Prevalence, genetics, and transmissibility in ferrets of Eurasian avian-like H1N1 swine influenza viruses

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Methods

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Methods

Swine influenza virus isolation and genetic analyses. Swine nasal swab collection, virus isolation, and subtyping were performed as previously reported (1). All of the individual samples were inoculated into both 10-day-old embryonated chicken eggs and MDCK cells for virus isolation. HI assays were performed by using antisera against the H1, H3, H5, and H9 subtypes of swine influenza viruses. The HA lineage of the H1 subtype viruses and NA subtypes were determined by direct sequencing of the PCR products. The viral genomes were amplified by PCR and sequenced by using segment-specific primers. Phylogenetic trees were constructed by using the neighbor-joining method with a bootstrap value of 1,000; 95% sequence identity cut-offs were used to categorize the groups of each gene segment in the phylogenetic trees.

Serologic analyses. Antigenic analyses of the H1N1 influenza viruses were performed by using HI tests with ferret antisera generated in our laboratory against the indicated viruses. The children's sera were selected from children who visited the hospital but did not have fever; the adult's sera were collected from apparently healthy adults and elderly adults during routine physical examination. Informed consent to use the sera for influenza antibody detection was obtained. Sera were treated with Vibrio cholerae receptor-destroying enzyme (Denka-Seiken) before being tested for the presence of HI antibody with 0.5% (vol/vol) turkey erythrocytes. The minimum cut-off value for the HI assay was 10. MNT assays were performed as reported previously (2, 3).

Solid-phase binding assay. Viral receptor-binding capability was examined by using a solid-phase binding assay with four different biotinylated glycans as described previously (4, 5). Chicken antisera against H1N1 SIV SW/HuN/30/13 virus (for EAH1N1 SIVs), H5N1 virus A/Goose/Guangdong/1/96 (for H5N1 virus), or H1N1 virus SC/1/09 (for SC/1/09 virus) were generated in our laboratory and used for this study.

Animal studies. Six-week-old female BALB/c mice (Vital River Laboratories, Beijing, China) were used in this study. Briefly, groups of 6-week-old female BALB/c mice (n=8 per group) were lightly anaesthetized with CO_2 and inoculated intranasally (i.n.) with $10^{6.0}$ EID_{50} of test virus in a 50-µl volume. Three mice were euthanized on day 3 post-infection (p.i.), and spleens, kidneys, brains, nasal turbinate, and lungs were harvested for virus titrations in eggs. The remaining five mice in each group were monitored daily for a total of 14 days for weight loss and survival.

Ferret replication and transmission studies were performed as reported elsewhere by using four-month-old female ferrets (Mustela putorius furo) (Wuxi Cay Ferret Farm, Jiangsu, China) that were serologically negative for influenza viruses. The animals were anesthetized via intramuscular injection of ketamine (20 mg/kg) and xylazine (1 mg/kg). To investigate virus replication, groups of two ferrets were anesthetized and inoculated i.n. with $10^{6.0}$ EID_{50} of test virus in a 500-µl volume (250 µl per nostril). The ferrets were euthanized on day 4 p.i and the nasal turbinate, tonsils, trachea, lung, spleen, kidneys, livers, and brains were collected for virus titration in eggs.

For the respiratory droplet transmission studies, groups of ferrets (n=3 per group) were inoculated i.n. with $10^{6.0}$ EID_{50} of test virus and housed in specially designed cages inside an isolator. Twenty-four hours later, three naïve animals were placed in an adjacent cage (4 cm away), separated by a double-layered net divider. These cages allow free passage of air. Nasal washes were collected at 2-day intervals, beginning on day 2 p.i. (1 day post-exposure) and were titrated in eggs. Sera were collected from all animals on day 21 p.i. for HI antibody detection. The

ambient conditions for these studies were set at 20–22 ℃ and 30%–40% relative humidity. The airflow in the isolator was horizontal with a speed of 0.1 m/s; the airflow direction was from the inoculated animals to the exposed animals.

Histological study. Lungs were collected from ferrets on day 4 after virus inoculation and were fixed in 10% neutral buffered formalin, embedded in paraffin, and cut into 4-μm sections. The sections were stained with hematoxylin-eosin (H&E) or used in immunohistochemical (IHC) assays with mouse antiserum against the SW/HuN/30/13 virus and goat anti-mouse IgG as the secondary antibody (KPL 16-18-06, Gaithersburg, MD, USA).

References

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4. Zhang Q, *et al.* (2013) H7N9 influenza viruses are transmissible in ferrets by respiratory droplet. *Science* 341(6144):410-414.
5. Li X, *et al.* (2014) Genetics, receptor binding property, and transmissibility in mammals of naturally isolated H9N2 Avian Influenza viruses. *PLoS Pathog* 10(11):e1004508.

Supporting Figures

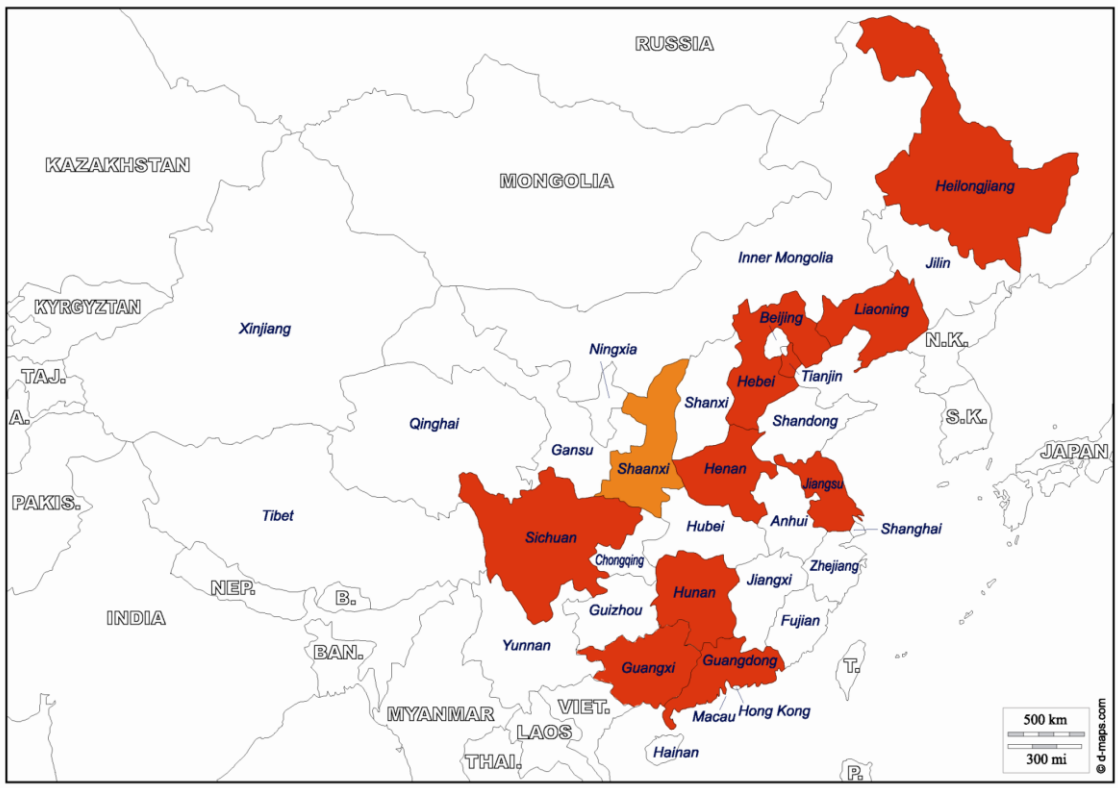

Figure S1. Map showing provinces from which EA H1N1 SIVs were isolated from 2010 through 2013. The red color represents the areas where the viruses were isolated in our study; the orange color depicts where EAH1N1 viruses were detected by others.

