Supplementary Information for

# Multiple polymerase gene mutations for human adaptation occurring in Asian H5N1 influenza virus clinical isolates

Yasuha Arai[a,b], Norihito Kawashita[c,d], Kozue Hotta[e,f], Phuong Vu Mai Hoang[g], Hang Le Khanh Nguyen[g], Thach Co Nguyen[g], Cuong Duc Vuong[g], Thanh Thi Le[g], Mai Thi Quynh Le[g], Kosuke Soda[h], Madiha S. Ibrahim[i], Tomo Daidoji[a], Tatsuya Takagi[d], Tatsuo Shioda[b], Takaaki Nakaya[a], Toshihiro Ito[h], Futoshi Hasebe[e], Yohei Watanabe[a,b]

[a]Department of Infectious Diseases, Graduate School of Medicine, Kyoto Prefectural University of Medicine, Kyoto, Japan, [b]Department of Viral Infections, Research Institute for Microbial Diseases, Osaka University, Osaka, Japan, [c]Graduate School of Science and Engineering, Kindai University, Osaka, Japan, [d]Graduate School of Pharmaceutical Science, Osaka University, Osaka, Japan, [e]Vietnam Research Station, Center for Infectious Disease Research in Asia and Africa, Institute of Tropical Medicine, Nagasaki University, Hanoi, Vietnam, [f]Laboratory of Veterinary Public Health, Department of Veterinary Medical Sciences, Graduate School of Agricultural and Life Science, The University of Tokyo, Tokyo, Japan, [g]Department of Virology, National Institute of Hygiene and Epidemiology, Hanoi, Vietnam, [h]Avian Zoonosis Research Center, Faculty of Agriculture, Tottori University, Tottori, Japan, [i]Department of Microbiology and Immunology, Faculty of Veterinary Medicine, Damanhour University, Damanhour, Egypt

