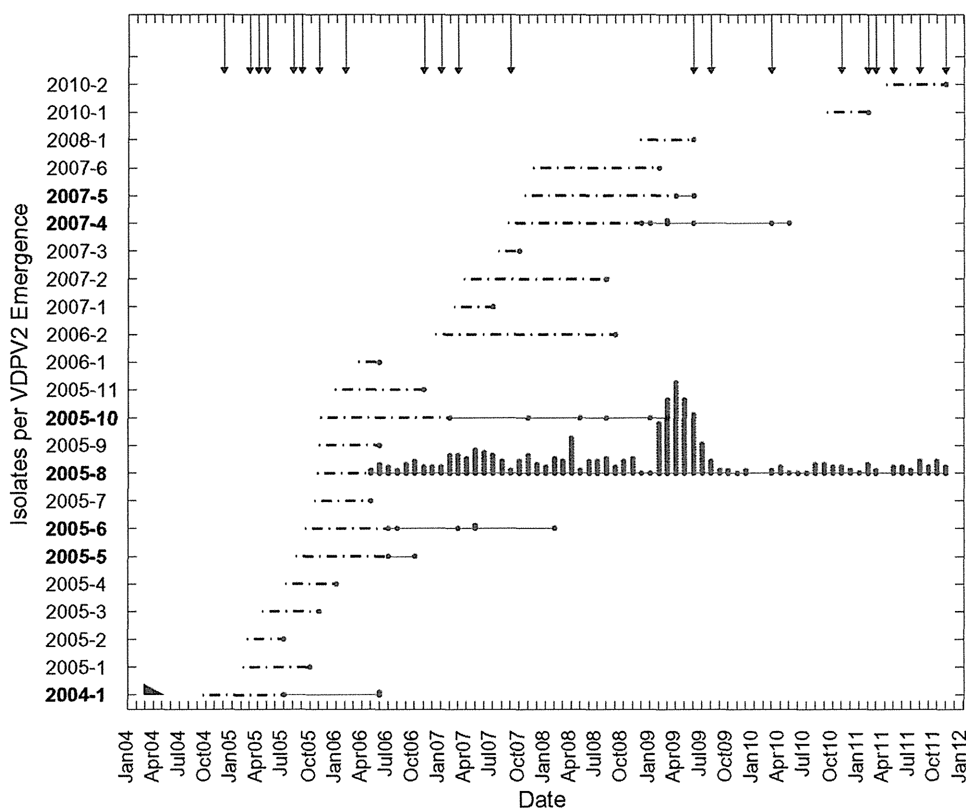

FIG 4 Timeline of independent VDPV2 emergences and spread in Nigeria from 2004 to 2011 based on the dates of VDPV2 specimen collection (specimens were collected 10 ± 6 days after onset of AFP). cVDPV2 lineage groups are indicated in boldface type. Dates of the initiating tOPV doses for the 14 emergences associated with single isolates are point estimates with indeterminate confidence intervals. Arrows indicate months of tOPV supplemental immunization activities (SIAs) (mass vaccination campaigns) in the northern states. The blue point symbolizes two superimposed points. The blue wedge in the lower left corner symbolizes the duration of type 2 vaccine-related poliovirus excretion in immunologically healthy primary-dose OPV recipients (30).

and permitted several new VDPV2 emergences to appear throughout the outbreak.

The most closely related isolates tended to cluster geographically (see Fig. S1 to S4 in the supplemental material). However, virus of the major lineage group, 2005-8, was disseminated widely across the northern states during its >6 years of transmission.

VDPV2 lineages transiting the low-transmission season for poliovirus. The occurrence of VDPV2 infections (represented in Fig. 4 by dates of collection of VDPV2-positive specimens) showed a distinct seasonality (45), with a peak during March to June and a low during December to January (Fig. 4 and 6). Seasonal curves based on the appearance of polio cases would be shifted to the left because specimens were collected 10 ± 6 days after onset of AFP. Seasonal curves based on the estimated times of exposure would be shifted further to the left (by a total of ~25 days) because paralysis usually occurs within 15 days of exposure to neurovirulent poliovirus (79). Seasonal variation is low in humid tropical climates and higher in dryer, more temperate zones (45) and may vary in its details from year to year. Superimposed on these natural cycles is the impact of immunization (including choice of vaccines, vaccine coverage rates, and population immunity profiles) on the virus population.