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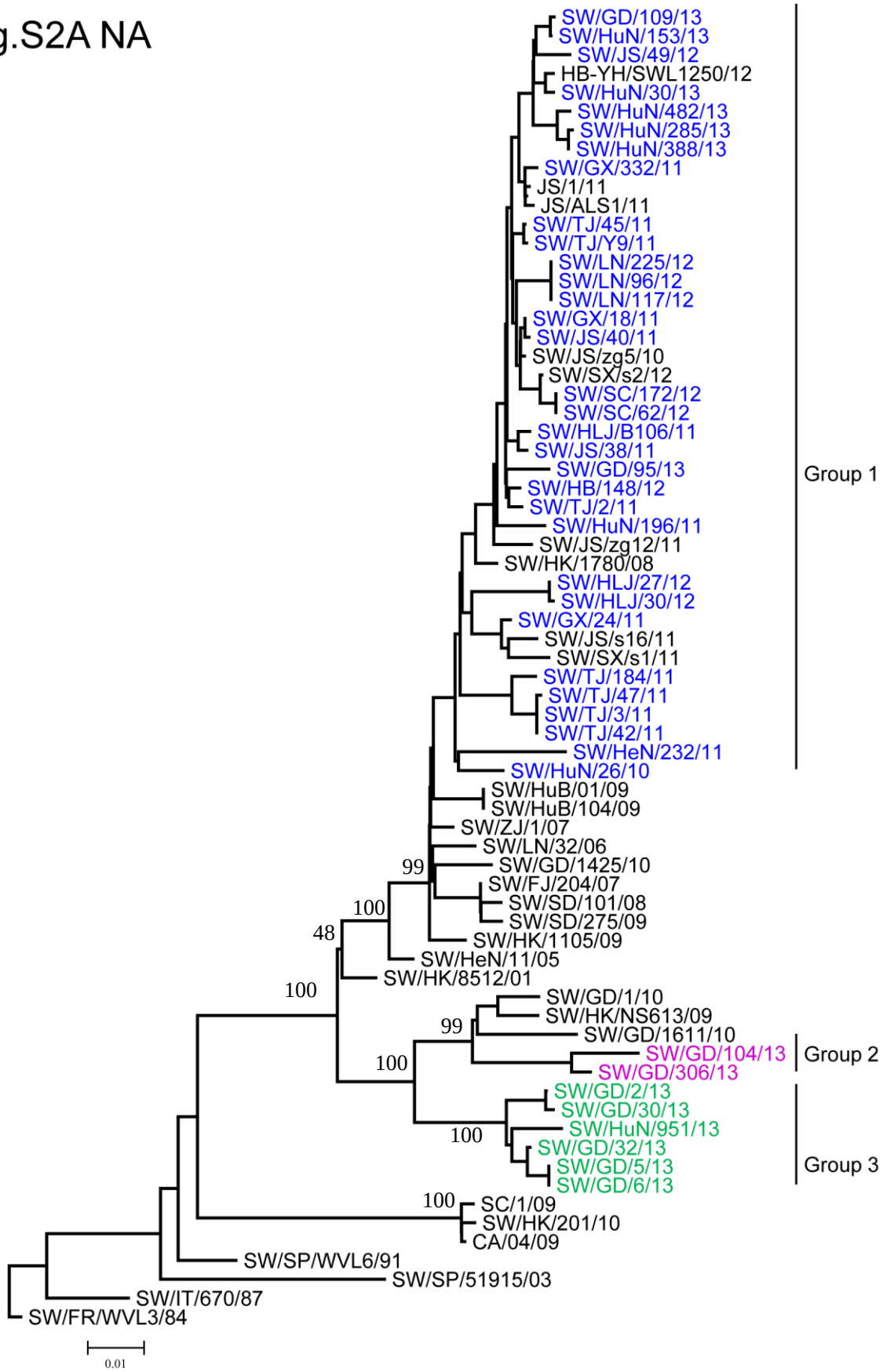
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Fig.S2B PB2

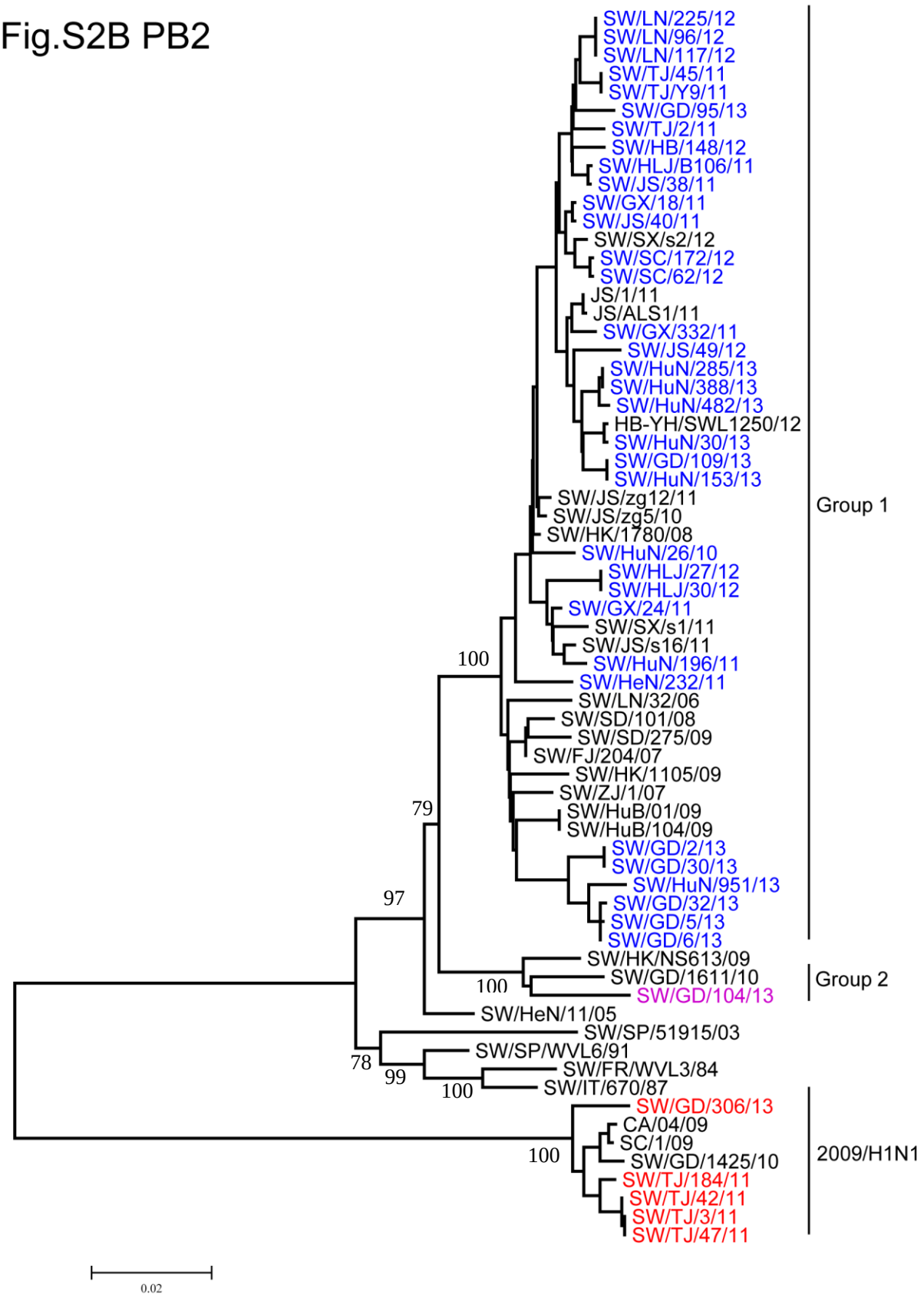
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Fig.S2C PB1

