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SUPPLEMENTARY INFORMATION

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Influenza A variants with reduced susceptibility to baloxavir isolated from Japanese patients are fit and transmit through respiratory droplets

Masaki Imai[1,17]*, Makoto Yamashita[1,17], Yuko Sakai-Tagawa[1,17], Kiyoko Iwatsuki-Horimoto[1,17], Maki Kiso[1], Jurika Murakami[1], Atsuhiro Yasuhara[1], Kosuke Takada[1], Mutsumi Ito[1], Noriko Nakajima[2], Kenta Takahashi[2], Tiago J. S. Lopes[1,3], Jayeeta Dutta[4], Zenab Khan[4], Divya Kriti[4], Harm van Bakel[4], Akifumi Tokita[5,6], Haruhisa Hagiwara[6,7], Naomi Izumida[6,8], Haruo Kuroki[9], Tamon Nishino[6,10], Noriyuki Wada[6,11], Michiko Koga[12], Eisuke Adachi[13], Daisuke Jubishi[1,14], Hideki Hasegawa[2,15] and Yoshihiro Kawaoka[1,3,16]*

[1]Division of Virology, Department of Microbiology and Immunology, Institute of Medical Science, University of Tokyo, Tokyo, Japan. [2]Department of Pathology, National Institute of Infectious Diseases, Tokyo, Japan. [3]Influenza Research Institute, Department of Pathobiological Sciences, School of Veterinary Medicine, University of Wisconsin, Madison, WI, USA. [4]Department of Genetics and Genomic Sciences, Icahn School of Medicine at Mount Sinai, New York, NY, USA. [5]Clinic Bambini, Tokyo, Japan. [6]Members of the Tokyo Pediatric Association Public Health Committee, Tokyo, Japan. [7]Hagiwara Clinic, Tokyo, Japan. [8]Akebonocho Clinic, Tokyo, Japan. [9]Sotobo Children's Clinic, Chiba, Japan. [10]Alpaca Kids Ent Clinic, Tokyo, Japan. [11]Wada Pediatric Clinic, Tokyo, Japan. [12]Division of Infectious Diseases, Advanced Clinical Research Center, Institute of Medical Science, University of Tokyo, Tokyo, Japan. [13]Department of Infectious Diseases and Applied Immunology, IMSUT Hospital of the Institute of Medical Science, University of Tokyo, Tokyo, Japan. [14]Nezu Clinic, Tokyo, Japan. [15]Influenza Virus Research Center, National Institute of Infectious Diseases, Tokyo, Japan. [16]Department of Special Pathogens, International Research Center for Infectious Diseases, Institute of Medical Science, University of Tokyo, Minato-ku, Tokyo, Japan. [17]These authors contributed equally: Masaki Imai, Makoto Yamashita, Yuko Sakai-Tagawa, Kiyoko Iwatsuki-Horimoto. *e-mail: mimai@ims.u-tokyo.ac.jp; yoshihiro.kawaoka@wisc.edu

Supplementary Information

Supplementary Discussion

In this study, we observed that seasonal A/H1N1pdm and A/H3N2 viruses possessing the PA-I38T mutation isolated from patients grew as efficiently as their baloxavir acid-sensitive counterparts in hCK cells (**Fig. 1a**). In addition, our data show that these mutant isolates replicate comparably to baloxavir acid-sensitive isolates in the respiratory tracts of hamsters and mice and retain their pathogenicity in these animals (**Fig. 2a,b, Extended Data Fig. 3, and Extended Data Fig. 4**). Importantly, the A/H1N1pdm and H3N2 mutant isolates were efficiently transmitted via respiratory droplets among ferrets (**Fig. 2c**). These findings suggest that the currently circulating seasonal influenza A viruses could maintain their viral fitness even though they have evolved to a status that is less susceptibility to baloxavir acid. Our *in vitro* and animal model data are consistent with the finding that in some patients the emergence of viruses with reduced susceptibility to baloxavir acid is associated with recurrent clinical manifestations.

In a Phase III trial, baloxavir marboxil-treated patients infected with PA-I38T/M mutant variants exhibited prolonged infectious viral shedding compared with the baloxavir marboxil-treated group infected with viruses that lacked these mutations and the placebo-treated group[1]. In addition, in the baloxavir marboxil-treated patients infected with PA-I38T/M mutant variants, the time to alleviation of influenza symptoms was longer than that for the baloxavir marboxil-treated group infected with viruses that lacked these mutations. In contrast, our study in ferrets showed that the duration of viral shedding was not significantly different between animals infected with either the PA-I38T variants or the wild-type parent viruses, suggesting that the I38T mutation itself does not appear to be related to

prolonged viral loads or increased duration of illness. In the clinical trial, a rapid decline in viral load was observed in patients treated with baloxavir marboxil within 24 hours of treatment[1]. These findings suggest the possibility that limited viral replication in baloxavir marboxil-treated patients does not stimulate effective innate and adaptive immune responses, which are essential for viral clearance, and thus allows the emergence of PA-I38T variants, thereby causing prolonged virus shedding. Further investigations are required to determine whether the host immune response to viral infections is effectively induced in these specific patients.

In this study, we observed that most of the patients infected with A/H1N1pdm and A/H3N2 virus variants carrying PA-I38T mutation were children under age 12. Similar findings for baloxavir marboxil-treated patients with A/H3N2 infection have been reported by Takashita et al.[2]. In the clinical studies of baloxavir marboxil, the frequency of PA-I38 variants in patients aged 12 to 64 years and in children aged <12 years was 9.7% (36/370) and 23.4% (18/77), respectively[1,3]. These findings indicate that the frequency of PA-I38 variants is higher in children than in adults. Limited immunity to circulating influenza viruses in children may allow prolonged viral replication, promoting to the emergence of PA-I38 variants. Importantly, our data show that the replicative abilities, pathogenicity, and transmissibility of PA-I38T variant isolates are comparable to those of wild-type isolates in animal models. Given that the emergence of these variants is associated with increased duration of illness, the use of baloxavir marboxil in pediatric patients should be carefully considered.

