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Antiviral susceptibility of influenza viruses isolated from patients pre- and post-administration of favipiravir

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ABSTRACT

Favipiravir, a viral RNA-dependent RNA polymerase inhibitor, has recently been approved in Japan for influenza pandemic preparedness. Here, we conducted a cell-based screening system to evaluate the susceptibility of influenza viruses to favipiravir. In this assay, the antiviral activity of favipiravir is determined by inhibition of virus-induced cytopathic effect, which can be measured by using a colorimetric cell proliferation assay. To demonstrate the robustness of the assay, we compared the favipiravir susceptibilities of neuraminidase (NA) inhibitor-resistant influenza A(H1N1)pdm09, A(H3N2), A(H7N9) and B viruses and their sensitive counterparts. No significant differences in the favipiravir susceptibilities were found between NA inhibitor-resistant and sensitive viruses. We, then, examined the antiviral susceptibility of 57 pairs of influenza viruses isolated from patients pre- and post-administration of favipiravir in phase 3 clinical trials. We found that there were no viruses with statistically significant reduced susceptibility to favipiravir or NA inhibitors, although two of 20 paired A(H1N1)pdm09, one of 17 paired A(H3N2) and one of 20 paired B viruses possessed amino acid substitutions in the RNA-dependent RNA polymerase subunits, PB1, PB2 and PA, after favipiravir administration. This is the first report on the antiviral susceptibility of influenza viruses isolated from patients after favipiravir treatment.

1. Introduction

In Japan, four neuraminidase (NA) inhibitors, oseltamivir, zanamivir, peramivir and laninamivir, are approved for therapeutic or prophylactic treatment against influenza virus infection. Because they are prescribed at the highest frequency in the world, we have been conducting nationwide monitoring for the antiviral susceptibility of influenza viruses since 1999 (Monto et al., 2006; Takashita et al., 2013; Tashiro et al., 2009; Ujike et al., 2010, 2011). After the (H1N1)2009 pandemic started, the detection rate of NA inhibitor-resistant A(H1N1)pdm09 viruses was low (approximately 1%) and no resistant A(H3N2) and B viruses were detected in Japan. However, between November 2013 and February 2014, a large cluster of mutant A(H1N1)pdm09 viruses cross-resistant to oseltamivir and peramivir with an H275Y substitution (N1 numbering) in the NA protein emerged in Hokkaido, Japan (Takashita et al., 2014, 2015a). The detection rate for this resistant virus reached 29% in this area. Our previous report suggested that the novel variant retained replication and transmission capability to spread among humans, due to the permissive substitutions V241I and N369K (N1 numbering) in the NA protein (Takashita et al., 2015a). Furthermore, significant numbers of oseltamivir and peramivir cross-resistant A(H1N1)pdm09 viruses were also detected in China and the United States (Huang et al., 2015; Okomo-Adhiambo et al., 2015; Takashita et al., 2015b). Therefore, the surveillance of antiviral-resistant influenza viruses is important to protect public health and aid in clinical management.

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A novel antiviral drug, favipiravir (T-705; 6-fluoro-3-hydroxy-2-pyrazinecarboxamide) inhibits the RNA-dependent RNA polymerase of influenza viruses (Furuta et al., 2005). Favipiravir is converted to 4-ribofuranosyl-5-triphosphate metabolite by intracellular enzymes and may be misincorporated into the nascent RNA strand as an adenosine and a guanosine analog, preventing incorporation of natural ATP and GTP for viral RNA synthesis (Jin et al., 2013; Sangawa et al., 2013; Furuta et al., 2013). Favipiravir is active against a broad range of influenza A, B and C viruses, including highly pathogenic avian A(H5N1) and novel avian A(H7N9) viruses (Kiso et al., 2010; Watanabe et al., 2013) and has antiviral activity against influenza viruses resistant to NA inhibitors or adamantane (Sleeman et al., 2010; Watanabe et al., 2013). Favipiravir also has broad-spectrum activities against various other RNA viruses, such as arenaviridae, bunyaviridae, caliciviridae, flaviviridae, picornaviridae, paramyxoviridae and togaviridae (Delang et al., 2014; Furuta et al., 2002, 2009, 2013; Rocha-Pereira et al., 2014). Recently, the efficacy of favipiravir against Ebola virus *in vitro* and *in vivo* was also reported (Oestereich et al., 2014; Smither et al., 2014). Subsequently, a clinical trial named the JIKI study, which is testing the efficacy of favipiravir in reducing mortality associated with Ebola virus infections in Guinea, is ongoing as of April 2015 (Kupferschmidt and Cohen, 2015). In Japan, favipiravir was approved for influenza pandemic preparedness on March 24, 2014 (http://www.toyama-chemical.co.jp/eng/news/news140324e.html). Therefore, there is a need to develop convenient assays that monitor favipiravir susceptibility of influenza viruses. The susceptibility of influenza viruses to favipiravir has been determined by using plaque reduction, yield reduction or focus inhibition assays in the previous reports (Furuta et al., 2002; Sidwell et al., 2007; Sleeman et al., 2010). Here, we conducted a cell-based screening system to evaluate favipiravir susceptibility, that is, a colorimetric cytopathic effect (CPE) reduction assay. Using our approach, we examined the susceptibility of a panel of NA inhibitor-resistant viruses and their sensitive counterparts to favipiravir. Furthermore, the antiviral susceptibility of 57 pairs of influenza A(H1N1) pdm09, A(H3N2) and B viruses isolated from patients pre- and post-administration of favipiravir in phase 3 clinical trials were determined.

