and PA E23K substitutions also reduce susceptibility to BXA, and a recombinant influenza B virus containing PA I38T/M showed a level of fitness similar to that of the wild-type virus [28]. We could not rescue a recombinant influenza B virus containing the PA E198K substitution in our reverse genetics system. In influenza B virus, the PA E198K substitution may reduce susceptibility to BXA, but it apparently reduces viral fitness.

We found that one human A/H3N2 strain (A/New York/12/2015) and one human B strain (B/Pernambuco/M3598-IEC/2016) containing the PA E198K substitution have been registered in the GISAID database, suggesting that viruses containing PA E198K substitutions have been isolated from clinical samples. However, the PA E198K substitution has not been identified in clinical samples from BXM-treated patients. The PA E198K substitution was not found in any of the reported sequences of H3N2 strains from BXM-treated patients [18] (data not shown). Viruses containing PA E198K were more susceptible to BXA than those containing PA I38T, and the growth rates of these two variants were comparable. Therefore, it is possible that PA I38T would be preferentially selected *in vivo* in the presence of BXM. Although PA E198K has not been identified in BXM-treated patients, our results will help to elucidate the mechanism by which PA substitutions reduce susceptibility to BXA and to clarify the viral transcription mechanism, which is initiated by cap-snatching from host mRNA.

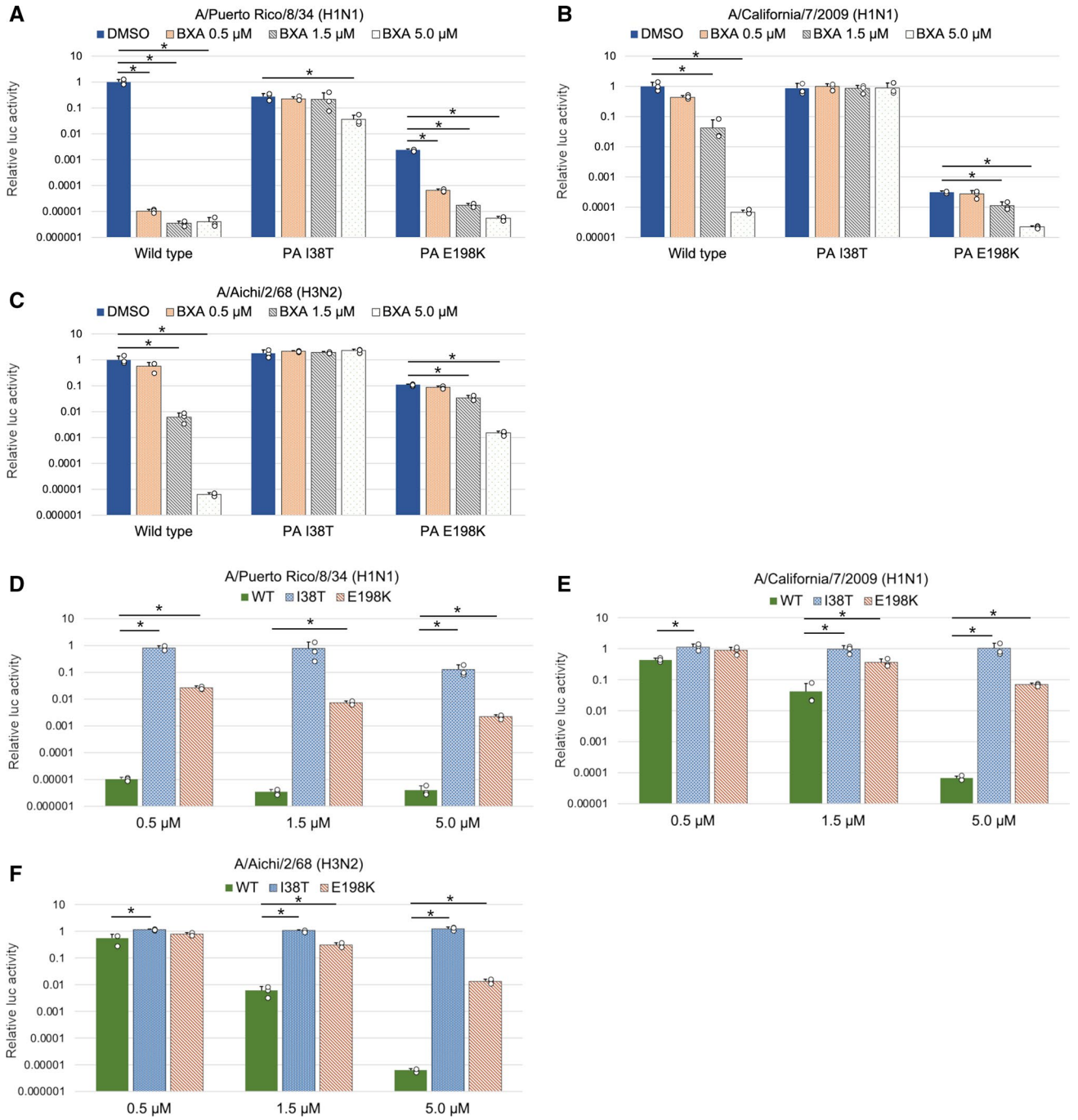

Fig. 2 Reduced BXA susceptibility of the viral polymerase complexes of H1N1 influenza A viruses containing PA E198K. A mini-replicon assay was performed using viral polymerase and NP derived from strains A/Puerto Rico/8/34 (H1N1) (A and D), A/California/7/2009 (H1N1) (B and E), and A/Aichi/2/68 (H3N2) (C and F). The relative luciferase activity was normalized to that of cells transfected with wild-type PA vector and not treated with BXA (A-C) or to that of infected cells without BXA treatment (D-F). The graph shows average values with standard deviations from three independent experiments. The circles show the values from each experiment. *P*-values were calculated using Student's *t*-test and were adjusted using Holm's method for multiple comparisons, and an asterisk indicates a p-value < 0.05. WT, wild-type

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Author contributions NT: conceptualization, data curation, writing—original draft preparation, funding acquisition. FM: resources, writing—reviewing and editing

Declarations

Conflict of interest The authors have no conflicts of interest to declare that are relevant to the contents of this article.

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