Address corresponding to Yohei Watanabe, nabe@koto.kpu-m.ac.jp

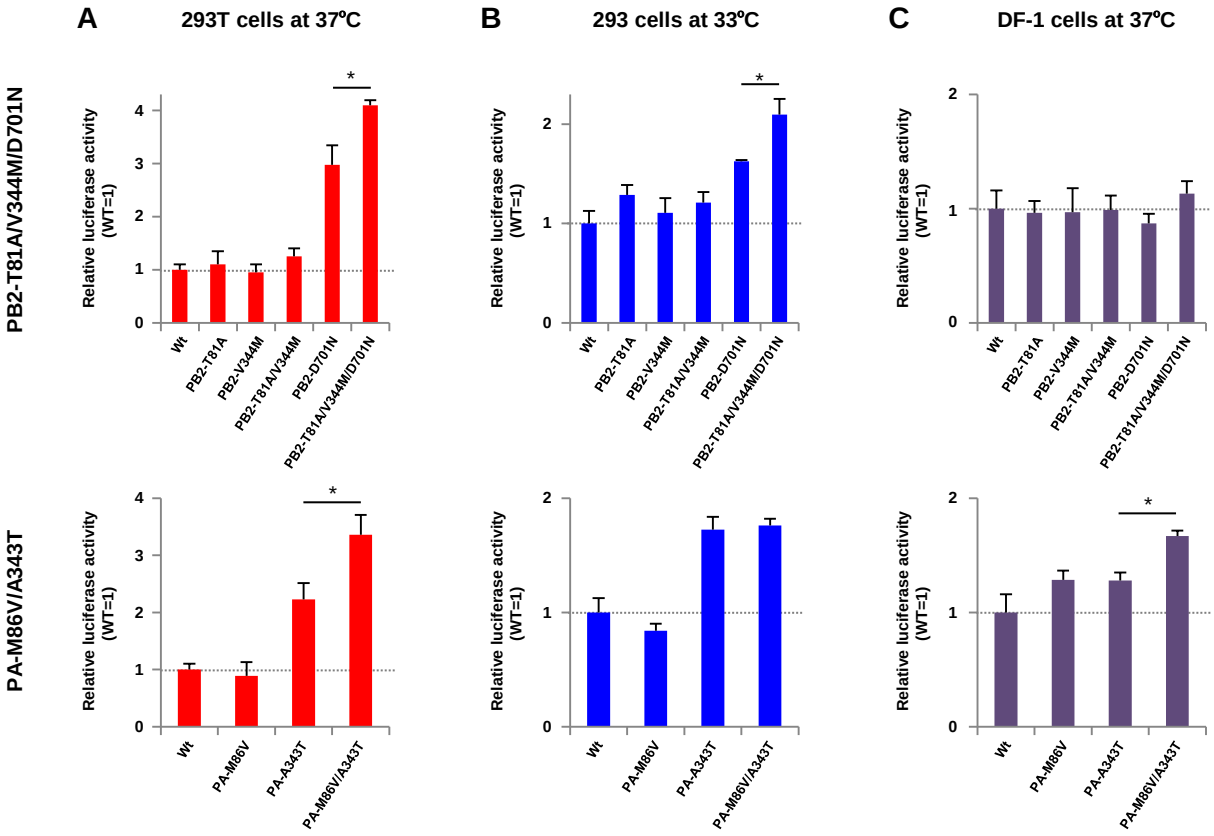


**Fig. S1. Synergistic effect of multiple mutations on polymerase activity.** To clarify the significance of the PB2-T81A/V344M/D701N and PA-M86V/A343T multiple mutations on polymerase activity, the polymerase activity data of the indicated VN/HN mutants (Fig. 1) were compared. (A) 293T cells at 37ºC. (B) 293T cells at 33ºC. (C) QT-6 cells at 37ºC. An asterisk indicates a *P* value < 0.01 (ANOVA with Tukey's multiple comparison test).