2. Materials and methods

2.1. Viruses

A panel of NA inhibitor-resistant viruses and their sensitive counterparts was used in this study (Table 1). A/Chiba/1017/2009 and A/Chiba/1016/2009 were isolated in Japan and used as reference H275Y mutant and 275H wild-type A(H1N1)pdm09 viruses, respectively (Ujike et al., 2011). A/Fukui/45/2004 and A/Fukui/20/2004 were kindly provided by the ISIRV Antiviral Group (http://www.isirv.org/site/index.php/reference-panel) and used as reference E119V mutant and 119E wild-type A(H3N2) viruses, respectively (World Health Organization, 2012a). A/Kagoshima/2/2012 and A/Kagoshima/4/2012 were used as reference R292K mutant and 292R wild-type A(H3N2) viruses, respectively. These A/Kagoshima strains were isolated from clinical specimens distributed by Kagoshima Prefectural Institute for Environmental Research and Public Health, Japan. Plaque purified A/Shanghai/1/2013-292K and A/Shanghai/1/2013-292R were used as reference R292K mutant and 292R wild-type A(H7N9) viruses, respectively (Watanabe et al., 2013). A/Shanghai/1/2013 was kindly provided by Dr. Yuelong Shu (Chinese Center for Disease Control and Prevention, China). All experiments with A(H7N9) viruses were carried out in approved biosafety level 3 (BSL-3) containment laboratories. Plaque purified B/Perth/211/2001-197E and B/Perth/211/2001-197D were kindly provided by the ISIRV Antiviral Group and used as reference D197E mutant and 197D wild-type B/Yamagata-lineage viruses, respectively (World Health Organization, 2012a). All viruses were propagated in MDCK (NBL-2) cells. Amino acid position numbering is A subtype and B type specific.

Nasal swabs were obtained from adult patients positive for influenza virus in phase 3 clinical trials of favipiravir between October 2009 and October 2010 according to the protocols JP312 and JP313 meeting IRB approval by each study site (http://www.clinicaltrials.jp/user/showCteDetailE.jsp?japicId=JapicCTI-090934). Subjects were administered favipiravir (1200/400 mg for 1 day + 400 mg bid for 4 days) and the specimens were collected pre- and 1 or 2 days post-administration of favipiravir. Influenza A(H1N1) pdm09, A(H3N2) and B viruses were isolated from the specimens by using MDCK (NBL-2) cells by Toyama Chemical Co., Ltd. (Toyama, Japan). Twenty pairs of A(H1N1)pdm09 and B viruses and 17 pairs of A(H3N2) viruses were sent to the National Institute of Infectious Diseases without the corresponding patient records and were propagated in MDCK (NBL-2) cells for further analysis. Since most B viruses isolated were B/Victoria-lineage, 19 of 20 pairs of B viruses were B/Victoria-lineage and one pair was B/Yamagata-lineage.

2.2. Antiviral compounds

Oseltamivir carboxylate and zanamivir were purchased from Sequoia Research Products (Pangbourne, United Kingdom). Favipiravir, oseltamivir carboxylate and zanamivir were dissolved in distilled water as 5 mM stocks and stored in aliquots at −20 °C.

2.3. Colorimetric CPE reduction assay

Antiviral activity of favipiravir was determined by inhibition of virus-induced CPE. A tetrazolium compound [3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium, inner salt; MTS] is converted by viable cells into a colored formazan product that is soluble in cell culture medium (Cory et al., 1991). The quantity of formazan product is proportional to the number of viable cells (Cory et al., 1991). We used a single solution consisting of the MTS tetrazolium compound and an electron coupling reagent (phenazine ethosulfate; PES) in the CellTiter 96 AQueous One Solution Reagent (Promega Corporation, Madison, WI, USA) for homogeneous screening assay to monitor the amount of CPE.

Confluent monolayers of MDCK (NBL-2) cells in 96-well tissue culture plates were inoculated with 50 μl of viruses. A multiplicity of infection (MOI) of 0.01 50% tissue culture infective dose ($TCID_{50}$)/cell was the most suitable for this assay, although we tested serial dilutions (MOI of 0.0001–10) of viruses. Virus adsorption was carried out in the absence of favipiravir for 1 h at 37 °C in a 5% CO_2 incubator and then 50 μl of culture medium containing serial dilutions (0.05–1000 μM) of favipiravir was added to each well in triplicate. The cytotoxicity of favipiravir was evaluated in parallel by using uninfected MDCK (NBL-2) cells and favipiravir showed no cytotoxicity in this concentration range (data not shown). We assessed the susceptibility to favipiravir when maximum CPE was observed in control cells containing virus with no favipiravir under a microscope, i.e., on days 3–5 post-infection, depending on the virus strain. To measure the extent of CPE, 20 μl of CellTiter 96 AQueous One Solution Reagent was added to each well and the cells were incubated for 2 h at 37 °C in a 5% CO_2 incubator. Absorbance was then measured at 490 nm with a reference wavelength of 650 nm. The susceptibilities of viruses to favipiravir were expressed as the 50% effective concentration (EC_{50}). The EC_{50} values were calculated by using GraphPad Prism version 5.04 for Windows (GraphPad Software, San Diego, CA, USA).

Table 1
The panel of influenza viruses used in this study.

Subtype	Isolate name	Amino acid substitution in[a]:		Susceptibility to:						
		NA	M2	Oseltamivir[b]	Zanamivir[b]	Peramivir[b]	Laninamivir[b]	Adamantane[c]	Favipiravir	
									EC_{50} [μM][d]	IC_{50} [μM][e]
A(H1N1) pdm09	A/Chiba/1017/2009	H275Y	S31N	HRI	NI	HRI	NI	Resistant	3.66 ± 0.04	0.43 ± 0.09
	A/Chiba/1016/2009	275H (Wild-type)	S31N	NI	NI	NI	NI	Resistant	3.55 ± 0.06	0.35 ± 0.16
A(H3N2)	A/Fukui/45/2004	E119V	31S (Wild-type)	RI	NI	NI	NI	Sensitive	9.83 ± 0.03	0.16 ± 0.07
	A/Fukui/20/2004	119E (Wild-type)	31S (Wild-type)	NI	NI	NI	NI	Sensitive	12.17 ± 0.08	0.14 ± 0.05
	A/Kagoshima/2/2012	R292K	S31N	HRI	RI	HRI	NI	Resistant	12.05 ± 0.05	0.22 ± 0.08
	A/Kagoshima/4/2012	292R (Wild-type)	S31N	NI	NI	NI	NI	Resistant	16.17 ± 0.04	0.22 ± 0.01
A(H7N9)	A/Shanghai/1/2013-292K	R292K	S31N	HRI	RI	HRI	RI	Resistant	38.72 ± 0.13	—[f]
	A/Shanghai/1/2013-292R	292R (Wild-type)	S31N	NI	NI	NI	NI	Resistant	20.65 ± 0.03	–
B	B/Perth/211/2001-197E	D197E	—[g]	RI	RI	RI	NI	—[g]	2.92 ± 0.05	0.45 ± 0.02
	B/Perth/211/2001-197D	197D (Wild-type)	–	NI	NI	NI	NI	–	3.28 ± 0.04	0.99 ± 0.01