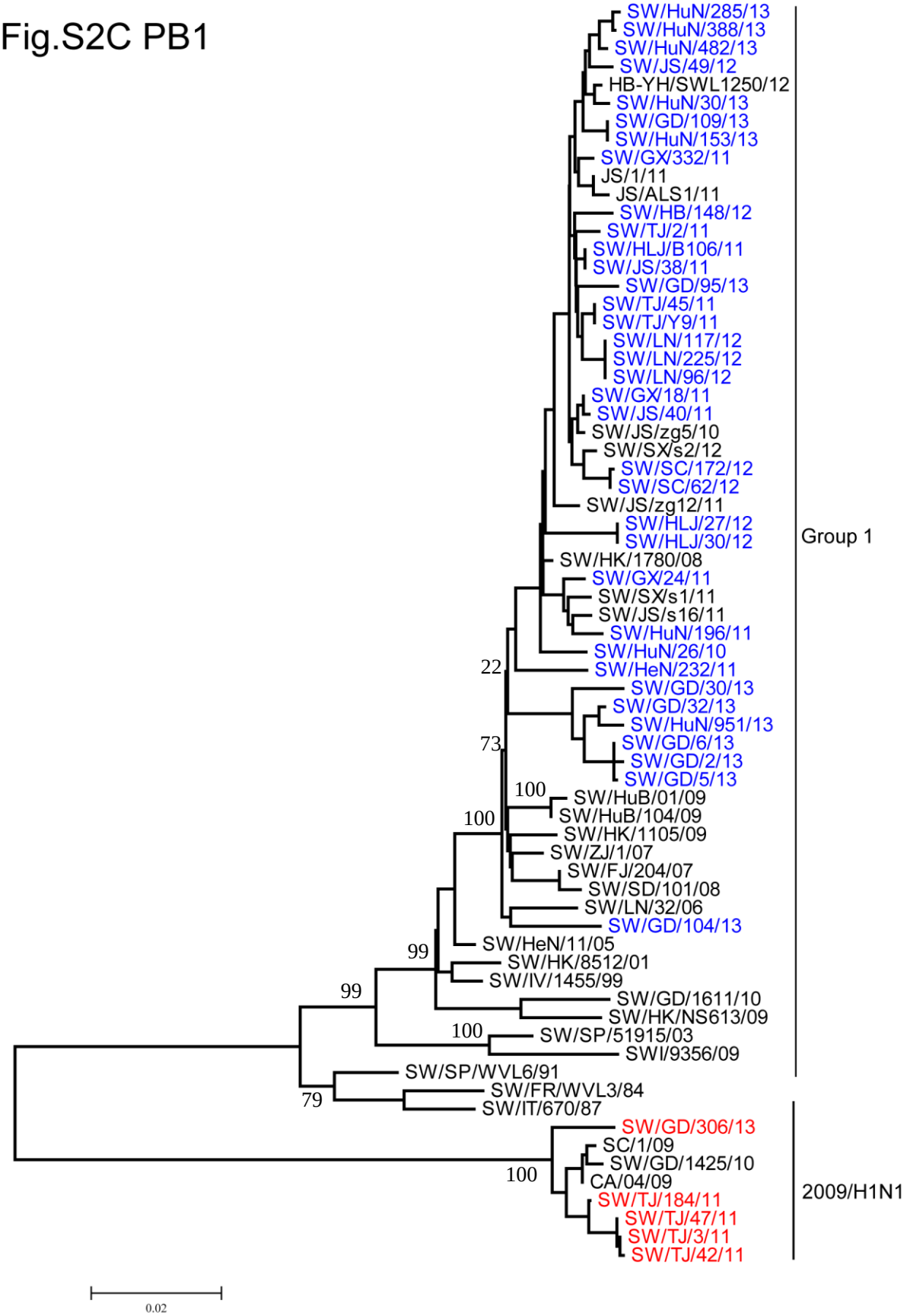
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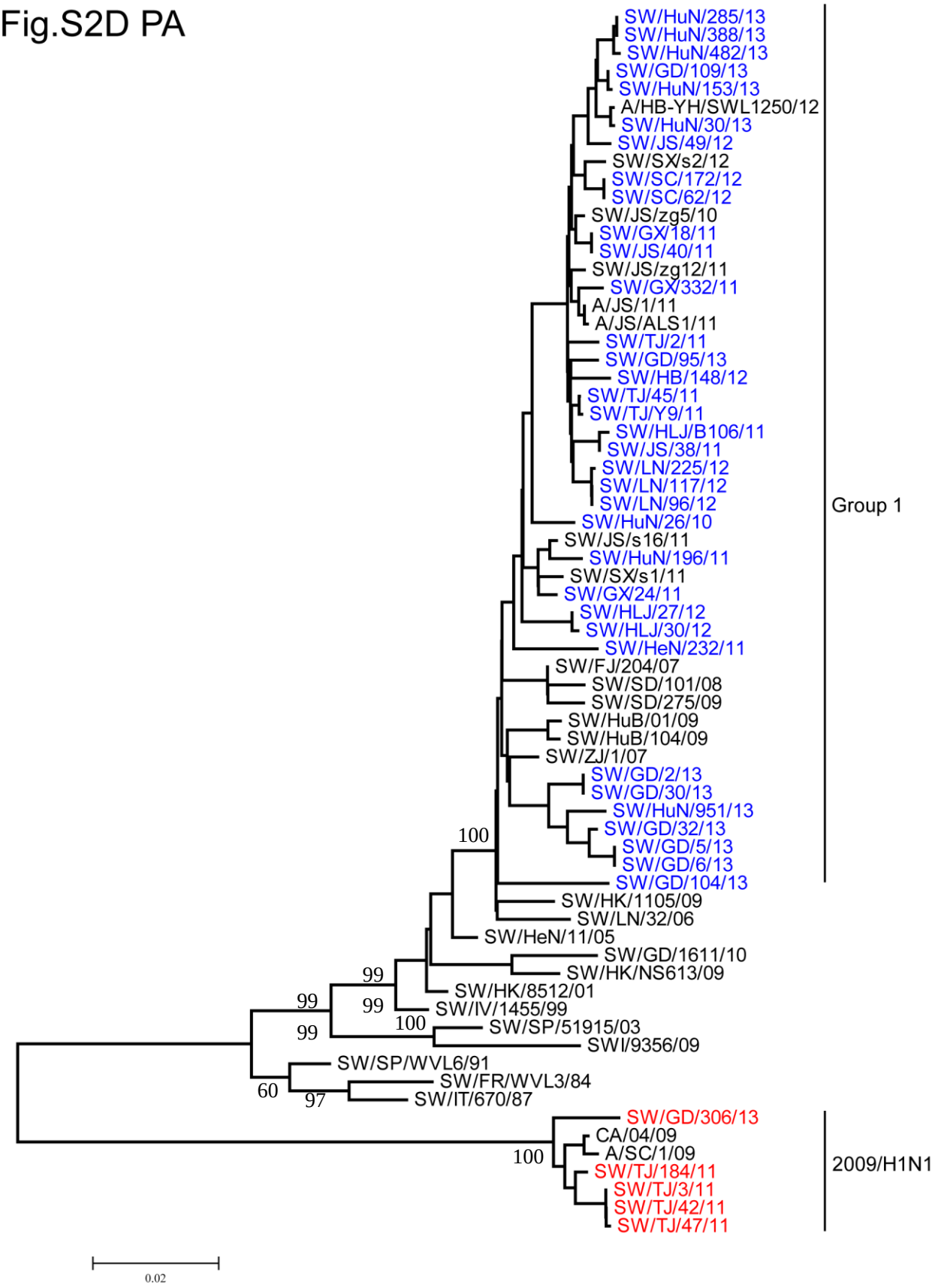
Fig.S2D PA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