Supplementary References

1. Hayden, F.G. *et al.* Baloxavir Marboxil for Uncomplicated Influenza in Adults and Adolescents. *N Engl J Med* **379**, 913-923 (2018).

2. Takashita, E. *et al.* Influenza A(H3N2) virus exhibiting reduced susceptibility to baloxavir due to a polymerase acidic subunit I38T substitution detected from a hospitalised child without prior baloxavir treatment, Japan, January 2019. *Euro Surveill* **24** (2019).

3. Omoto, S. *et al.* Characterization of influenza virus variants induced by treatment with the endonuclease inhibitor baloxavir marboxil. *Sci Rep* **8**, 9633 (2018).

Supplementary Table 1. Detection of influenza A/H1pdm viruses with a mutation at position 38 of PA in patients prior to drug treatment[a].

Patient ID	Sex	Age, y	Sample ID	Subtype	Amino acid at position 38 of PA	
					[b]Sanger sequencing	[c]Deep sequencing
AC65	Female	7	AC65-0	H1pdm	I	I:99%
BB165	Male	8	BB165-0	H1pdm	I	I:100%
BB166	Male	8	BB166-0	H1pdm	I	I:100%
BB169	Male	7	BB169-0	H1pdm	I	I:100%
BB170	Male	4	BB170-0	H1pdm	I	I:100%
BB171	Female	3	BB171-0	H1pdm	I	I:100%
BB173	Male	9	BB173-0	H1pdm	I	I:100%
BB175	Male	6	BB175-0	H1pdm	I	I:100%
BB176	Male	4	BB176-0	H1pdm	I	I:100%
BB177	Female	10	BB177-0	H1pdm	I	I:100%
BB178	Male	5	BB178-0	H1pdm	I	I:100%
BB179	Female	4	BB179-0	H1pdm	I	I:100%
BB180	Female	7	BB180-0	H1pdm	I	I:100%
BB181	Male	11	BB181-0	H1pdm	I	I:100%
BB182	Female	1	BB182-0	H1pdm	I	I:100%
BB183	Female	10	BB183-0	H1pdm	I	I:100%
BB185	Female	9	BB185-0	H1pdm	I	I:100%
BB186	Male	3	BB186-0	H1pdm	I	I:100%
BB188	Female	10	BB188-0	H1pdm	I	I:100%
BB190	Male	6	BB190-0	H1pdm	I	I:100%
BB191	Male	9	BB191-0	H1pdm	I	I:100%
BB192	Male	6	BB192-0	H1pdm	I	I:100%
BB195	Female	7	BB195-0	H1pdm	I	I:100%
BB202	Female	9	BB202-0	H1pdm	I	I:100%
BB205	Female	10	BB205-0	H1pdm	I	I:100%
BB206	Male	5	BB206-0	H1pdm	I	I:100%
BB210	Female	8	BB210-0	H1pdm	I	I:100%
BB236	Male	9	BB236-0	H1pdm	I	I:100%
BB243	Male	8	BB243-0	H1pdm	I	I:100%
BB245	Male	6	BB245-0	H1pdm	I	I:100%
BB246	Male	8	BB246-0	H1pdm	I	I:100%
KK003	Male	11	KK003-0	H1pdm	I	I:100%
KK005	Female	12	KK005-0	H1pdm	I	I:100%
KK009	Female	8	KK009-0	H1pdm	I	I:100%
KK010	Female	21	KK010-0	H1pdm	I	I:100%

KK011	Female	12	KK011-0	H1pdm	I	I:100%
KK012	Male	9	KK012-0	H1pdm	I	I:100%
KK013	Female	10	KK013-0	H1pdm	I	I:100%
KK014	Female	12	KK014-0	H1pdm	I	I:100%
KK016	Female	5	KK016-0	H1pdm	I	I:100%
KK017	Female	7	KK017-0	H1pdm	I	I:100%
KK018	Male	13	KK018-0	H1pdm	I	I:100%
KK019	Female	44	KK019-0	H1pdm	I	I:100%
KK021	Female	7	KK021-0	H1pdm	I	I:100%
LK004	Male	7	LK004-0	H1pdm	I	I:100%
LK006	Male	7	LK006-0	H1pdm	I	I:100%
LK009	Female	9	LK009-0	H1pdm	I	I:100%
LK010	Female	3	LK010-0	H1pdm	I	I:100%
LK014	Female	8	LK014-0	H1pdm	I	I:100%
LK019	Female	7	LK019-0	H1pdm	I	I:100%
LK021	Female	7	LK021-0	H1pdm	I	I:100%
LK023	Female	2	LK023-0	H1pdm	I	I:100%
LK025	Male	5	LK025-0	H1pdm	I	I:100%
LK031	Male	7	LK031-0	H1pdm	I	I:100%
LK032	Male	9	LK032-0	H1pdm	I	I:100%
LK036	Female	3	LK036-0	H1pdm	I	I:100%
LK039	Male	9	LK039-0	H1pdm	I	I:100%
WD039	Male	8	WD039	H1pdm	I	I:100%
WD041	Male	9	WD041-0	H1pdm	I	I:100%
WD046	Male	11	WD046-0	H1pdm	I	I:100%
WD047	Male	8	WD047-0	H1pdm	I	I:100%
WD052	Female	4	WD52-0	H1pdm	I	I:100%
GR068	Female	3	GR068-0	H1pdm	I	I:100%
GR069	Female	24	GR069-0	H1pdm	I	I:100%
GR070	Male	9	GR070-0	H1pdm	I	I:100%
GR071	Female	65	GR071-0	H1pdm	I	I:100%
GR075	Male	8	GR075-0	H1pdm	I	I:100%
GR076	Female	45	GR076-0	H1pdm	I	I:100%
GR077	Female	15	GR077-0	H1pdm	I	I:100%
GR079	Female	25	GR079-0	H1pdm	I	I:100%
GR080	Female	33	GR080-0	H1pdm	I	I:100%
GR081	Male	22	GR081-0	H1pdm	I	I:100%
GR087	Female	16	GR087-0	H1pdm	I	I:100%