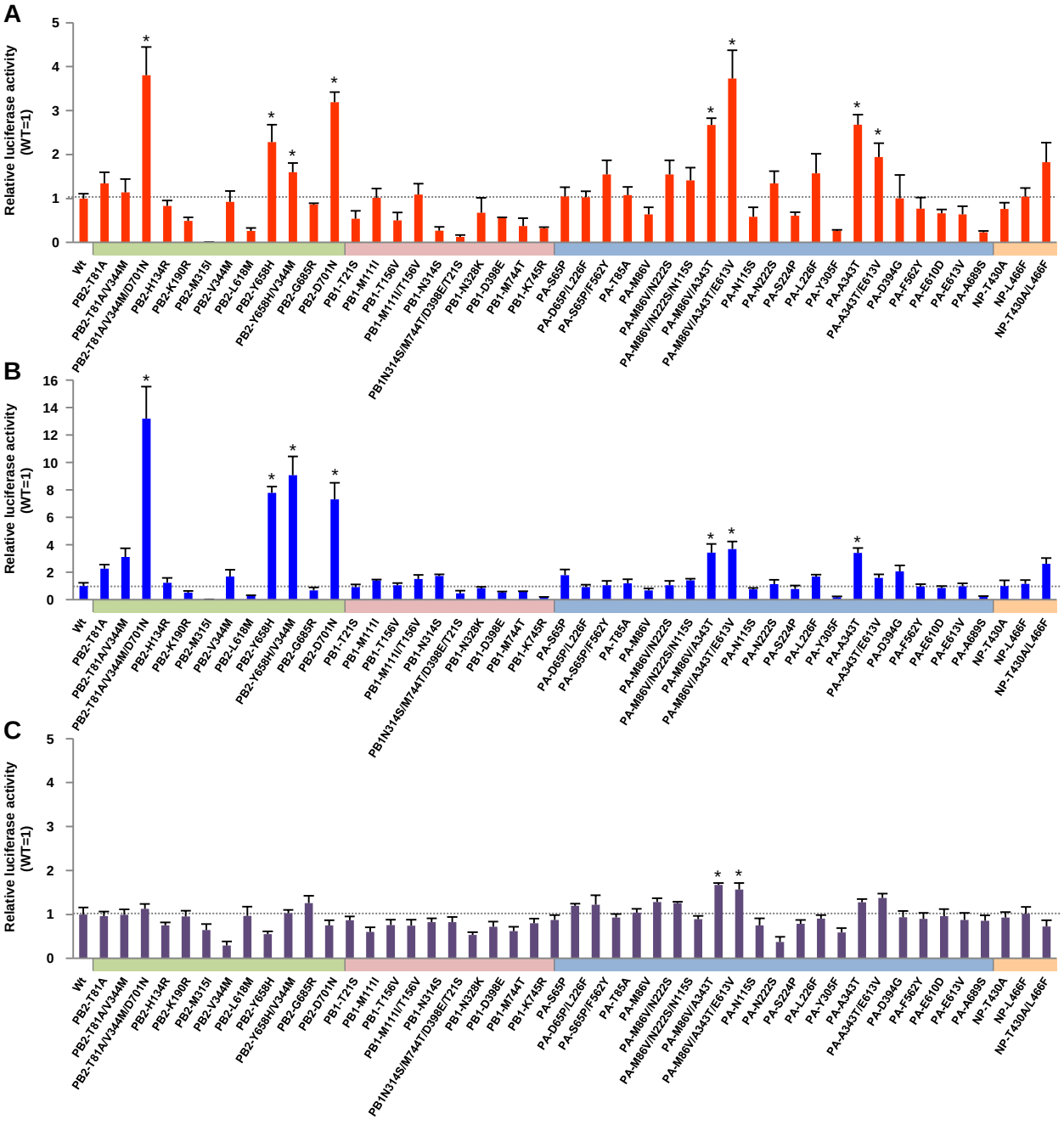


**Fig. S2. Effect of mutations on H5N1 clade 2.2.1 virus polymerase activity..** Human 293T cells and avian DF-1 cells were transfected with plasmids expressing EG/D1 PB2, PB1, PA or NP with the indicated single or multiple mutations, a human or chicken polymerase I-driven plasmid expressing a vRNA-oriented luciferase reporter gene, and a plasmid expressing *Renilla* luciferase as an internal control. After 48 h incubation at 33 or 37ºC, luciferase activities were measured, normalized to the internal *Renilla* luciferase activity, and expressed relative to the results for VN/HN (wt). (A) EG/D1 polymerase activity at 37ºC in 293T cells. (B) EG/D1 polymerase activity at 33ºC in 293T cells. (C) EG/D1 polymerase activity at 37 ºC in DF-1 cells. Colors on each x-axis indicate the different virus genes. An asterisk indicates a *P* value < 0.01 (ANOVA with Tukey's multiple comparison test). Asterisks for mutations with negative effects on polymerase activity were omitted for clarity.