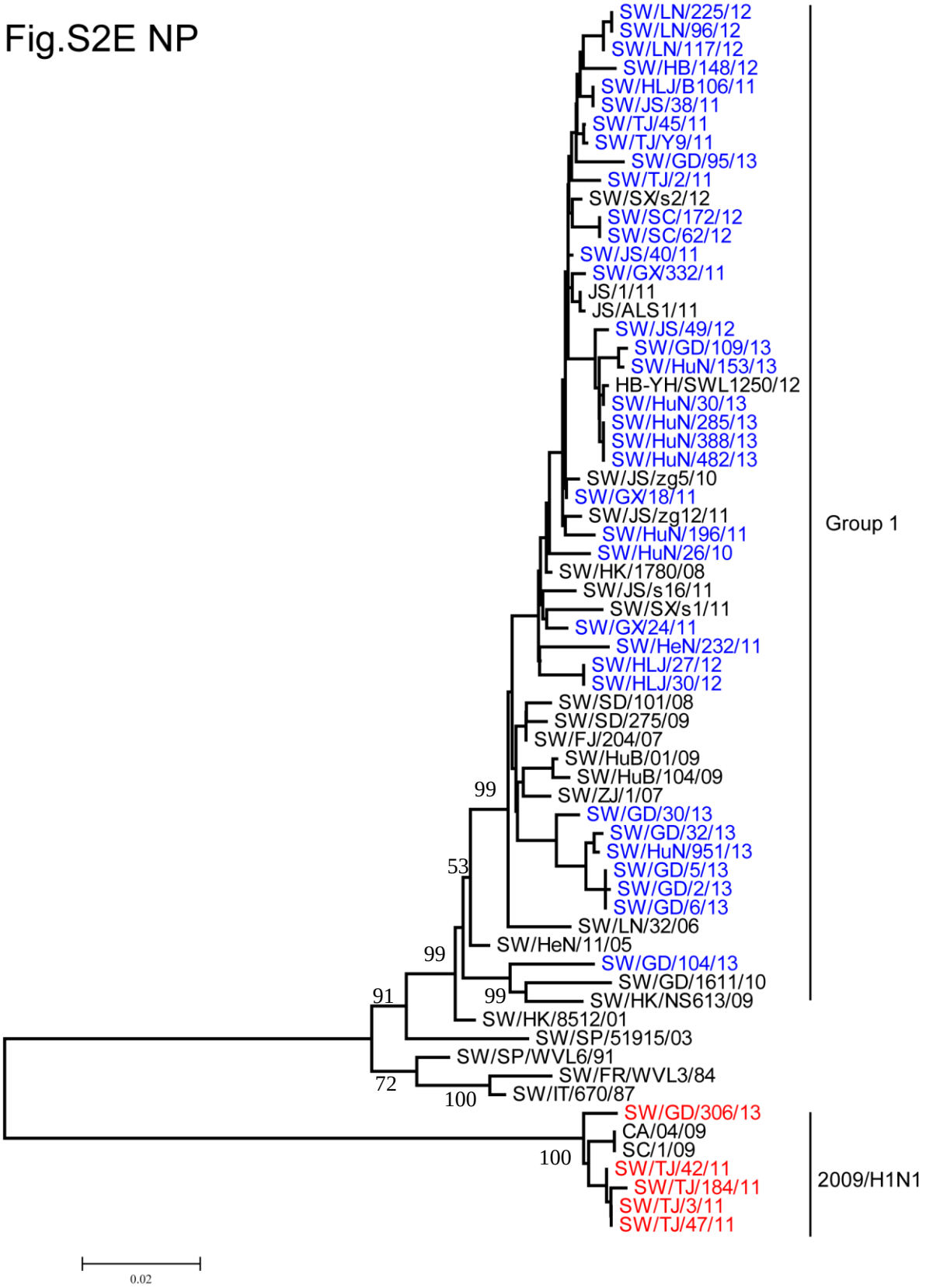
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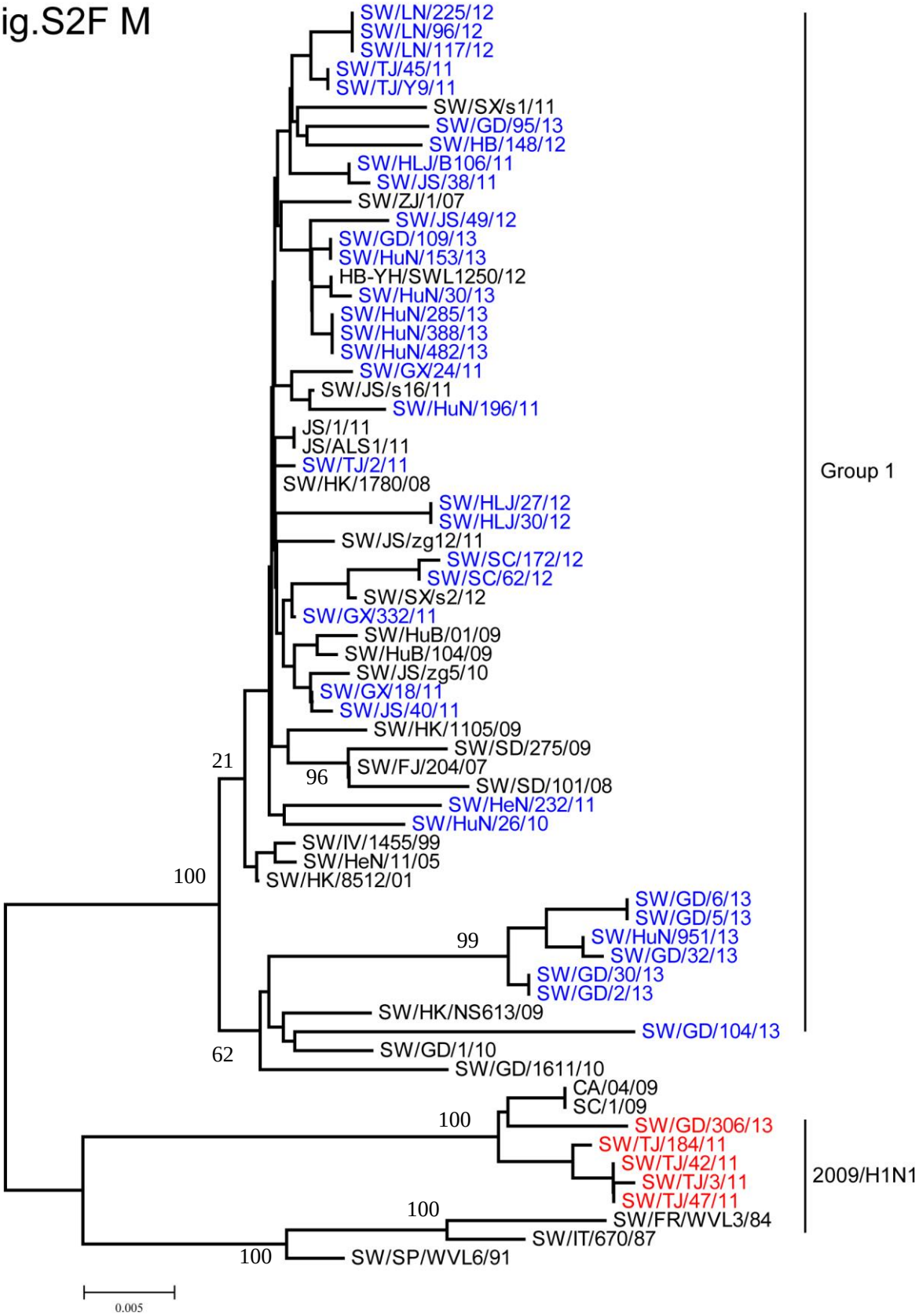