GR088	Male	4	GR088-0	H1pdm	I	I:100%
GR089	Male	2	GR089-0	H1pdm	I	I:100%
GR093	Male	5	GR093-0	H1pdm	I	I:100%
GR094	Male	8	GR094-0	H1pdm	I	I:100%
GR096	Male	10	GR096-0	H1pdm	I	I:100%
GR102	Male	9	GR102-0	H1pdm	I	I:100%
GR105	Female	10	GR105-0	H1pdm	I	I:100%
GR106	Female	2	GR106-0	H1pdm	I	I:100%
GR107	Male	0	GR107-0	H1pdm	I	I:100%
GR108	Male	13	GR108-0	H1pdm	I	I:100%
GR116	Female	11	GR116-0	H1pdm	I	I:100%
GR118	Male	12	GR118-0	H1pdm	I	I:100%
GR119	Female	10	GR119-0	H1pdm	I	I:100%
GR126	Female	13	GR126-0	H1pdm	I	I:100%
GR140	Female	2	GR140-0	H1pdm	I	I:100%
HP92	Male	36	HP92-0	H1pdm	I	I:100%
HP94	Male	50	HP94-0	H1pdm	I	I:100%
HP95	Male	26	HP95-0	H1pdm	I	I:100%
HP96	Male	50	HP96-0	H1pdm	I	I:100%
HP98	Female	51	HP98-0	H1pdm	I	I:100%
HP104	Female	35	HP104-0	H1pdm	I	I:100%
HP108	Female	34	HP108-0	H1pdm	I	I:100%
HP110	Female	85	HP110-0	H1pdm	I	I:99%

[a]The sequences of the PA gene of the A/H3 viruses in the clinical specimens were determined by means of Sanger sequencing and deep sequencing. [b]The level of mutation frequencies was estimated based on the height of the waves at each position on the sequencing chromatogram. The detection limit for a minor population was 10%–20%. [c]Residues present at a frequency of more than 5% are shown.

Supplementary Table 2. Detection of influenza A/H3 viruses with a mutation at position 38 of PA in patients prior to drug treatment[a].

Patient ID	Sex	Age, y	Sample ID	Subtype	Amino acid at position 38 of PA	
					[b]Sanger sequencing	[c]Deep sequencing
AC41	Female	49	AC41-0	H3	I	I:100%
AC42	Female	49	AC42-0	H3	I	I:100%
AC43	Female	45	AC43-0	H3	I	I:100%
AC44	Male	20	AC44-0	H3	I	I:100%
AC45	Male	23	AC45-0	H3	I	I:100%
AC47	Female	54	AC47-0	H3	I	I:100%
AC48	Male	35	AC48-0	H3	I	I:100%
AC49	Female	42	AC49-0	H3	I	I:100%
AC50	Female	10	AC50-0	H3	I	I:100%
AC51	Female	17	AC51-0	H3	I	I:100%
AC52	Male	16	AC52-0	H3	I	I:100%
AC53	Female	22	AC53-0	H3	I	I:100%
AC54	Male	35	AC54-0	H3	I	I:100%
AC55	Female	19	AC55-0	H3	I	I:100%
AC56	Male	40	AC56-0	H3	I	I:100%
AC57	Male	50	AC57-0	H3	I	I:100%
AC60	Male	33	AC60-0	H3	I	I:100%
AC61	Male	43	AC61-0	H3	I	I:100%
AC62	Male	5	AC62-0	H3	I	I:100%
AC63	Female	44	AC63-0	H3	I	I:100%
AC64	Female	21	AC64-0	H3	I	I:100%
AC66	Male	25	AC66-0	H3	I	I:100%
AC67	Male	43	AC67-0	H3	I	N/A[d]
AC68	Female	8	AC68-0	H3	I	I:100%
AC69	Male	13	AC69-0	H3	I	I:100%
BB168	Female	8	BB168-0	H3	I	I:100%
BB172	Female	6	BB172-0	H3	I	I:100%
BB184	Female	3	BB184-0	H3	I	I:100%
BB187	Male	9	BB187-0	H3	I	I:97%
BB189	Female	12	BB189-0	H3	I	I:100%
BB193	Male	2	BB193-0	H3	I	I:100%
BB194	Female	4	BB194-0	H3	I	I:100%
BB196	Female	0	BB196-0	H3	I	I:100%
BB198	Male	4	BB198-0	H3	I	I:99%
BB203	Male	3	BB203-0	H3	I	I:100%

BB208	Male	6	BB208-0	H3	I	I:100%
BB212	Male	7	BB212-0	H3	I	I:100%
BB213	Female	10	BB213-0	H3	I	I:100%
BB214	Female	11	BB214-0	H3	I	I:99%
BB215	Female	4	BB215-0	H3	I	I:100%
BB216	Female	10	BB216-0	H3	I	I:100%
BB217	Female	3	BB217-0	H3	I	I:97%
BB218	Male	5	BB218-0	H3	I	I:99%
BB220	Male	4	BB220-0	H3	I	I:99%
BB221	Female	7	BB221-0	H3	I	I:100%
BB222	Female	8	BB222-0	H3	I	I:100%
BB223	Male	9	BB223-0	H3	I	I:100%
BB224	Female	9	BB224-0	H3	I	I:99%
BB225	Female	10	BB225-0	H3	I	I:100%
BB231	Male	6	BB231-0	H3	I	I:100%
BB233	Female	7	BB233-0	H3	**T**	**T:100%**
BB235	Male	8	BB235-0	H3	I	I:100%
BB239	Female	5	BB239-0	H3	I	I:100%
BB240	Female	8	BB240-0	H3	I	I:100%
KK006	Male	17	KK006-0	H3	I	I:100%
KK007	Female	7	KK007-0	H3	I	I:100%
KK008	Male	12	KK008-0	H3	I	I:100%
KK015	Male	5	KK015-0	H3	I	I:100%
KK020	Female	12	KK020-0	H3	I	I:100%
LK002	Male	3	LK002-0	H3	I	I:100%
LK003	Male	7	LK003-0	H3	I	I:99%
LK005	Male	4	LK005-0	H3	I	I:100%
LK007	Male	2	LK007-0	H3	I	I:100%
LK008	Female	7	LK008-0	H3	I	I:100%
LK011	Female	10	LK011-0	H3	I	I:100%
LK012	Female	5	LK012-0	H3	I	I:100%
LK013	Male	3	LK013-0	H3	I	I:100%
LK015	Female	8	LK015-0	H3	I	I:100%
LK016	Male	10	LK016-0	H3	I	I:100%
LK017	Female	12	LK017-0	H3	I	I:100%
LK018	Male	8	LK018-0	H3	I	I:100%
LK020	Female	4	LK020-0	H3	I	I:100%
LK022	Female	1	LK022-0	H3	I	I:100%