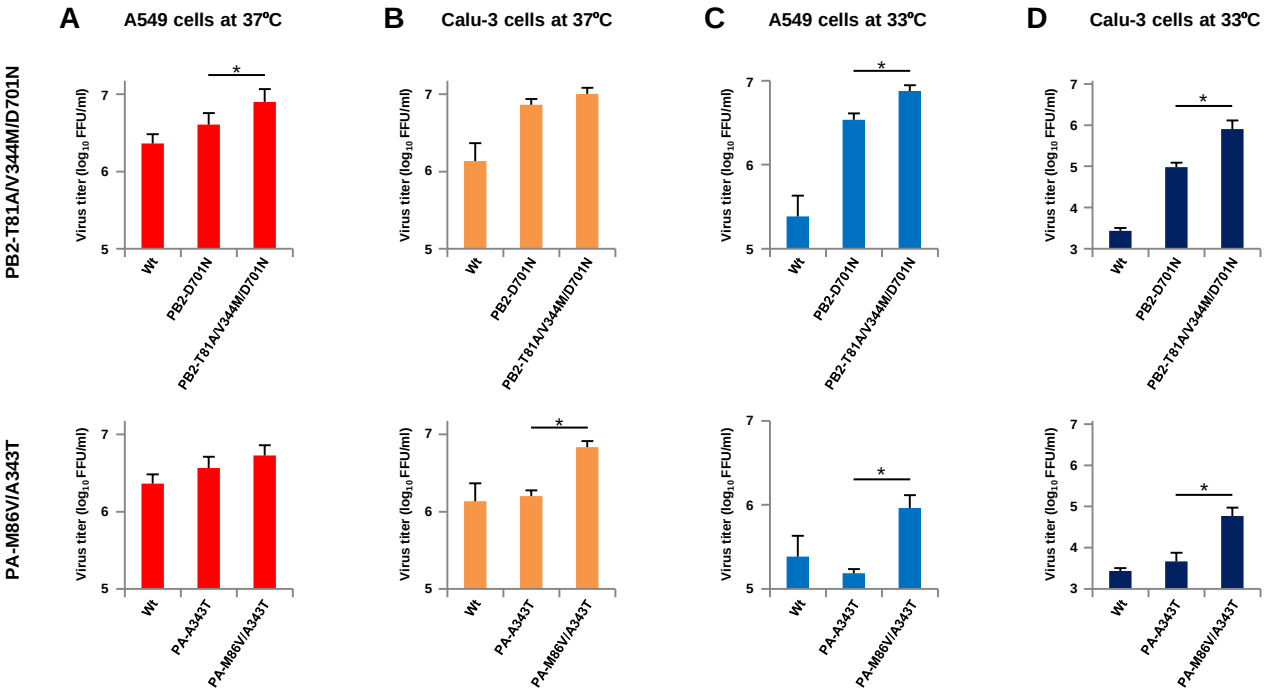


**Fig. S3. Synergistic effect of multiple mutations on viral replication in human airway epithelial cells.** To clarify the significance of the PB2-T81A/V344M/D701N and PA-M86V/A343T multiple mutations in viral replication, virus titer data at 48 or 72 h post-infection for the indicated VN/HN mutants (Fig. 2) were compared. (A) A549 cells at 37ºC. (B) Calu-3 cells at 37ºC. (C) A549 cells at 33ºC. (D) Calu-3 cells at 33ºC. An asterisk indicates a *P* value < 0.01 (ANOVA with Tukey's multiple comparison test).

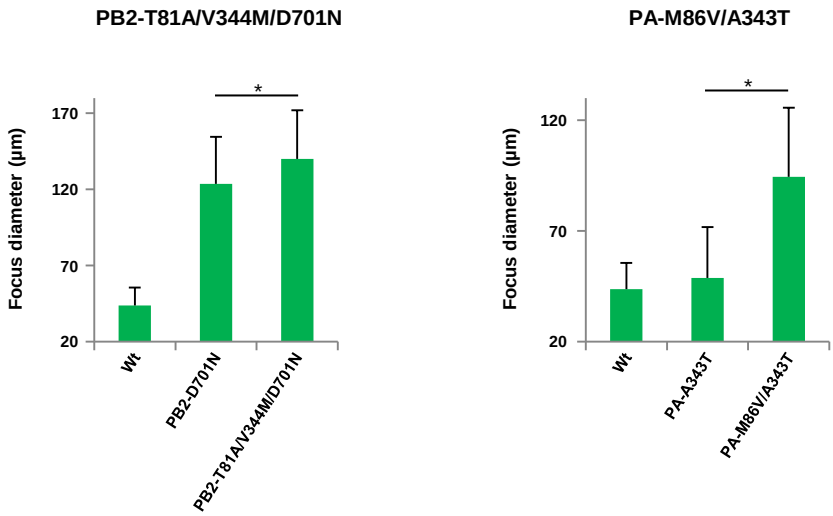


**Fig. S4. Synergistic effect of multiple mutations on foci sizes.** To clarify the significance of the PB2-T81A/V344M/D701N and PA-M86V/A343T multiple mutations on foci sizes, foci size data for the indicated VN/HN mutants (Fig. 4) were compared. An asterisk indicates a *P* value < 0.01 (ANOVA with Tukey's multiple comparison test).