[a] Amino acid position numbering is A subtype and B type specific.
[b] HRI = highly reduced inhibition, RI = reduced inhibition, NI = normal inhibition.
[c] Adamantane resistance is associated with M2 S31N amino acid substitution.
[d] EC_{50} values were determined by the use of the colorimetric cytopathic effect reduction assay. The values are presented as the mean ± SD of at least three reactions.
[e] IC_{50} values were determined by the use of the plaque reduction assay. The values are presented as the mean ± SD of six reactions.
[f] Not tested.
[g] Not applicable.

2.4. Plaque reduction assay

Antiviral activity of favipiravir was also determined by using a conventional plaque reduction assay as previously described (Furuta et al., 2002). Confluent monolayers of MDCK (NBL-2) cells in 6-well tissue culture plates were inoculated with 50 PFU/well of viruses. Virus adsorption was carried out in the absence of favipiravir for 1 h at 37 °C in a 5% CO_2 incubator and then the inoculum was removed. A 0.8% agarose in culture medium containing serial dilutions (0.05–1000 μM) of favipiravir was added to each well in triplicate. The cells were incubated for 3 days and the plaque numbers were counted. The 50% inhibitory concentration (IC_{50}) values were calculated by using GraphPad Prism.

2.5. NA inhibition assay

The susceptibilities of the viruses to NA inhibitors, oseltamivir and zanamivir, were determined by using a chemiluminescent NA inhibition assay with the NA-XTD Influenza Neuraminidase Assay Kit (Applied Biosystems, Foster City, CA, USA). The IC_{50} values were calculated by using GraphPad Prism. To interpret NA inhibitor susceptibility, we used the World Health Organization criteria, which are based on the fold-change in IC_{50} compared to the median for viruses from the same type/subtype/lineage showing normal inhibition (World Health Organization, 2012b). Reduced inhibition is defined as a 10- to 100-fold increase in IC_{50} for influenza A viruses, or a 5- to 50-fold increase in IC_{50} for influenza B viruses. Viruses showing highly reduced inhibition are influenza A viruses with >100-fold increase in IC_{50} or influenza B viruses with >50-fold increase in IC_{50} (World Health Organization, 2012b).

2.6. Sanger sequencing

Viral RNA was prepared from isolates by using the QIAamp viral RNA kit (Qiagen, Düsseldorf, Germany). The full-length polymerase basic subunit 1 (PB1), polymerase basic subunit 2 (PB2), polymerase acidic subunit (PA) and nucleoprotein (NP) genes were amplified from viral RNA by using SuperScript III One-step RT-PCR system with Platinum Taq (Invitrogen, Carlsbad, CA). Nucleotide sequences were determined by using the BigDye terminator v3.1 cycle sequencing kit (Applied Biosystems) with Applied Biosystems 3730 DNA Analyzer. Primer sequences are available upon request.

2.7. Deep sequencing

A cDNA library was prepared from viral RNA by using NEBNext Ultra RNA Library Prep Kit for Illumina and NEBNext Singleplex Oligos for Illumina (New England Biolabs, Ipswich, MA, USA), followed by purification by using Agencourt AMPure XP (Beckman Coulter, Brea, CA, USA) according to the manufacturer's instruction. The library was sequenced by using MiSeq Reagent Kits v2 with MiSeq (Illumina, San Diego, CA, USA). Sequence reads were aligned to the reference sequence of A/California/07/2009 (H1N1)pdm09 by using CLC Genomics Workbench 8 (CLC bio, Aarhus, Denmark). The threshold of 10% was used for the detection of single nucleotide polymorphisms.

2.8. Mini-genome assay

Luciferase-based mini-genome assay was performed as previously described (Octaviani et al., 2010). Expression plasmids for PB1, PB2, PA and NP proteins were constructed from those of A/California/04/2009(H1N1)pdm09 (Octaviani et al., 2010). The plasmid pPolINP(0)luc2(0) (20 ng), which expresses a firefly luciferase gene between the noncoding regions of the NP gene, was cotransfected into 293T cells in 96-well plates, along with the protein expression plasmids for PB1, PB2, PA and NP (20 ng) and pRL-null renilla luciferase control reporter vector (4 ng; Promega Corporation), by using the TransIT-293 Transfection Reagent (Mirus Bio, Madison, WI, USA) (3 μl/μg of plasmid). To determine the IC_{50} values of the polymerase complexes, serial dilutions (0.05–1000 μM) of favipiravir were added to the cells. The luciferase activity in the transfected cells was measured by using the Dual-Glo Luciferase Assay System (Promega Corporation) at 24 h post-transfection. The relative firefly luciferase activity, normalized to the renilla luciferase activity, was calculated. The IC_{50} values were calculated by using GraphPad Prism.

2.9. Statistical analysis

Statistical analyses were performed using GraphPad Prism. Grubb's test was used to detect significant outliers. Statistically significant differences between groups were determined by using Paired *t*-test and Fisher's exact test. *P* values of <0.05 were considered statistically significant.