LK024	Female	4	LK024-0	H3	I	I:100%
LK026	Male	8	LK026-0	H3	I	I:98%
LK028	Female	11	LK028-0	H3	I	I:100%
LK029	Female	2	LK029-0	H3	I	I:100%
LK030	Female	1	LK030-0	H3	I	I:100%
LK033	Male	10	LK033-0	H3	I	I:100%
LK034	Male	7	LK034-0	H3	I	I:100%
LK035	Female	5	LK035-0	H3	I	I:99%
LK037	Female	1	LK037-0	H3	I	I:100%
LK038	Female	7	LK038-0	H3	I	I:100%
LK040	Male	8	LK040-0	H3	I	I:100%
LK041	Male	3	LK041-0	H3	I	I:100%
WD034	Male	33	WD034-0	H3	I	I:100%
WD035	Female	6	WD035-0	H3	I	I:96%
WD037	Female	9	WD037-0	H3	I	I:100%
WD038	Male	8	WD038-0	H3	I	I:100%
WD040	Male	14	WD040-0	H3	I	I:100%
WD042	Male	11	WD042-0	H3	I	I:100%
WD043	Female	9	WD043-0	H3	I	I:100%
WD044	Male	9	WD044-0	H3	I	I:99%
WD045	Male	9	WD045-0	H3	I	I:100%
WD048	Male	8	WD048-0	H3	I	I:99%
WD051	Male	8	WD051-0	H3	I	I:100%
GR072	Male	42	GR072-0	H3	I	I:100%
GR084	Male	4	GR084-0	H3	I	I:100%
GR085	Male	36	GR085-0	H3	I	I:100%
GR086	Female	46	GR086-0	H3	I	I:100%
GR091	Male	6	GR091-0	H3	I	I:100%
GR092	Female	3	GR092-0	H3	I	I:100%
GR095	Female	5	GR095-0	H3	I	I:100%
GR097	Male	27	GR097-0	H3	I	I:100%
GR098	Male	5	GR098-0	H3	I	I:100%
GR099	Female	12	GR099-0	H3	I	I:100%
GR100	Female	1	GR100-0	H3	I	I:99%
GR101	Male	21	GR101-0	H3	I	I:100%
GR103	Female	5	GR103-0	H3	I	I:100%
GR104	Male	7	GR104-0	H3	I	I:100%
GR109	Female	5	GR109-0	H3	I	I:100%

GR110	Male	0	GR110-0	H3	I	I:100%
GR111	Male	24	GR111-0	H3	I	I:100%
GR113	Male	13	GR113-0	H3	I	I:100%
GR115	Male	6	GR115-0	H3	I	N/A[d]
GR117	Male	5	GR117-0	H3	I	I:100%
GR120	Female	3	GR120-0	H3	I	I:100%
GR121	Female	16	GR121-0	H3	I	I:100%
GR123	Male	2	GR123-0	H3	I	I:100%
GR124	Male	9	GR124-0	H3	I	I:100%
GR125	Male	11	GR125-0	H3	I	I:99%
GR128	Male	12	GR128-0	H3	I	I:100%
GR129	Male	11	GR129-0	H3	I	I:100%
GR130	Female	1	GR130-0	H3	I	I:100%
GR131	Male	12	GR131-0	H3	I	I:100%
GR133	Female	5	GR133-0	H3	I	I:100%
GR134	Female	11	GR134-0	H3	I	I:100%
GR135	Male	1	GR135-0	H3	I	I:100%
GR136	Female	6	GR136-0	H3	I	I:100%
GR137	Female	16	GR137-0	H3	I	I:100%
GR138	Female	5	GR138-0	H3	I	I:100%
GR141	Male	1	GR141-0	H3	I	I:100%
GR142	Female	3	GR142-0	H3	**T**	**T:100%**
GR143	Male	1	GR143-0	H3	I	I:100%
GR144	Male	10	GR144-0	H3	I	I:100%
GR145	Male	11	GR145-0	H3	I	I:100%
GR147	Male	8	GR147-0	H3	I	I:100%
GR149	Male	9	GR149-0	H3	I	I:100%
GR151	Male	8	GR151-0	H3	I	I:100%
GR152	Male	7	GR152-0	H3	I	I:100%
HP93	Male	33	HP93-0	H3	I	I:100%
HP97	Male	45	HP97-0	H3	I	I:100%
HP101	Male	16	HP101-0	H3	I	N/A[d]
HP102	Female	9	HP102-0	H3	I	I:100%
HP105	Female	88	HP105-0	H3	I	I:100%
HP106	Male	41	HP106-0	H3	I	I:100%
HP107	Female	83	HP107-0	H3	I	I:100%
HP112	Male	36	HP112-0	H3	I	I:100%
NZ006	Male	66	NZ006-0	H3	I	I:100%

NZ007	Male	60	NZ007-0	H3	I	I:100%
NZ008	Male	86	NZ008-0	H3	I	I:100%
DA34	Male	2	DA34-0	H3	I	I:100%
DA37	Male	45	DA37-00	H3	I	I:100%
AC70	Male	33	AC70-0	H3	I	I:100%
AC71	Female	9	AC71-0	H3	I	I:100%
AC72	Male	8	AC72-0	H3	I	I:100%
AC73	Male	43	AC73-0	H3	I	I:100%

[a]The sequences of the PA gene of the A/H3 viruses in the clinical specimens were determined by means of Sanger sequencing and deep sequencing. [b]The level of mutation frequencies was estimated based on the height of the waves at each position on the sequencing chromatogram. The detection limit for a minor population was 10%–20%. [c]Residues present at a frequency of more than 5% are shown. [d]N/A, not available.