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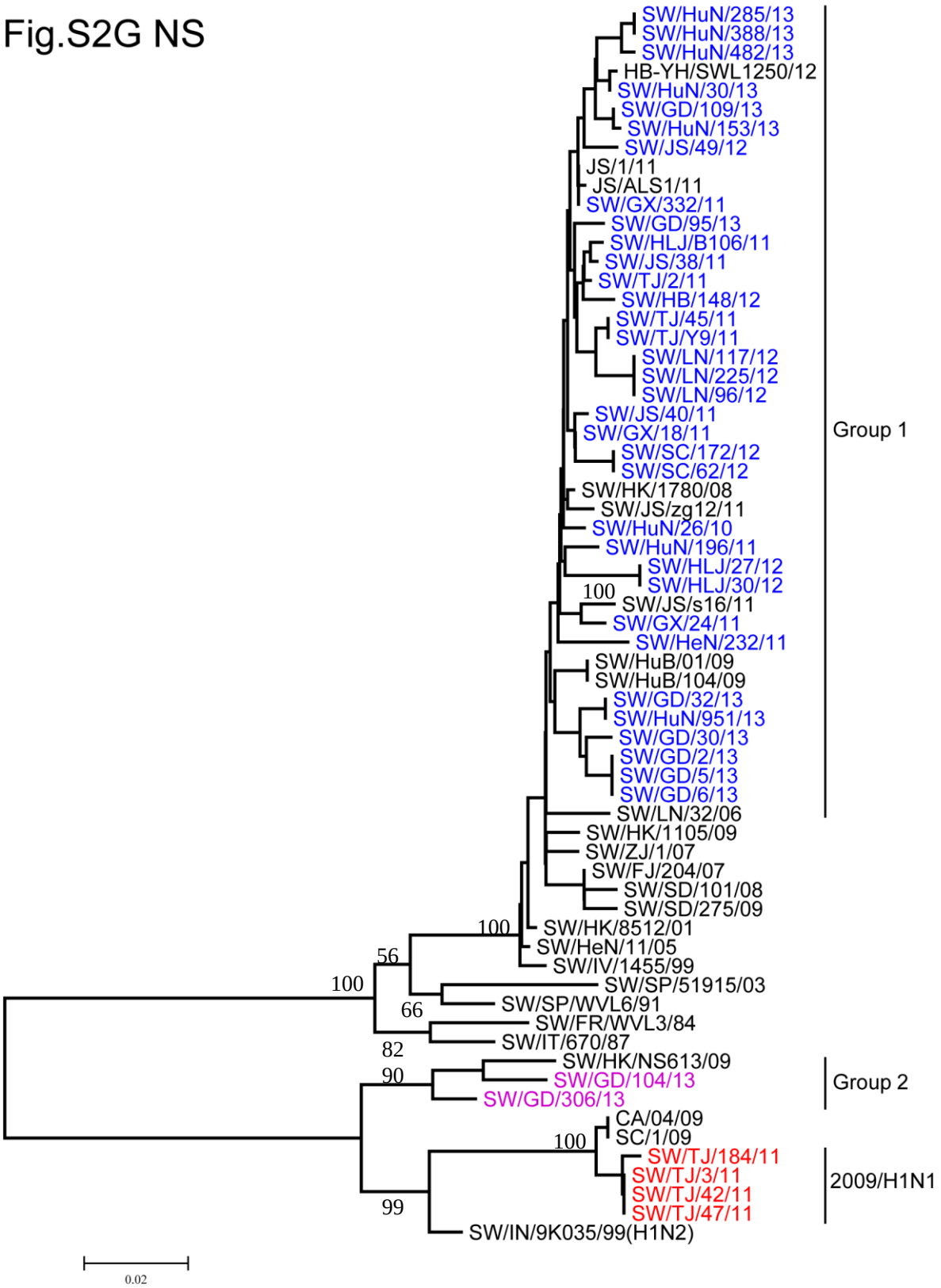
Fig.S2G NS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Group 1
Group 2
2009/H1N1
0.02