3. Results

3.1. Favipiravir susceptibility of NA inhibitor-resistant influenza viruses

Using the CPE reduction assay, we compared the favipiravir susceptibilities of NA inhibitor-resistant viruses and their sensitive counterparts. A panel of representative human influenza A(H1N1) pdm09, A(H3N2), A(H7N9) and B viruses was tested. The A(H1N1) pdm09-H275Y, A(H3N2)-E119V or -R292K, A(H7N9)-R292K and influenza B-D197E viruses exhibited highly reduced inhibition or reduced inhibition against at least one of the four NA inhibitors and most of them were also resistant to adamantane, which blocks the M2 ion channel (Table 1). Since no favipiravir-resistant influenza viruses have been reported to date, positive controls for resistance to this drug do not exist.

The EC_{50} values of the NA inhibitor-resistant and wild-type viruses to favipiravir were determined by using the CPE reduction assay (Table 1). The IC_{50} values of the same viruses were determined by using the conventional plaque reduction assay (Table 1). Not only the number but also the size of plaques decreased as favipiravir concentration increased. No significant differences in the favipiravir susceptibilities were found between NA inhibitor-resistant and wild-type viruses by either assay, consistent with a previous study (Sleeman et al., 2010).

3.2. Favipiravir susceptibility of influenza viruses isolated from patients pre- and post-administration of favipiravir

To examine the favipiravir susceptibility of influenza viruses after favipiravir administration, we determined the EC_{50} values of the viruses isolated before or on days 1 or 2 post-administration of favipiravir. The level of susceptibility was evaluated by comparing the difference in EC_{50} values between pre- and post-administration samples. We could not analyze the viruses isolated after day 3 post-administration because the virus titers in the clinical specimens were low. EC_{50} values of 20 pairs of A(H1N1)pdm09, 17 pairs of A(H3N2) and 20 pairs of B viruses are shown in Tables 2–4, respectively. The median EC_{50}s of the viruses isolated pre- and 1 or 2 days post-administration were 0.99, 1.02 and 0.90 μM for A(H1N1)pdm09 (Table 2), 8.40, 14.24 and 10.97 μM for A(H3N2) (Table 3) and 3.51, 2.52 and 5.82 μM for B (Table 4), respectively. *P* values between pre and post day 1 or day 2 administration were 0.25 and 0.55 for A(H1N1)pdm09, 0.36 and 0.42 for A(H3N2) and 0.30 and 0.11 for B, respectively. These results demonstrated that none of the viruses acquired statistically significant reduced susceptibility to favipiravir during favipiravir administration.

The EC_{50} fold-change of the virus isolated post-administration compared to the virus isolated pre-administration ranged from 0.4 to 3.3 for A(H1N1)pdm09 (Table 2), from 0.4 to 4.8 for A(H3N2) (Table 3) and from 0.4 to 3.9 for B (Table 4), respectively. To evaluate the significance of slightly higher EC_{50} fold-changes (i.e., 3.3–4.8-fold changes), IC_{50} fold-changes of the same viruses were determined by using the plaque reduction assay. The IC_{50} fold-changes of the viruses were 2.4-fold increase for A(H1N1)pdm09 showing 3.3-fold increase in EC_{50}, 1.5-fold increase for A(H3N2) showing 4.8-fold increase in EC_{50}, 0.9-fold increase for B showing 3.9-fold increase in EC_{50} and 1.5-fold increase for B showing 3.6-fold increase in EC_{50}, respectively (data not shown). Since a difference in IC_{50} values less than 2.4-fold is clearly within experimental variation, the above described results suggest that at least 4.8-fold increase in EC_{50} does not translate to a clinically important difference.

3.3. Comparison of RNA-dependent RNA polymerase gene sequences of influenza viruses isolated pre- and post-administration of favipiravir

Although none of the viruses tested exhibited reduced susceptibility to favipiravir, we compared the gene sequences of the RNA-dependent RNA polymerase subunits, PB1, PB2 and PA, of influenza viruses isolated pre- and post-administration of favipiravir by using Sanger sequencing. We found that two of 20 paired A(H1N1) pdm09, one of 17 paired A(H3N2) and one of 20 paired B viruses possessed at least one amino acid substitutions after favipiravir administration (Table 5).

To find increase in the substitution rates associated with favipiravir treatment, the frequency of these substitutions among circulating influenza viruses was analyzed by using the Analyze Sequence Variation (SNP) tool in the Influenza Research Database (IRD) (Squires et al., 2012). Of 5202 PB1 sequences from A(H1N1) pdm09 viruses, 16 (0.3%) contained V728I substitution and one (0.02%) contained R734Q substitution. Four (0.08%) of 5301 PB2 sequences and one (0.02%) of 5369 PA sequences from A(H1N1) pdm09 viruses contained an A221T or L666F substitution, respectively. Of 5660 PA sequences from A(H3N2) viruses, 75 (1.3%) contained a L550I substitution. None of 2000 PB2 sequences from influenza B viruses contained an I356V substitution. Compared with these background levels of circulating viruses, favipiravir administration was shown to increase the mutation frequency in the viral polymerase complex similar to a previous *in vitro* study (Baranovich et al., 2013).

3.4. Polymerase activity of the A(H1N1)pdm09 virus carrying three amino acid substitutions in the RNA-dependent RNA polymerase subunits after favipiravir administration

An A(H1N1)pdm09 virus carried three amino acid substitutions, V728I in the PB1, A221T in the PB2 and L666F in the PA, after favipiravir administration, as determined by Sanger sequencing (Table 5). Deep sequencing analysis revealed that PB1 V728I, PB2 A221T and PA L666F substitutions of the virus were detected in 77%, 72% and 72%, respectively, of mixed populations with wild-type. To examine the effect of these amino acid substitutions on the polymerase activity, a luciferase-based mini-genome assay was performed. The 'pre-favipiravir' (i.e., wild-type) virus polymerase complex exhibited higher luciferase activity than the 'post-favipiravir' virus polymerase complex (encoding the three mutations listed above) (Fig. 1). The pre-favipiravir virus polymerase complex with PB1 or PB2 of the post-favipiravir virus exhibited substantial luciferase activity, whereas a polymerase complex of the pre-favipiravir virus with PA of the post-favipiravir virus showed low luciferase activity. The IC_{50} values of the polymerase complexes were 46.67 μM for pre-favipiravir, 41.48 μM for PB1 V728I, 41.46 μM for PB2 A221T, 37.62 μM for PA L666F and 19.82 μM for post-favipiravir, respectively. These results showed that the PA L666F substitution reduced the polymerase activity, at least in *in vitro* mini-genome assay, consistent with a previous report that the PA F666L substitution increased the polymerase activity (de Wit et al., 2010).