Supplementary Table 3. Detection of influenza A/H1pdm viruses with a mutation at position 38 of PA in patients treated with baloxavir marboxil[a].

	Patient ID	Sex	Age, y	Sample ID	Subtype	Days post-administration of baloxavir marboxil	Amino acid at position 38 of PA	
							[b]Sanger sequencing	[c]Deep sequencing
1	BB210	Female	8	BB210-0	H1pdm	0	I	I:100%
				BB210-3		3	**T/I**[d]	**T/I[d]:86/14%**
2	BB245	Male	6	BB245-0	H1pdm	0	I	I:100%
				BB245-3		3	I	I:100%
3	BB246	Male	8	BB246-0	H1pdm	0	I	I:100%
				BB246-3		3	I	I:100%
4	KK003	Male	11	KK003-0	H1pdm	0	I	I:100%
				KK003-2		2	I	I:100%
				KK003-4		4	**T/N/S/I**[e]	**T/N/I/S[f]:48/21/18/13%**
				KK003-5		5	**T/S/I**[g]	**T/S/I[g]:47/43/9%**
5	KK005	Female	12	KK005-0	H1pdm	0	I	I:100%
				KK005-1		1	I	N/A[h]
				KK005-2		2	I	I:100%
				KK005-4		4	**T/I**[d]	**I/T[i]:74/26%**
				KK005-5		5	**T/I**[d]	**I/T[i]:57/43%**
6	KK010	Female	21	KK010-0	H1pdm	0	I	I:100%
				KK010-1		1	I	I:100%
				KK010-2		2	I	I:100%
				KK010-3		3	I	I:100%
7	KK011	Female	12	KK011-0	H1pdm	0	I	I:100%
				KK011-2		2	I	I:100%
8	KK012	Male	9	KK012-0	H1pdm	0	I	I:100%
				KK012-1		1	I	I:100%
				KK012-2		2	I	I:100%
				KK012-3		3	I	I:100%
				KK012-4		4	**T/I**[d]	**T/F/I[j]:77/11/10%**
				KK012-5		5	**T/I**[d]	**T/I/F[k]:84/11/5%**
				KK012-6		6	**T/I**[d]	**T/F/I[j]:80/13/7%**
9	KK013	Female	10	KK013-0	H1pdm	0	I	I:100%
				KK013-1		1	I	I:100%
10	KK017	Female	7	KK017-0	H1pdm	0	I	I:100%
				KK017-1		1	I	I:100%
11	KK018	Male	13	KK018-0	H1pdm	0	I	I:100%
				KK018-2		2	I	I:100%

				KK018-3		3	I	I:100%
				KK018-5		5	I	I:100%
				KK018-6		6	I	I:100%
12	KK019	Female	44	KK019-0	H1pdm	0	I	I:100%
				KK019-2		2	I	I:100%
13	KK021	Female	7	KK021-0	H1pdm	0	I	I:100%
				KK021-1		1	I	I:100%
				KK021-3		3	I	I:100%
				KK021-5		5	I	I:99%
				KK021-6		6	I	I:100%
14	WD039	Male	8	WD039-0	H1pdm	0	I	I:100%
				WD039-1		1	I	I:100%
				WD039-2		2	I	I:100%
				WD039-3		3	I	I:100%
				WD039-4		4	I	I:100%
				WD039-5		5	I	I:100%
				WD039-6		6	I	I:100%
15	GR087	Female	16	GR087-0	H1pdm	0	I	I:100%
				GR087-3		3	I	I:100%
16	GR096	Male	10	GR096-0	H1pdm	0	I	I:100%
				GR096-1		1	I	I:100%
17	GR105	Female	10	GR105-0	H1pdm	0	I	I:100%
				GR105-2		2	I	I:100%
18	GR108	Male	13	GR108-0	H1pdm	0	I	I:100%
				GR108-1		1	I	I:100%
				GR108-2		2	I	I:100%
				GR108-3		3	I	I:100%
				GR108-4		4	I	I:100%
				GR108-5		5	I	I:100%
				GR108-6		6	I	I:100%
19	HP92	Male	36	HP92-0	H1pdm	0	I	I:100%
				HP92-1		1	I	I:100%
				HP92-2		2	I	I:100%
				HP92-3		3	I	I:100%
				HP92-7		7	I	I:96%
20	HP95	Male	26	HP95-0	H1pdm	0	I	I:100%
				HP95-1		1	**T/I^{d}**	**T/I^{d}:53/47%**
21	HP96	Male	50	HP96-0	H1pdm	0	I	I:100%
				HP96-1		1	I	I:100%

				HP96-2		2	I	I:100%
				HP96-4		4	I	I:100%
				HP98-0		0	I	I:100%
				HP98-1		1	I	I:100%
22	HP98	Female	51	HP98-2	H1pdm	2	I	I:100%
				HP98-3		3	I	I:100%
				HP98-4		4	I	I:100%
				KK001-1		1	I	I:100%
				KK001-2		2	I	I:100%
				KK001-3		3	**T/I**[d]	**T/I/F**[k]**:72/21/6%**
23	KK001	Female	13	KK001-4	H1pdm	4	**T/I**[d]	**T:91%**
				KK001-5		5	**T/I**[d]	**I/T**[i]**:84/16%**
				KK001-6		6	**T/I**[d]	**I/T**[i]**:95/5%**
24	BB234	Female	6	BB234-5	H1pdm	5	I	I:100%

[a]The sequences of the PA gene of the A/H1pdm viruses in the clinical specimens were determined by means of Sanger sequencing and deep sequencing. [b]The level of mutation frequencies was estimated based on the height of the waves at each position on the sequencing chromatogram. The detection limit for a minor population was 10%–20%. [c]Residues present at a frequency of more than 5% are shown. [d]T/I, mixture of threonine and isoleucine. [e]T/N/S/I, mixture of threonine, asparagine, serine, and isoleucine. [f]T/N/I/S, mixture of threonine, asparagine, isoleucine, and serine. [g]T/S/I, mixture of threonine, serine, and isoleucine. [h]N/A, not available. [i]I/T, mixture of isoleucine and threonine. [j]T/F/I, mixture of threonine, phenylalanine, and isoleucine. [k]T/I/F, mixture of threonine, isoleucine, and phenylalanine.