Figure S2. Phylogenetic analyses of the EAH1N1 SIVs. The unrooted trees were based on nucleotides (nt) 21 to 1430 for NA (**A**), 28 to 2307 for PB2 (**B**), 25 to 2298 for PB1 (**C**), 25 to 2175 for PA (**D**), 46 to 1542 for NP (**E**), 26 to 1007 for M (**F**), and 27 to 864 for NS (**G**). Sequences of viruses with names in black were downloaded from available databases; viruses with names in other colors were sequenced in this study. Abbreviations are as follows: SW, swine; CA, California; FR, France; FJ, Fujian; GD, Guangdong; GX, Guangxi; HB, Hebei; HB-YH, Hebei-Yuhua; HLJ, Heilongjiang; HeN, Henan; HK, Hong Kong; HuB, Hubei; HuN, Hunan; IN, Indiana; IO, Iowa; IT, Italy; IV, Ille et Vilaine; JS, Jiangsu; LN, Liaoning; NC, Nanchang; SC, Sichuan; SD, Shandong; SP, Spain; SX, Shanxi; SWI, Switzerland; TJ, Tianjin; and ZJ, Zhejiang.

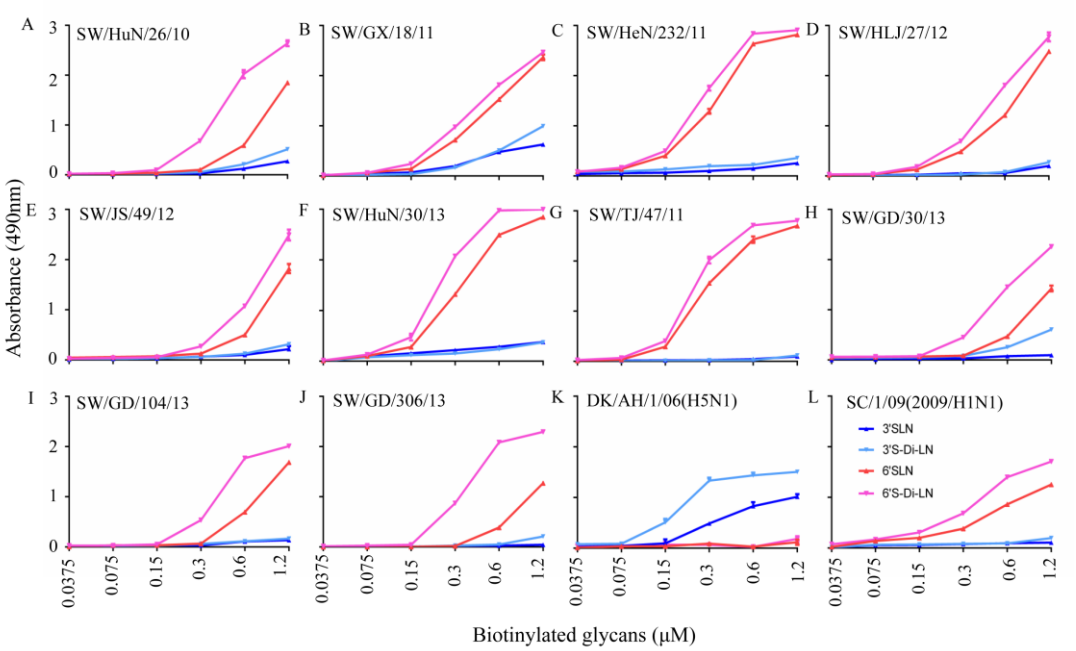

Figure S3. Characterization of the receptor-binding properties of the EAH1N1 SIVs. The binding of the viruses to four different biotinylated glycans (two α-2,3 glycans, blue and Cambridge blue; two α-2,6 glycans, red and pink) was tested. (**A**) SW/HuN/26/10; (**B**) SW/GX/18/11; (**C**) SW/HeN/232/11; (**D**) SW/HLJ/27/12; (**E**) SW/JS/49/12; (**F**) SW/HuN/30/13; (**G**) SW/TJ/47/11; (**H**) SW/GD/30/13; (**I**) SW/GD/104/13; (**J**) SW/GD/306/13; (**K**) DK/AH/1/06 (H5N1); and (**L**) SC/1/09 (2009/H1N1). Data shown are the mean of three repeats; the error bars indicate standard deviations.

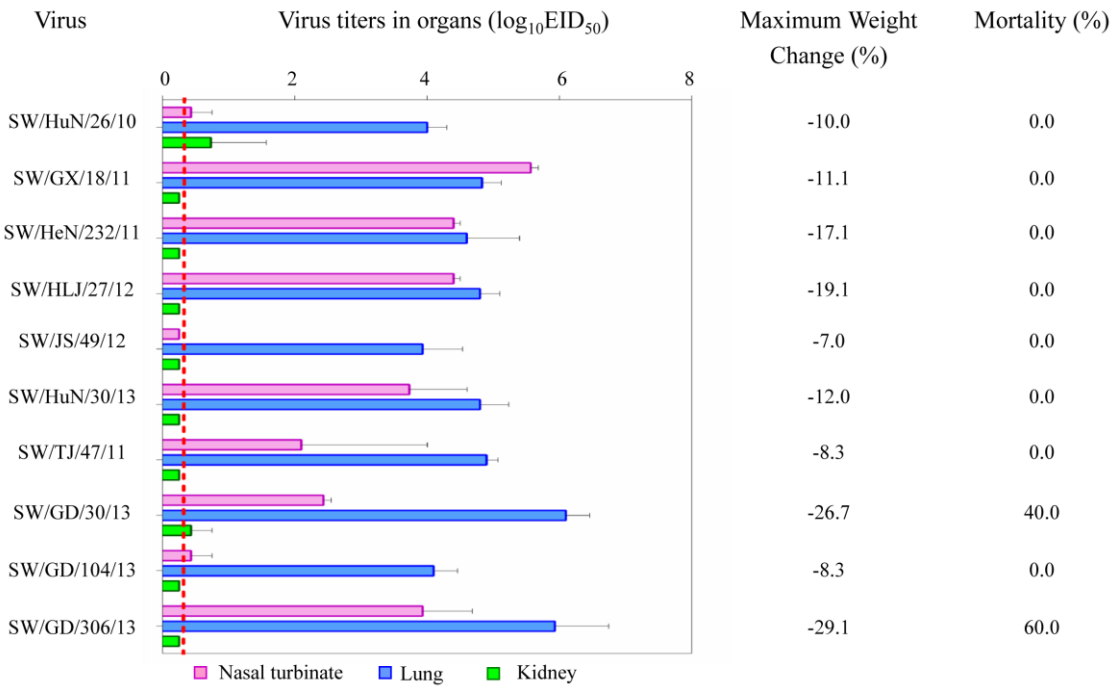

Figure S4. Replication and virulence of EAH1N1 SIVs in mice. Virus replication was tested as described in the Materials and Methods section. Groups of eight mice were inoculated with EAH1N1 virus at dose of 10^6 EID_{50}. The virus titers shown are the mean titers of three mice. A value of 0.3 was assigned if the virus was not detected from the undiluted sample. The maximum weight change and mortality are shown as percentages. The red dashed line indicates the lower limit of detection.

Table S1. Specifics of samples collected for SIVs isolation from 2010–2013.

Province or city	Sampling site	Number of Farms (Number of virus-positive farms)	Total # of samples collected	Influenza virus-positive samples (number of farms of virus presented)						
				Total	H1N1 subtype			Other subtypes		
					Avian-like H1N1	pH1N1/2009	Classical H1N1	H1N2	H3N2	H9N2
Anhui	Slaughterhouse	15	216	0	0	0	0	0	0	0
	Farm	4	96	0	0	0	0	0	0	0
Chongqing	Slaughterhouse	0	0	0	0	0	0	0	0	0
	Farm	2	50	0	0	0	0	0	0	0
Fujian	Slaughterhouse	12	412	0	0	0	0	0	0	0
	Farm	4 (1)	80	1	0	0	0	1 (1)	0	0
Guangdong	Slaughterhouse	114 (7)	5473	7 (7)	3 (3)	1 (1)	0	3 (3)	0	0
	Farm	15 (10)	590	15 (10)	9 (6)	0	2 (1)	4 (3)	0	0
Guangxi	Slaughterhouse	172 (19)	10246	98 (19)	43 (3)	21 (6)	0	32 (8)	0	2 (2)
	Farm	14 (3)	400	3 (3)	0	1 (1)	0	2 (2)	0	0
Hebei	Slaughterhouse	10 (1)	424	1 (1)	1 (1)	0	0	0	0	0
	Farm	4	100	0	0	0	0	0	0	0
Heilongjiang	Slaughterhouse	45 (5)	2820	15 (5)	9 (3)	6 (2)	0	0	0	0
	Farm	13 (1)	272	1 (1)	0	1 (1)	0	0	0	0
Henan	Slaughterhouse	10	463	0	0	0	0	0	0	0
	Farm	4 (1)	80	1 (1)	1 (1)	0	0	0	0	0
Hubei	Slaughterhouse	12	414	0	0	0	0	0	0	0
	Farm	4	80	0	0	0	0	0	0	0
Hunan	Slaughterhouse	89 (14)	5466	42 (14)	35 (7)	7 (7)	0	0	0	0
	Farm	15 (3)	614	7 (3)	3 (1)	4 (2)	0	0	0	0
Inner Mongolia	Slaughterhouse	6	395	0	0	0	0	0	0	0
	Farm	5	226	0	0	0	0	0	0	0