3.5. NA inhibitor susceptibility of influenza viruses isolated from patients pre- and post-administration of favipiravir

To assess the susceptibilities of influenza viruses to NA inhibitors after favipiravir administration, we compared the IC_{50} values of the viruses isolated pre- and post-administration of favipiravir to NA inhibitors, oseltamivir and zanamivir. The IC_{50} values of 20 pairs of A(H1N1)pdm09, 17 pairs of A(H3N2) and 20

Table 2
Favipiravir and neuraminidase inhibitor susceptibility of influenza A(H1N1)pdm09 viruses isolated from patients pre- and post-administration of favipiravir.

Patient ID	Susceptibility to:								
	Favipiravir (EC_{50} [μM])[a]			Oseltamivir (IC_{50} [nM])[b]			Zanamivir (IC_{50} [nM])[b]		
	Pre-treatment	Day 1 or 2 post-treatment	Fold change	Pre-treatment	Day 1 or 2 post-treatment	Fold change	Pre-treatment	Day 1 or 2 post-treatment	Fold change
1	1.08 ± 0.43	3.56 ± 1.19	3.3	0.32 ± 0.08	0.24 ± 0.07	0.8	0.22 ± 0.07	0.37 ± 0.04	1.7
2	1.19 ± 0.51	1.72 ± 0.76	1.5	0.26 ± 0.07	0.18 ± 0.02	0.7	0.28 ± 0.11	0.24 ± 0.07	0.9
3	1.70 ± 1.01	1.50 ± 0.87[c]	0.9	0.13 ± 0.06	0.20 ± 0.06[c]	1.5	0.16 ± 0.06	0.34 ± 0.13[c]	2.1
4	2.24 ± 1.39	1.34 ± 0.69[c]	0.6	0.19 ± 0.004	0.23 ± 0.04[c]	1.2	0.26 ± 0.07	0.31 ± 0.14[c]	1.2
5	1.27 ± 0.24	1.18 ± 0.19	0.9	0.19 ± 0.05	0.20 ± 0.08	1.1	0.39 ± 0.07	0.26 ± 0.09	0.7
6	0.71 ± 0.41	0.82 ± 0.41[c]	1.2	0.16 ± 0.05	0.21 ± 0.10[c]	1.3	0.26 ± 0.08	0.31 ± 0.10[c]	1.2
7	0.94 ± 0.50	2.02 ± 1.71	2.1	0.13 ± 0.06	0.16 ± 0.08	1.2	0.25 ± 0.08	0.36 ± 0.16	1.4
8	0.39 ± 0.16	0.60 ± 0.30[c]	1.5	0.25 ± 0.05	0.10 ± 0.03[c]	0.4	0.20 ± 0.09	0.30 ± 0.04[c]	1.5
9	1.12 ± 0.48	0.87 ± 0.23	0.8	0.11 ± 0.05	0.16 ± 0.07	1.5	0.37 ± 0.13	0.29 ± 0.13	0.8
10	0.51 ± 0.07	1.26 ± 0.28	2.5	0.26 ± 0.09	0.17 ± 0.04	0.7	0.30 ± 0.11	0.23 ± 0.11	0.8
11	0.65 ± 0.44	0.46 ± 0.17	0.7	0.14 ± 0.06	0.11 ± 0.04	0.8	0.16 ± 0.07	0.31 ± 0.11	1.9
12	1.51 ± 0.90	0.96 ± 0.42	0.6	0.26 ± 0.10	0.19 ± 0.06	0.7	0.17 ± 0.08	0.33 ± 0.16	1.9
13	2.16 ± 0.85	0.87 ± 0.75	0.4	0.17 ± 0.07	0.22 ± 0.09	1.3	0.32 ± 0.04	0.17 ± 0.08	0.5
14	0.74 ± 0.55	0.93 ± 0.32	1.3	0.13 ± 0.02	0.25 ± 0.12	1.9	0.15 ± 0.05	0.24 ± 0.11	1.6
15	0.71 ± 0.41	0.47 ± 0.26	0.7	0.15 ± 0.07	0.11 ± 0.04	0.7	0.15 ± 0.02	0.18 ± 0.06	1.2
16	0.47 ± 0.30	0.53 ± 0.05[c]	1.1	0.11 ± 0.02	0.08 ± 0.04[c]	0.7	0.25 ± 0.11	0.28 ± 0.13[c]	1.1
17	0.58 ± 0.19	0.49 ± 0.20	0.8	0.21 ± 0.09	0.24 ± 0.10	1.1	0.13 ± 0.01	0.15 ± 0.04	1.2
18	0.54 ± 0.19	0.79 ± 0.53	1.5	0.26 ± 0.02	0.19 ± 0.09	0.7	0.29 ± 0.11	0.23 ± 0.08	0.8
19	1.04 ± 0.56	0.53 ± 0.24	0.5	0.25 ± 0.09	0.20 ± 0.07	0.8	0.25 ± 0.10	0.15 ± 0.04	0.6
20	2.06 ± 0.89	1.22 ± 0.71[c]	0.6	0.25 ± 0.12	0.13 ± 0.03[c]	0.5	0.35 ± 0.10	0.31 ± 0.04[c]	0.9

[a] EC_{50} values were determined by the use of the colorimetric cytopathic effect reduction assay. The values are presented as the mean ± SD of triplicate reactions.
[b] IC_{50} values were determined by the use of a chemiluminescent neuraminidase inhibition assay. The values are presented as the mean ± SD of triplicate reactions.
[c] Viruses isolated from patients on day 1 post-treatment.

Table 3
Favipiravir and neuraminidase inhibitor susceptibility of influenza A(H3N2) viruses isolated from patients pre- and post-administration of favipiravir.