Supplementary Table 4. Detection of influenza A/H3 viruses with a mutation at position 38 of PA in patients treated with baloxavir marboxil[a].

	Patient ID	Sex	Age, y	Sample ID	Subtype	Days post-administration of baloxavir marboxil	Amino acid at position 38 of PA	
							[b]Sanger sequencing	[c]Deep sequencing
1	BB212	Male	7	BB212-0	H3	0	I	I:100%
				BB212-4		4	I	I:99%
2	BB213	Female	10	BB213-0	H3	0	I	I:100%
				BB213-3		3	I	I:100%
3	BB218	Male	5	BB218-0	H3	0	I	I:99%
				BB218-1		1	I	I:100%
4	BB235	Male	8	BB235-0	H3	0	I	I:100%
				BB235-2		2	I	I:100%
5	KK006	Male	17	KK006-0	H3	0	I	I:100%
				KK006-1		1	I	I:100%
				KK006-4		4	I	N/A[d]
				KK006-6		6	I	I:100%
6	KK015	Male	5	KK015-0	H3	0	I	I:100%
				KK015-1		1	I	I:100%
				KK015-2		2	I	I:99%
				KK015-3		3	**T/I**[e]	**I/T[f]:76/20%**
7	KK020	Female	12	KK020-0	H3	0	I	I:100%
				KK020-1		1	I	I:100%
8	GR099	Female	12	GR099-0	H3	0	I	I:100%
				GR099-1		1	I	I:97%
				GR099-2		2	I	I:100%
9	GR104	Male	7	GR104-0	H3	0	I	I:100%
				GR104-1		1	I	I:98%
				GR104-2		2	I	I:98%
				GR104-3		3	I	I:98%
				GR104-4		4	I	I:98%
				GR104-5		5	I	I:99%
				GR104-6		6	**I/M**[g]	**I/M[g]:68/31%**
10	GR113	Male	13	GR113-0	H3	0	I	I:100%
				GR113-1		1	I	I:99%
				GR113-2		2	I	I:99%
				GR113-3		3	**T/I**[e]	**I/T[f]:58/41%**
				GR113-4		4	**T/I**[e]	**I/T[f]:57/42%**
				GR113-5		5	I	I:97%
11	GR117	Male	5	GR117-0	H3	0	I	I:100%

				GR117-5		5	**T/I[e]**	**T/I[e]:67/33%**
12	GR121	Female	16	GR121-0	H3	0	I	I:100%
				GR121-1		1	I	I:100%
				GR121-2		2	I	I:99%
				GR121-3		3	I	I:99%
13	GR131	Male	12	GR131-0	H3	0	I	I:100%
				GR131-1		1	I	I:99%
				GR131-2		2	I	I:99%
				GR131-3		3	I	I:99%
				GR131-5		5	I	I:99%
				GR131-6		6	I	I:99%
				GR131-7		7	I	I:99%
				GR131-8		8	I	I:99%
14	GR134	Female	11	GR134-0	H3	0	I	I:100%
				GR134-1		1	I	I:99%
15	HP101	Male	16	HP101-0	H3	0	I	N/A[d]
				HP101-1		1	I	N/A[d]
				HP101-3		3	I	N/A[d]
16	DA37	Male	45	DA37-00	H3	0	I	I:100%
				DA37-2		2	I	I:100%
17	BB230	Male	6	BB230-5	H3	5	**T**	**T/I[e]:94/6%**
18	BB232	Male	10	BB232-4	H3	4	**T/I[e]**	**I/T[f]:76/24%**
19	WD049	Female	39	WD049-1	H3	1	I	I:100%
				WD049-5		5	I	I:100%
20	GR148	Male	8	GR148-7	H3	7	**T/I[e]**	**T/I[e]:84:16%**

[a]The sequences of the PA gene of the A/H3 viruses in the clinical specimens were determined by means of Sanger sequencing and deep sequencing. [b]The level of mutation frequencies was estimated based on the height of the waves at each position on the sequencing chromatogram. The detection limit for a minor population was 10%–20%. [c]Residues present at a frequency of more than 5% are shown. [d]N/A, not available. [e]T/I, mixture of threonine and isoleucine. [f]I/T, mixture of isoleucine and threonine. [g]I/M, mixture of isoleucine and methionine.

Supplementary Table 5. Seasonal influenza A/H1N1pdm and A/H3N2 virus isolated from patients and used in this study.

Clinical sample			Virus			
Patient ID	Sample ID	Days post-administration of baloxavir marboxil	Virus name	Subtype	Amino acid at position 38 of PA	Abbreviation
KK001	KK001-1	1	A/Isumi/UT-KK001-1/2018	H1N1pdm	I	WT-KK001-I38
	KK001-3	3	A/Isumi/UT-KK001-3/2018	H1N1pdm	T	MUT-KK001-I38T
KK003	KK003-0	0	A/Isumi/UT-KK003-0/2018	H1N1pdm	I	WT-KK003-I38
	KK003-4	4	A/Isumi/UT-KK003-4/2018	H1N1pdm	T	MUT-KK003-I38T
KK015	KK015-0	0	A/Isumi/UT-KK015-0/2019	H3N2	I	WT-KK015-I38
	KK015-3	3	A/Isumi/UT-KK015-3/2019	H3N2	T	MUT-KK015-I38T
GR117	GR117-0	0	A/Tokyo/UT-GR117-0/2019	H3N2	I	WT-GR117-I38
	GR117-5	5	A/Tokyo/UT-GR117-5/2019	H3N2	T	MUT-GR117-I38T

Supplementary Table 6. Mutations detected in virus stocks of the A/H1N1pdm and A/H3N2 variants isolated from patients treated with baloxavir marboxil.