Jiangsu	slaughterhouse	19 (2)	777	5 (2)	5 (2)	0	0	0	0	0
	Farm	6 (1)	120	7 (1)	7 (1)	0	0	0	0	0
Jiangxi	Slaughterhouse	0	0	0	0	0	0	0	0	0
	Farm	6	132	0	0	0	0	0	0	0
Liaoning	Slaughterhouse	13 (3)	510	7 (3)	7 (3)	0	0	0	0	0
	Farm	5	176	0	0	0	0	0	0	0
Qinghai	Slaughterhouse	4 (1)	370	2 (1)	0	0	0	0	2 (1)	0
	Farm	1	30	0	0	0	0	0	0	0
Shandong	Slaughterhouse	12	210	0	0	0	0	0	0	0
	Farm	2	40	0	0	0	0	0	0	0
Shanghai	Slaughterhouse	10	480	0	0	0	0	0	0	0
	Farm	2	40	0	0	0	0	0	0	0
Shanxi	Slaughterhouse	11	214	0	0	0	0	0	0	0
	Farm	2	40	0	0	0	0	0	0	0
Sichuan	Slaughterhouse	18 (2)	602	2 (2)	2 (2)	0	0	0	0	0
	Farm	6	140	0	0	0	0	0	0	0
Tianjin	slaughterhouse	24 (6)	914	12 (6)	12 (6)	0	0	0	0	0
	Farm	8 (1)	180	2 (1)	2 (1)	0	0	0	0	0
Tibet	Slaughterhouse	0	0	0	0	0	0	0	0	0
	Farm	4	205	0	0	0	0	0	0	0
Xinjiang	Slaughterhouse	1	100	0	0	0	0	0	0	0
	Farm	5	710	0	0	0	0	0	0	0
Yunnan	Slaughterhouse	15	1000	0	0	0	0	0	0	0
	Farm	8	270	0	0	0	0	0	0	0
Zhejiang	Slaughterhouse	6 (2)	200	2 (2)	0	0	0	2 (2)	0	0
	Farm	2	40	0	0	0	0	0	0	0
Total		759 (83)	36,417	228	139 (40)	39 (20)	2 (1)	44 (19)	2 (1)	2 (2)

Table S2. Amino acid differences in the antigenic sites of the HA molecule of the H1N1 viruses (H3 numbering).

Virus	HA group	Amino acid in the antigenic site																						
		Sa				Sb						Ca1						Ca2				Cb		
		156	158	160	166	187	188	189	190	193	198	169	171	172	208	238	239	140	142	145	225	74	75	77
SC/1/09	2009/H1N1	K	G	S	K	T	S	A	D	S	A	I	D	K	R	E	P	P	A	K	D	S	T	S
SW/GX/18/11	1	K	G	S	K	T	D	S	D	T	N	T	N	K	K	D	Q	S	S	N	E	L	T	N
SW/HuN/26/10	1	K	G	S	K	T	D	S	D	T	N	T	N	K	K	D	Q	S	S	N	E	L	T	N
SW/TJ/47/11	1	K	G	S	K	T	D	S	V	T	N	T	N	K	K	D	Q	S	S	N	E	L	T	N
SW/HeN/232/11	1	K	G	S	K	T	D	S	D	T	N	T	N	K	K	D	Q	S	S	N	E	L	T	N
SW/HLJ/27/12	1	K	G	S	K	T	D	S	D	T	N	T	N	K	K	D	Q	S	S	N	E	L	T	N
SW/JS/49/12	1	K	G	S	K	T	Y	S	D	T	N	T	N	K	K	D	Q	S	S	N	E	L	T	N
SW/HuN/30/13	1	K	G	S	K	T	D	S	D	T	N	T	N	K	K	D	Q	S	S	N	E	L	T	N
SW/GD/30/13	1	K	G	S	K	T	D	S	D	T	N	T	N	R	K	D	Q	S	S	N	G	L	K	N
SW/GD/104/13	2	Q	E	S	N	N	Y	R	D	A	N	T	N	K	K	D	Q	S	S	K	E	L	T	D
SW/GD/306/13	2	Q	E	A	N	N	Y	S	N	A	N	T	N	K	K	D	Q	S	S	N	E	L	T	D

Table S3. Mutations detected in the EAH1N1 SIVs that contribute to increased binding to human-type receptors, transmission, replication, and virulence in mammals, as well as to resistance to amantadine and rimantadine.

Virus[a]	Passage history[b]	Genotype	HA			PB2				M2
			E190D	G225E	R226Q	T271A	Q591R	E627K	D701N	S31N
SW/HuN/26/10	C1E2	1	D	E	Q	T	Q	E	N	N
SW/GX/18/11	E3	1	D	E	Q	T	Q	E	N	N
SW/GX/24/11	E3	1	D	E	Q	T	Q	E	N	N
SW/GX/332/11	E3	1	D	E	Q	T	Q	E	N	N
SW/HLJ/B106/11	E3	1	V	E	Q	T	Q	E	N	N
SW/HeN/232/11	E3	1	D	E	Q	T	Q	V	N	N
SW/HuN/196/11	E3	1	D	E	Q	T	Q	E	N	N
SW/JS/38/11	E3	1	D	G	Q	T	Q	E	N	N
SW/JS/40/11	E4	1	V	E	Q	T	Q	E	N	N
SW/TJ/2/11	C1E2	1	D	E	Q	T	Q	E	N	N
SW/TJ/45/11	C1E2	1	D	E	Q	T	Q	E	N	N
SW/TJ/Y9/11	C1E2	1	N	E	Q	T	Q	E	N	N
SW/HB/148/12	E3	1	D	E	Q	T	Q	E	N	N
SW/HLJ/27/12	C1E2	1	D	G	Q	T	Q	E	N	N
SW/HLJ/30/12	C1E2	1	D	E	Q	T	Q	E	N	N
SW/JS/49/12	C1E2	1	D	E	Q	T	Q	E	N	N
SW/LN/117/12	E4	1	D	G	Q	T	Q	E	N	N
SW/LN/225/12	E4	1	D	G	Q	T	Q	E	N	N
SW/LN/96/12	E4	1	D	E	Q	T	Q	E	N	N
SW/SC/172/12	C1E2	1	N	E	Q	T	Q	E	N	N
SW/SC/62/12	C1E2	1	D	G	Q	T	Q	E	N	N
SW/GD/109/13	E3	1	D	G	Q	T	Q	E	N	N
SW/GD/95/13	E3	1	D	E	Q	T	Q	E	N	N
SW/HuN/153/13	E3	1	D	G	Q	T	Q	E	N	N
SW/HuN/285/13	E3	1	D	G	Q	T	Q	E	N	N
SW/HuN/30/13	E3	1	D	E	Q	T	Q	E	N	N
SW/HuN/388/13	E3	1	V	E	Q	T	Q	E	N	N