Patient ID	Susceptibility to:								
	Favipiravir (EC_{50} [μM])[a]			Oseltamivir (IC_{50} [nM])[b]			Zanamivir (IC_{50} [nM])[b]		
	Pre-treatment	Day 1 or 2 post-treatment	Fold change	Pre-treatment	Day 1 or 2 post-treatment	Fold change	Pre-treatment	Day 1 or 2 post-treatment	Fold change
1	2.69 ± 1.72	6.74 ± 4.79	2.5	0.07 ± 0.02	0.13 ± 0.04	1.9	0.29 ± 0.10	0.49 ± 0.10	1.7
2	17.45 ± 10.13	14.24 ± 12.83[c]	0.8	0.13 ± 0.06	0.18 ± 0.03[c]	1.4	0.27 ± 0.10	0.24 ± 0.09[c]	0.9
3	18.10 ± 16.96	13.46 ± 13.17	0.7	0.16 ± 0.06	0.11 ± 0.03	0.7	0.61 ± 0.23	0.56 ± 0.16	0.9
4	5.75 ± 1.80	3.55 ± 0.86[c]	0.6	0.18 ± 0.05	0.07 ± 0.02[c]	0.4	0.81 ± 0.30	0.33 ± 0.16[c]	0.4
5	12.37 ± 7.17	12.43 ± 3.34	1.0	0.11 ± 0.04	0.15 ± 0.04	1.4	1.40 ± 0.45	0.68 ± 0.24	0.5
6	4.13 ± 1.93	9.51 ± 2.60	2.3	0.11 ± 0.04	0.12 ± 0.04	1.1	1.46 ± 0.39	0.57 ± 0.19	0.4
7	2.15 ± 0.72	4.37 ± 1.96	2.0	0.11 ± 0.03	0.09 ± 0.01	0.8	0.39 ± 0.08	0.73 ± 0.16	1.9
8	11.03 ± 5.29	5.35 ± 2.37[c]	0.5	0.10 ± 0.02	0.10 ± 0.03[c]	1.0	0.91 ± 0.14	0.79 ± 0.05[c]	0.9
9	2.16 ± 1.23	4.14 ± 2.37[c]	1.9	0.10 ± 0.03	0.19 ± 0.04[c]	1.9	0.49 ± 0.18	0.98 ± 0.06[c]	2.0
10	5.21 ± 3.52	25.13 ± 10.03[c]	4.8	0.12 ± 0.004	0.10 ± 0.01[c]	0.8	0.36 ± 0.06	1.06 ± 0.12[c]	2.9
11	20.23 ± 9.50	21.26 ± 2.29[c]	1.1	0.12 ± 0.06	0.12 ± 0.04[c]	1.0	0.50 ± 0.19	0.37 ± 0.09[c]	0.7
12	8.96 ± 5.42	4.03 ± 0.54[c]	0.4	0.08 ± 0.03	0.11 ± 0.02[c]	1.4	0.60 ± 0.22	0.53 ± 0.17[c]	0.9
13	10.12 ± 1.74	20.57 ± 5.07	2.0	0.17 ± 0.04	0.15 ± 0.05	0.9	0.79 ± 0.08	0.93 ± 0.09	1.2
14	8.40 ± 3.82	16.98 ± 2.24[c]	2.0	0.17 ± 0.04	0.09 ± 0.01[c]	0.5	0.85 ± 0.10	0.43 ± 0.15[c]	0.5
15	8.37 ± 4.69	12.68 ± 5.05	1.5	0.13 ± 0.02	0.13 ± 0.03	1.0	0.62 ± 0.16	0.78 ± 0.09	1.3
16	12.99 ± 3.53	5.23 ± 2.07	0.4	0.19 ± 0.02	0.06 ± 0.01	0.3	0.86 ± 0.16	0.39 ± 0.06	0.5
17	8.03 ± 2.54	16.92 ± 5.60[c]	2.1	0.18 ± 0.04	0.06 ± 0.02[c]	0.3	0.71 ± 0.09	0.46 ± 0.09[c]	0.6

[a] EC_{50} values were determined by the use of the colorimetric cytopathic effect reduction assay. The values are presented as the mean ± SD of triplicate reactions.
[b] IC_{50} values were determined by the use of a chemiluminescent neuraminidase inhibition assay. The values are presented as the mean ± SD of triplicate reactions.
[c] Viruses isolated from patients on day 1 post-treatment.

pairs of B viruses are also shown in Tables 2–4, respectively. The IC_{50} fold-change to oseltamivir of viruses isolated before or after treatment with favipiravir ranged from 0.4 to 1.9 for A(H1N1)pdm09 (Table 2), from 0.3 to 1.9 for A(H3N2) (Table 3) and from 0.4 to 1.5 for B (Table 4), respectively. The IC_{50} fold-change to zanamivir ranged from 0.5 to 2.1 for A(H1N1)pdm09 (Table 2), from 0.4 to 2.9 for A(H3N2) (Table 3) and from 0.4 to 2.4 for B (Table 4), respectively. The median IC_{50} values to oseltamivir of viruses isolated pre- and 1 or 2 days post-administration of favipiravir were 0.19, 0.17 and 0.19 μM for A(H1N1)pdm09 (Table 2), 0.12, 0.10 and 0.13 μM for A(H3N2) (Table 3) and 2.03, 1.97 and 1.84 μM for B (Table 4), respectively. *P* values between pre and post day 1 or day 2 administration were 0.60 and 0.36 for A(H1N1)pdm09, 0.44 and 0.55 for A(H3N2) and 0.41 and 0.45 for B, respectively. The median IC_{50}s to zanamivir were 0.25, 0.31 and 0.24 μM for A(H1N1)pdm09 (Table 2), 0.62, 0.46 and 0.63 μM for A(H3N2) (Table 3) and 1.11, 0.95 and 0.99 μM for B (Table 4), respectively. *P* values between pre and post day 1 or day 2 administration were 0.09 and 0.83 for A(H1N1)pdm09, 0.81 and 0.37 for A(H3N2) and 0.31 and 0.98 for B, respectively. These results suggest that favipiravir administration did not affect the susceptibility of influenza viruses to NA inhibitors, oseltamivir and zanamivir.