**Table S1. Mutations in the polymerase sequence of 24 H5N1 viruses isolated from patients in Asia and identified by an NCBI database search.**

| Clade | Virus Strain | Subtype | Passage history† | Collection date | Mutation in gene | | | |
|---|---|---|---|---|---|---|---|---|
| | | | | | PB2 | PB1 | PA | NP |
| 2.3.2.1 | A/Guangxi/1/2009 | H5N1 | E1 | 2009-01-19 | | M111I/N328K | L226F | |
| 2.3.2.1 | A/Hong Kong/6841/2010 | H5N1 | - | 2010-11-16 | G685R | | N115S | |
| 2.3.2.1 | A/Hubei/1/2010 | H5N1 | E1 | 2010-05-21 | | | | |
| 2.3.4 | A/Guangdong/1/2008 | H5N1 | E2 | 2008-02-16 | T81A/V344M | | | |
| 2.3.4 | A/Guangxi/1/2008 | H5N1 | E2 | 2008-02-12 | M315I/L618M | M111I/T156V/K745R | L226F | |
| 2.3.4 | A/Hunan/1/2008 | H5N1 | E2 | 2008-01-16 | T81A/K190R/V344M/D701N | | A343T/E613V/A689S | |
| 2.3.4 | A/Shandong/1/2009 | H5N1 | E1 | 2009-01-05 | T81A/V344M | N328K | D394G | |
| 2.3.4 | A/Xinjiang/1/2009 | H5N1 | E2 | 2009-01-10 | | | N222S/F562Y | |
| 2.3.4.1 | A/Guizhou/1/2009 | H5N1 | E2 | 2009-01-15 | Y658H | T21S/N314S/D398E/M744T | N115S/N222S | T430A/L466F |
| 2.3.4.1 | A/Hunan/1/2009 | H5N1 | E3 | 2009-01-08 | K190R/Y658H | | T85A/Y305F | |
| 2.3.4.1 | A/Hunan/2/2009 | H5N1 | E2 | 2009-01-23 | V344M/Y658H | T21S/N314S/D398E/M744T | | T430A/L466F |
| 2.3.4 | A/Beijing/1/2009 | H5N1 | E3 | 2008-12-04 | | | T85A/Y305F | |
| 2.3.4.3 | A/Vietnam/HN31432M/2008 | H5N1 | C2 | 2008-02-21 | | | D394G/F562Y | |
| 2.3.4.3 | A/Vietnam/UT31394II/2008 | H5N1 | C2 | 2008-01-17 | M315I/L618M | M111I/T156V/K745R | | |
| 2.3.4.3 | A/Vietnam/UT31412II/2008 | H5N1 | C2 | 2008-02-09 | L618M | K745R | A343T/E613V/A689S | T430A |
| 2.3.4.3 | A/Vietnam/UT31413II/2008 | H5N1 | C2 | 2008-02-13 | L618M | K745R | A343T/E613V | |
| 1.1 | A/Cambodia/S1211394/2008 | H5N1 | - | 2008-12-11 | G685R | | A343T/E613V | |
| 1.1 | A/Cambodia/U0417030/2010 | H5N1 | - | 2010-04-18 | | | D394G | |
| 1.1 | A/Cambodia/V0203306/2011 | H5N1 | - | 2011-02-03 | | | | |
| 1.1 | A/Cambodia/V0219301/2011 | H5N1 | - | 2011-02-17 | | | | |
| 1.1 | A/Cambodia/V0401301/2011 | H5N1 | - | 2011-03-31 | | | S224P | |
| 1.1 | A/Cambodia/V0417301/2011 | H5N1 | - | 2011-04-16 | | | | |
| 1.1 | A/Cambodia/V0606311/2011 | H5N1 | - | 2011-06-06 | | | S224P | |
| 1.1 | A/Cambodia/W0112303/2012 | H5N1 | - | 2012-01-10 | D701N | | | |

† C, cultured cells; E, eggs. The number after each letter indicates the number of passages of the strain.
- No information