Segment	A/Isumi/UT-KK001-3/2018 (H1N1pdm)		A/Isumi/UT-KK003-4/2018 (H1N1pdm)		A/Isumi/UT-KK015-3/2019 (H3N2)		A/Tokyo/UT-GR117-5/2019 (H3N2)	
	Nucleotides	Amino acids	Nucleotides	Amino acids	Nucleotides	Amino acids	Nucleotides	Amino acids
PB2	—[a]	—	—	—	a1575g	Syn[b]	—	—
PB1	a561g/a[c]	Syn	—	—	—	—	—	—
PA	t113c	I38T	t113c g/t1862t[d]	I38T S/I621I[e]	t113c	I38T	t113c	I38T
HA[f]	—	—	—	—	g/a1216g	E/K390E[g]	c282a	Syn
NP	—	—	—	—	—	—	—	—
NA[h]	g346a/g[i] t927c/t[k]	V116I/V[j] Syn	—	—	—	—	—	—
M	—	—	—	—	—	—	—	—
NS	—	—	—	—	—	—	—	—

[a]—, No mutation was detected compared to the sequences from the parental wild-type virus. [b]Syn, synonymous substitution. [c]g/a, mixture of guanine and adenine at position 561. [d]g/t, mixture of guanine and thymine at position 1862. [e]S/I, mixture of serine and isoleucine at position 621. [f]H3 numbering. [g]E/K, mixture of glutamic acid and lysine at position 390. [h]N2 numbering. [i]a/g, mixture of adenine and guanine at position 346. [j]I/V, mixture of isoleucine and valine at position 116. [k]c/t, mixture of cytosine and thymine at position 927.

Supplementary Table 7. Virus sensitivity to baloxavir acid *in vitro*[a].

Virus name	Amino acid at position 38 of PA	Mean EC_{50} (nM) ± SD[b]	Fold change[c]
A/Isumi/UT-KK001-1/2018 (H1N1pdm)	I	4.6 ± 3.2	N/A
A/Isumi/UT-KK001-3/2018 (H1N1pdm)	T	490 ± 330	107
A/Isumi/UT-KK003-0/2018 (H1N1pdm)	I	5.6 ± 4.3	N/A
A/Isumi/UT-KK003-4/2018 (H1N1pdm)	T	376 ± 342	67
A/Isumi/UT-KK015-0/2019 (H3N2)	I	0.55 ± 0.24	N/A
A/Isumi/UT-KK015-3/2019 (H3N2)	T	128 ± 81	233
A/Tokyo/UT-GR117-0/2019 (H3N2)	I	1.4 ± 0.8	N/A
A/Tokyo/UT-GR117-5/2019 (H3N2)	T	99 ± 50	71

[a]Baloxavir acid is the active form of baloxavir marboxil. [b]Antiviral activities of baloxavir acid against influenza A viruses in a plaque reduction assay on hCK cells. Data are presented as the mean ± standard deviation from three independent experiments. [c]Fold difference in EC_{50} determined by dividing the EC_{50} for the PA-I38T variant virus by that for the parental wild-type virus. N/A, not applicable.

Supplementary Table 8. Transmissibility of A/H3N2 viruses in ferrets.

Virus	Pair	Viral neutralization titer[a]			
		Infected[b]		Exposed[c]	
		Pre-	Post-	Pre-	Post-
A/Tokyo/UT-GR117-0/2019 (H3N2)	7	<10	80	<10	320
	8	<10	320	<10	160
	9	<10	160	<10	160
	10	<10	160	<10	160
	11	<10	80	<10	160
A/Tokyo/UT-GR117-5/2019 (H3N2)	12	<10	160	<10	160
	13	<10	160	<10	320
	14	<10	160	<10	80
	15	<10	160	<10	<10
	16	<10	160	<10	160

[a]Viral neutralization assays were carried out with A/Tokyo/UT-GR117-0/2019 (H3N2).

[b]Sera were collected on day 17 post-inoculation. [c]Sera were collected on day 16 post-exposure.

Supplementary Table 9. List of primers used in this study.