Table 4
Favipiravir and neuraminidase inhibitor susceptibility of influenza B viruses isolated from patients pre- and post-administration of favipiravir.

Patient ID	Susceptibility to:								
	Favipiravir (EC_{50} [μM])[a]			Oseltamivir (IC_{50} [nM])[b]			Zanamivir (IC_{50} [nM])[b]		
	Pre-treatment	Day 1 or 2 post-treatment	Fold change	Pre-treatment	Day 1 or 2 post-treatment	Fold change	Pre-treatment	Day 1 or 2 post-treatment	Fold change
1	0.69 ± 0.26	0.67 ± 0.18	1.0	2.28 ± 0.72	0.92 ± 0.43	0.4	1.13 ± 0.30	0.83 ± 0.30	0.7
2	3.92 ± 2.59	4.34 ± 2.19[d]	1.1	1.55 ± 0.39	0.94 ± 0.25[d]	0.6	0.90 ± 0.19	0.56 ± 0.25[d]	0.6
3[c]	2.79 ± 2.13	7.89 ± 7.16	2.8	3.15 ± 1.19	2.59 ± 0.46	0.8	2.46 ± 0.10	2.46 ± 0.48	1.0
4	2.60 ± 1.79	6.08 ± 4.70	2.3	1.70 ± 0.18	1.69 ± 0.79	1.0	0.50 ± 0.20	0.94 ± 0.35	1.9
5	4.99 ± 4.31	12.57 ± 3.65	2.5	1.75 ± 0.35	2.07 ± 0.23	1.2	1.14 ± 0.30	1.13 ± 0.08	1.0
6	1.21 ± 1.11	1.46 ± 0.47[d]	1.2	4.13 ± 0.11	3.23 ± 0.90[d]	0.8	3.95 ± 0.45	2.46 ± 1.13[d]	0.6
7	2.86 ± 1.54	2.54 ± 1.01	0.9	2.01 ± 0.48	1.87 ± 0.50	0.9	1.27 ± 0.61	1.11 ± 0.24	0.9
8	5.75 ± 3.78	2.49 ± 1.44	0.4	1.57 ± 0.55	1.73 ± 0.44	1.1	1.09 ± 0.35	2.65 ± 0.30	2.4
9	1.61 ± 1.37	2.20 ± 1.03[d]	1.4	1.33 ± 0.23	2.00 ± 0.08[d]	1.5	0.74 ± 0.34	0.75 ± 0.37[d]	1.0
10	0.81 ± 0.43	1.55 ± 0.77	1.9	2.02 ± 0.26	1.43 ± 0.26	0.7	1.51 ± 0.32	1.20 ± 0.44	0.8
11	8.68 ± 8.30	5.82 ± 4.32	0.7	2.06 ± 0.76	1.92 ± 0.56	0.9	0.65 ± 0.25	0.66 ± 0.23	1.0
12	8.17 ± 4.64	12.03 ± 5.67	1.5	2.03 ± 0.56	1.84 ± 0.44	0.9	0.71 ± 0.07	0.60 ± 0.09	0.8
13	0.71 ± 0.29	2.80 ± 1.04	3.9	1.08 ± 0.33	1.57 ± 0.20	1.5	1.22 ± 0.43	0.52 ± 0.12	0.4
14	5.96 ± 4.89	8.04 ± 4.92	1.3	1.83 ± 0.38	1.71 ± 0.42	0.9	0.68 ± 0.11	0.64 ± 0.12	0.9
15	4.13 ± 3.56	4.12 ± 2.09	1.0	2.11 ± 0.32	2.20 ± 0.60	1.0	0.75 ± 0.13	0.73 ± 0.08	1.0
16	5.23 ± 1.71	7.05 ± 2.01	1.3	3.45 ± 0.98	4.21 ± 0.48	1.2	2.69 ± 1.11	2.50 ± 0.88	0.9
17	4.71 ± 3.29	3.41 ± 2.45	0.7	2.48 ± 0.40	2.31 ± 0.50	0.9	0.98 ± 0.07	0.99 ± 0.11	1.0
18	3.10 ± 2.64	2.52 ± 0.91[d]	0.8	2.19 ± 0.23	1.97 ± 0.44[d]	0.9	1.04 ± 0.10	0.95 ± 0.06[d]	0.9
19	1.54 ± 1.12	5.55 ± 1.38[d]	3.6	2.07 ± 0.28	1.92 ± 0.22[d]	0.9	1.15 ± 0.13	1.34 ± 0.10[d]	1.2
20	9.33 ± 2.64	9.74 ± 5.87	1.0	1.81 ± 0.46	1.79 ± 0.14	1.0	1.31 ± 0.48	1.08 ± 0.32	0.8

[a] EC_{50} values were determined by the use of the colorimetric cytopathic effect reduction assay. The values are presented as the mean ± SD of triplicate reactions.
[b] IC_{50} values were determined by the use of a chemiluminescent neuraminidase inhibition assay. The values are presented as the mean ± SD of triplicate reactions.
[c] B/Yamagata-lineage virus.
[d] Viruses isolated from patients on day 1 post-treatment.

Table 5
Amino acid substitutions in the RNA-dependent RNA polymerase subunits of influenza viruses detected after favipiravir administration.

Subtype	No. of patients tested	Patient ID	Amino acid substitution in[a]		
			PB1	PB2	PA
A(H1N1)pdm09	20	1	V728I	A221T	L666F
		12	R734Q	—[b]	—
A(H3N2)	17	9	—	—	L550I
B	20	12	—	I356V	—

[a] Amino acid position numbering is A subtype and B type specific.
[b] Not detected.