SW/HuN/482/13	E3	1	D	E	Q	T	Q	E	N	N
SW/TJ/184/11	C1E2	2	D	E	Q	A	R	E	D	N
SW/TJ/3/11	C1E2	2	D	E	Q	A	R	E	D	N
SW/TJ/42/11	C1E2	2	D	E	Q	A	R	E	D	N
SW/TJ/47/11	C1E2	2	V	E	Q	A	R	E	D	N
SW/GD/2/13	E3	3	N	E	Q	T	Q	E	N	N
SW/GD/30/13	E3	3	D	G	Q	T	Q	E	N	N
SW/GD/32/13	E3	3	D	E	Q	T	Q	E	N	N
SW/GD/5/13	E3	3	D	E	Q	T	Q	E	N	N
SW/GD/6/13	E3	3	D	G	Q	T	Q	E	N	N
SW/HuN/951/13	E3	3	D	G	Q	T	Q	E	N	N
SW/GD/104/13	E3	4	D	E	Q	T	Q	E	N	N
SW/GD/306/13	E3	5	N	E	Q	A	R	E	D	N

[a]Viruses in red were selected for antigenic analysis, receptor binding assays, and mouse and ferret studies.

[b]C, cell culture; E, eggs; the number indicated the number of passages.

Table S4. Body temperature increases and body weight changes of ferrets inoculated with or exposed to different EAH1N1 SIVs or the 2009/H1N1 pandemic virus.

Virus (genotype)	Animal pair	Body temperature increase (℃)		Body weight change (%)[a]	
		Inoculated	Exposed	Inoculated	Exposed
SW/HuN/26/10 (1)	1	1	1.4	-0.8	+9.5
	2	2.2	1.4	-0.2	+5.7
	3	2.2	1.3	-0.3	-5.6
SW/GX/18/11 (1)	1	1.7	0.8	-17.9	-14
	2	0.8	0.9	-8.4	-6.9
	3	1.1	0.7	-6.0	-21
SW/HeN/232/11 (1)	1	1.9	1.1	-18.6	-7.4
	2	1.4	1.2	-12.5	-4.5
	3	1.7	0.9	-8	-14
SW/HLJ/27/12 (1)	1	0.8	0.3	-11.8	+11.8
	2	1	0.6	-15.2	-11.7
	3	0.7	0.2	-4.5	+5.8
SW/JS/49/12 (1)	1	1.5	0.5	-5.6	-7
	2	2.1	0.1	-1.4	+8
	3	0.9	1.2	-4	-12.3
SW/HuN/30/13 (1)	1	0.9	1.2	-3.4	-2.8
	2	0.8	0.8	-0.1	-9.4
	3	1.5	0.9	-2.8	-2.9
SW/TJ/47/11 (2)	1	0.7	1	-5.4	-4.5
	2	0.7	0.8	-12.9	-6.7
	3	1.4	0.7	-3.2	-7.1
SW/GD/30/13 (3)	1	0.8	0.2	-3.2	+13.8
	2	1.0	0.5	-1.4	-4.6
	3	0.7	0.2	-0.8	+3.3
SW/GD/104/13 (4)	1	1.0	0.6	-1.8	-1.0
	2	0.6	0.5	-0.7	+11.1
	3	0.9	0.3	-1.3	+11.7

SW/GD/306/13 (5)	1	0.9	0.4	-2.4	+3.0
	2	0.7	0.6	-2.9	+1.4
	3	1.6	0.5	-10.3	+9.5
SC/1/09	1	1.7	0.6	-10.1	-10.2
	2	0.7	1.3	-6.1	-3.7
	3	2.2	2.3	-9.6	-21.2

[a]The maximam body weight change of each ferret observed during the three-week observation period is shown. +, body weight increase; -, body weight loss.

Table S5. Cross-reactive antibody response against influenza A(H1N1) viruses of human sera collected in north China in 2015.

Age group (year)	Antigen	%HI titer ≥ 40 (95% CI[a])	%NT titer ≥ 40 for children or ≥ 80 for adults (95% CI[a])
≤10, n=55	SC/1/09	9.1 (1.5-16.7)	12.7 (3.9–21.5)
	SW/GX/18/11	5.5 (0-11.5)	3.6 (0–8.6)
	SW/GD/104/13	0	0
25-53, n=52	SC/1/09	7.7 (0.5-14.9)	1.9 (0–5.7)
	SW/GX/18/11	11.5 (2.9-20.2)	0
	SW/GD/104/13	3.8 (0-9.1)	0
≥60, n=52	SC/1/09	1.9 (0-5.7)	0
	SW/GX/18/11	3.8 (0-9.1)	13.4 (4.2–22.7)
	SW/GD/104/13	0	0

[a]Confidence intervals.

Table S6. Risk scores for the emergence of a pandemic strain among six animal influenza viruses[a].

Element	Weight (W) [b]	Avian H1N1		H7N9		H3N2v		H9N2		H5N1		EAH1N1	
		Risk score (RS)[c]	W×RS	RS	W×RS	RS	W×RS	RS	W×RS	RS	W×RS	RS	W×RS
Human infections	0.2929	2.3	0.674	5	1.465	5.8	1.699	3	0.879	5	1.465	5	1.466
Transmission in laboratory animals	0.1929	2	0.386	7	1.350	7.5	1.447	8	1.543	2	0.386	9.5	1.833
Receptor binding	0.1429	2	0.286	6.3	0.900	8.6	1.229	9	1.286	3	0.429	9	1.286
Population immunity (To vaccine)	0.1096	3	0.329	9	0.986	4.1	0.449	9	0.986	9	0.986	7	0.767
Infections in animals	0.0846	2	0.169	4.7	0.398	7.4	0.626	8	0.677	8	0.677	8	0.678
Genomic variation	0.0646	3	0.194	8.6	0.556	7.6	0.491	2	0.129	5	0.323	7	0.452
Antigenic relatedness	0.0479	2	0.096	3.7	0.177	5.5	0.263	3.5	0.168	3.5	0.168	5	0.240
Global distribution animals	0.0336	2.5	0.084	4.7	0.158	5.8	0.195	7	0.235	7	0.235	7	0.235
Disease severity	0.0211	2.25	0.047	8.5	0.179	4.8	0.101	7	0.148	9	0.190	7	0.148
Antiviral and treatment options	0.001	2.25	0.002	5.8	0.006	3.9	0.004	5.8	0.006	5.8	0.006	5.8	0.006
Summary risk score			2.27		6.18		6.5		6.06		4.87		7.11

[a] Avian H1N1, A/duck/New York/1996; H7N9, A/pigeon/Shanghai/S1421/2013; H3N2v, A/Indiana/08/2011(H3N2); H9N2, A/chick/Jiangsu/C4258/2012; H5N1, A/duck/Guangxi/35/2001; EAH1N1, A/swine/Guangxi/18/2011.

[b] The calculated weight of each element is described in the Influenza Risk Assessment Tool (IRAT) developed by CDC (USA) (reference 41, main text).

[c] The risk score numbers are the averages provided by five experts, who estimated the scores by using the framework of risk element definitions and scoring criteria described in the IRAT.