Primer	Sequence (5' - 3')[a]	Orientation	Purpose
H1N1pdmPA-ATG(-1)F	AATGGAAGACTTTGTGCG	Forward	Amplification and sequencing of A/H1N1pdm PA fragment
H1N1pdmPA-ATG478R	TCTTCGTCAAGGGTGTAGTC	Reverse	Amplification of A/H1N1pdm PA fragment
H1N1pdmPA-ATG238R	GATTCGGTCTCTTCCTTC	Forward	Sequencing of A/H1N1pdm PA fragment
H3N2PA-ATG1F	ATGGAAGATTTTGTGCGAC	Reverse	Amplification and sequencing of A/H3N2 PA fragment
H3N2PA-ATG582R	GTTTCTTCGCCTCTTTCG	Forward	Amplification of A/H3N2 PA fragment
H3N2PA-ATG278R	CCAGTAGTGTTGCAGATAC	Reverse	Sequencing of A/H3N2 PA fragment
Opti1-H1N1pdm-PA F1	GTTACGCGCCAATGGAAGACTTTGTGCG	Forward	Amplification of A/H1N1pdm PA fragment
Opti1-H1N1pdm-PA478-R1	GTTACGCGCCTCTTCGTCAAGGGTGTAGTC	Reverse	Amplification of A/H1N1pdm PA fragment
Opti1-H3N2-PA-F	GTTACGCGCCATGGAAGATTTTGTGCGAC	Forward	Amplification of A/H3N2 PA fragment
Opti1-H3N2-PA582-R	GTTACGCGCCGTTTCTTCGCCTCTTTCG	Reverse	Amplification of A/H3N2 PA fragment
Bm-PB2-1	TATTCGTCTCAGGGAGCGAAAGCAGGTC	Forward	Amplification of A/H3N2 PB2 segment
Bm-PB2-2341R	ATATCGTCTCGTATTAGTAGAAACAAGGTCGTTT	Reverse	Amplification of A/H3N2 PB2 segment
Bm-PB1-1	TATTCGTCTCAGGGAGCGAAAGCAGGCA	Forward	Amplification of A/H3N2 PB1 segment
Bm-PB1-2341R	ATATCGTCTCGTATTAGTAGAAACAAGGCATTT	Reverse	Amplification of A/H3N2 PB1 segment
Bm-PA-1	TATTCGTCTCAGGGAGCGAAAGCAGGTAC	Forward	Amplification of A/H3N2 PA segment
Bm-PA-2233R	ATATCGTCTCGTATTAGTAGAAACAAGGTACTT	Reverse	Amplification of A/H3N2 PA segment
Bm-HA-1	TATTCGTCTCAGGGAGCAAAAGCAGGGG	Forward	Amplification of A/H3N2 HA segment
Bm-NP-1	TATTCGTCTCAGGGAGCAAAAGCAGGGTA	Forward	Amplification of A/H3N2 NP segment
Bm-NP-1565R	ATATCGTCTCGTATTAGTAGAAACAAGGGTATTTTT	Reverse	Amplification of A/H3N2 NP segment
Bm-NA-1	TATTCGTCTCAGGGAGCAAAAGCAGGAGT	Forward	Amplification of A/H3N2 NA segment
Bm-NA-1413R	ATATCGTCTCGTATTAGTAGAAACAAGGAGTTTTTT	Reverse	Amplification of A/H3N2 NA segment

Bm-M-1	TATTCGTCTCAGGGAGCAAAAGCAGGTAG	Forward	Amplification of A/H3N2 M segment
Bm-M-1027R	ATATCGTCTCGTATTAGTAGAAACAAGGTAGTTTTT	Reverse	Amplification of A/H3N2 M segment
Bm-NS-1	TATTCGTCTCAGGGAGCAAAAGCAGGGTG	Forward	Amplification of A/H3N2 NS segment
Bm-NS-890R	ATATCGTCTCGTATTAGTAGAAACAAGGGTGTTTT	Reverse	Amplification of A/H3N2 NS and HA segments
U12+18F	AGCGAAAGCAGGTCAAATATATTCAATATG	Forward	Amplification of A/H1N1pdm PB2 segment
U13+18R	AGTAGAAACAAGGTCGTTTTTAAACAATTCG	Reverse	Amplification of A/H1N1pdm PB2 segment
U12+15F	AGCAAAAGCAGGCAAACCATTTGAATG	Forward	Amplification of A/H1N1pdm PB1 segment
U13+16R	AGTAGAAACAAGGCATTTTTTCATGAAGG	Reverse	Amplification of A/H1N1pdm PB1 segment
U12+15F	AGCAAAAGCAGGTACTGATCCAAAATG	Forward	Amplification of A/H1N1pdm PA segment
U13+20R	AGTAGAAACAAGGTACTTTTTCGGACAGTATGG	Reverse	Amplification of A/H1N1pdm PA segment
U12+16F	AGCAAAAGCAGGGGAAAATAAAAGCAAC	Forward	Amplification of A/H1N1pdm HA segment
U13+16R	AGTAGAAACAAAGGGTGTTTTTTCTCATG	Reverse	Amplification of A/H1N1pdm HA segment
U12+18F	AGCAAAAGCAGGGTAGATAATCACTCAATG	Forward	Amplification of A/H1N1pdm NP segment
U13+13R	AGTAGAAACAAGGGTATTTTTCCTCA	Reverse	Amplification of A/H1N1pdm NP segment
U12+15F	AGCAAAAGCAGGAGTTCAAAATGAATC	Forward	Amplification of A/H1N1pdm NA segment
U13+17R	TTATATGGTCTCGTATTAGTAGAAACAAGG	Reverse	Amplification of A/H1N1pdm NA segment
U12+16F	AGCAAAAGCAGGTAGATATTTAAAGATG	Forward	Amplification of A/H1N1pdm M segment
U13+7R	AGTAGAAACAAGGTAGTTTT	Reverse	Amplification of A/H1N1pdm M segment
U12+14F	AGCAAAAGCAGGGTGACAAAGACATA	Forward	Amplification of A/H1N1pdm NS segment
U13+17R	AGTAGAAACAAGGGTGTTTTTTATCA	Reverse	Amplification of A/H1N1pdm NS segment
qPCR-A/H1N1pdm-F	AGAAAAGAATGTAACAGTAACACACTCTGT	Forward	Real-time RT-PCR primer for detection of A/H1pdm virus HA
qPCR-A/H1N1pdm-R	TGTTTCCACAATGTARGACCAT	Reverse	Real-time RT-PCR primer for detection of A/H1pdm virus HA
qPCR-A/H3N2-F	CTATTGGACAATAGTAAAACCGGGRGA	Forward	Real-time RT-PCR primer for detection of A/H3 virus HA

qPCR-A/H3N2-R	GTCATTGGGRATGCTTCCATTTGG	Reverse	Real-time RT-PCR primer for detection of A/H3 virus HA
FAM-A/H1N1pdm-HA-Probe	(FAM) CAGCCAGCAATRTTRCATTTACC (BHQ-1)[a]		Real-time RT-PCR probe for detection of A/H1pdm virus HA
HEX-A/H3N2-HA-Probe	(HEX) AAGTAACCCCKAGGAGCAATTAG (BHQ-1)[a]		Real-Time RT-PCR probe for detection of A/H3 virus HA

[a]FAM, 6-carboxyfluorescein; HEX, hexacholoro-6-carboxyfluorescein; BHQ-1, black hole quencher.