4. Discussion

In Japan, favipiravir has been approved for influenza pandemic preparedness. Here, we conducted a CPE reduction assay to evaluate the favipiravir susceptibility of influenza viruses for antiviral susceptibility surveillance. The susceptibility of the viruses to favipiravir was expressed as the EC_{50}. Since these values are assay-specific, they cannot be compared with results published by others (Smee et al., 2001). However, our data from the CPE reduction assay, together with the conventional plaque reduction assay, that viruses resistant to NA inhibitors are sensitive to favipiravir match those published by Sleeman et al. (2010). These results suggest that the CPE reduction assay is an option to screen influenza viruses for susceptibility to favipiravir.

Influenza A(H1N1) or A(H1N1)pdm09 viruses with reduced susceptibility to favipiravir have not been detected even after sequential passages in MDCK cells in the presence of favipiravir (Baranovich et al., 2013; Daikoku et al., 2014; Sangawa et al., 2013). We examined the favipiravir susceptibility of 57 pairs of influenza A(H1N1)pdm09, A(H3N2) and B viruses isolated from patients pre- and post-administration of favipiravir enrolled in its clinical trials and found no viruses with statistically significant reduced susceptibility to favipiravir after its administration. These results support

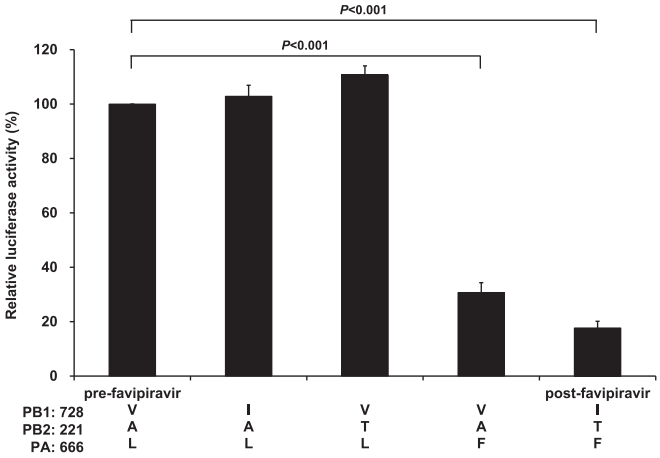

Fig. 1. Polymerase activities of the A(H1N1)pdm09 viral polymerase complex carrying amino acid substitutions in the RNA-dependent RNA polymerase subunits. Minigenome assays were performed by transfecting 293T cells with protein expression plasmids (PB1, PB2, PA and NP), pPolINP(0)luc2(0) for the production of vRNA encoding a firefly luciferase gene, and pRL-null renilla luciferase control reporter vector. At 24 h posttransfection, cells were assayed for luciferase activity. The relative firefly luciferase activities, normalized to the renilla luciferase activities, are shown. Error bars indicate the standard deviations from three independent experiments in triplicates.

the possibility that mutation(s) causing resistance to favipiravir is fatal for influenza virus replication as previously described (Baranovich et al., 2013; Daikoku et al., 2014).

A previous report suggested that favipiravir increases the mutation frequency in the influenza viral genome beyond the biological tolerance threshold (Baranovich et al., 2013). In this study, two of 20 paired A(H1N1)pdm09, one of 17 paired A(H3N2) and one of 20 paired B viruses possessed at least one amino acid substitutions in the RNA-dependent RNA polymerase subunits, PB1, PB2 or PA, after favipiravir administration (Table 5). In a previous study, several amino acid substitutions in the PB1, PB2 or PA of influenza

A/PR/8/34(H1N1) virus were also detected during sequential passages in MDCK cells in the presence of favipiravir (Daikoku et al., 2014), but they were no identical substitutions with this study. These amino acid substitutions did not affect susceptibilities of the viruses to favipiravir.

After favipiravir treatment, an A(H1N1)pdm09 virus possessed three amino acid substitutions, PB1 V728I, PB2 A221T and PA L666F. *In vitro* mini-genome assay showed that the PA L666F substitution reduced the polymerase activity, consistent with a previous study of an A(H7N7) virus (de Wit et al., 2010). The L666 is located in the C-terminal domain of PA and makes hydrophobic contact with PB1 (He et al., 2008; Obayashi et al., 2008). Mutation of residue L666 disrupts the binding to PB1 and the synthesis of vRNA, cRNA and mRNA (He et al., 2008; Obayashi et al., 2008). Thus, the PA L666F substitution might cause similar disruptions, resulting in reduced polymerase activity.

Susceptibility of influenza A(H1N1) and A(H1N1)pdm09 viruses to NA inhibitors, oseltamivir and zanamivir, was not altered by serial passage in MDCK cells with or without favipiravir (Baranovich et al., 2013). To assess susceptibility of influenza A(H1N1)pdm09, A(H3N2) and B viruses to oseltamivir and zanamivir after favipiravir administration, we compared IC_{50}s of 57 pairs of influenza A(H1N1)pdm09, A(H3N2) and B viruses isolated from patients pre- and post-administration of favipiravir. None of these viruses displayed reduced susceptibility to NA inhibitors, suggesting that favipiravir administration did not alter NA inhibitor susceptibility of influenza viruses.

We previously reported that 55 of 516 influenza A(H1N1)pdm09 viruses isolated from patients prophylactically or therapeutically treated with oseltamivir showed resistance to oseltamivir (Ujike et al., 2011). Six of 55 oseltamivir-resistant A(H1N1)pdm09 viruses were detected by 2 days post-administration of oseltamivir (Ujike et al., 2011). In contrast, we found no favipiravir-resistant viruses at least by 2 days post-administration of favipiravir. Therefore, the frequency of emergence of antiviral-resistant viruses in clinical settings is most likely much lower for favipiravir compared with that for oseltamivir.

In summary, we conducted a CPE reduction assay to evaluate the susceptibility of influenza viruses to a novel antiviral drug, favipiravir. The assay will be an option to monitor the favipiravir susceptibility of influenza viruses. Favipiravir administration for 1 or 2 days (1200/400 mg for 1 day + 400 mg bid during the following day) did not affect the susceptibility of influenza A(H1N1)pdm09, A(H3N2) and B viruses to favipiravir and NA inhibitors.

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