The natural seasonality of polio reflects the fluctuation in the number of chains of poliovirus transmission during the year (80). To estimate of the number of lineages (representing observed chains of transmission) that survived the seasonal bottlenecks in December to January, we counted the number of branches that transited across year boundaries (Fig. 4; see also Fig. S1 to S4 in the supplemental material), excluding branches that appeared to have terminated in January. The number of observed lineages estimated to have survived the seasonal bottlenecks rose sharply in 2006 to 2007 (~63 lineages), declined slightly in 2007 to 2008 (~48), peaked in 2008 to 2009 (~144), declined sharply in 2009 to 2010 (~22), and stabilized in 2010 to 2011 (~25) (Fig. 7). Lineage group 2005-8 was predominant by 2006 to 2007, peaked at ~135 lineages transiting the 2008-2009 seasonal low, and declined in 2009 to 2010 (~21) and 2010 to 2011 (~23) (Fig. 7).

VDPV2 population dynamics during the outbreak. The dynamics of virus populations throughout the year can be modeled by coalescent methods in which multiple lineages of a population are traced back in time through successive joining (coalescent) events to the most recent common ancestor (81). Virus population size and rates of growth and decline can be inferred from the topologies of phylogenetic trees (representing genealogies) constructed from temporally ordered sequences (66, 81). In rapidly evolving viruses, variation in the number of lineages can be used to estimate changes over time in the effective virus population size (N_e), a parameter used to model the fixation of "neutral" mutations in actual populations. Virus population dynamics can be visualized through Bayesian skyline plots (66) of the time dependence of N_e (approximately proportional to nucleotide diversity). We used the program BEAST (60) to analyze the population dy-

TABLE 1 Independent VDPV2 emergences, Nigeria, 2004 to 2011[g]

Emergence[a]	Lineage group[b]	Earliest isolate[c]	Earliest isolate specimen date (day, mo, yr)	No. of nt substitutions of earliest isolate		Estimated date of initiating tOPV dose (day, mo, yr) (95% HPD)[d]	Latest isolate specimen date (day, mo, yr)[e]	Total no. of isolates	Estimated duration of replication (mo) (95% HPD)[f]
				VP1	P1				
1	**2004-1**	BAS05-01	05 July 05	10	21	22 Sep 04 (03 Apr 04–20 Apr 05)	08 June 06	3	21 (14–26)
2	2005-1	SOS05-01	05 Oct 05	7	19	[07 Feb 05]	—	1	[8]
3	2005-2	LAS05-01	22 July 05	6	12	[21 Feb 05]	—	1	[5]
4	2005-3	KDS05-01	03 Nov 05	7	16	[16 Apr 05]	—	1	[7]
5	2005-4	KBS06-01	14 Jan 06	8	16	[06 July 05]	—	1	[7]
6	**2005-5**	JIS06-06	17 July 06	15	28	13 Aug 05 (10 Apr–06 Dec 05)	17 Oct 06	2	14 (10–20)
7	**2005-6**	BOS06-03	05 July 06	9	19	14 Sep 05 (15 Apr 05–01 Feb 06)	07 Feb 08	6	29 (24–34)
8	2005-7	BOS06-01	31 May 06	9	18	[16 Oct 05]	—	1	[7]
9	**2005-8**	JIS06-01	19 May 06	6	16[h]	26 Oct 05 (22 July 05–12 Jan 06)	17 Dec 11	361	74 (70–77)
10	2005-9	JIS06-04	15 June 06	9	18	[31 Oct 05]	—	1	[7]
11	**2005-10**	SOS07-01	26 Feb 07	13	27	03 Dec 05 (11 May 05–16 July 06)	05 Mar 09	6	40 (33–47)
12	2005-11	BAS06-01	07 Nov 06	11	25	[27 Dec 05]	—	1	[10]
13	2006-1	BOS06-02	15 June 06	6	7	[19 Mar 06]	—	1	[3]
14	2006-2	SOS08-09	02 Sep 08	20	50	[11 Dec 06]	—	1	[21]
15	2007-1	NIS07-01	30 July 07	9	13	[16 Feb 07]	—	1	[5]
16	2007-2	BAS08-06	07 Aug 08	10	40	[22 Mar 07]	—	1	[17]
17	2007-3	ANS07-01	27 Oct 07	6	8	[18 July 07]	—	1	[3]
18	**2007-4**	KBS08-03	01 Dec 08	12	30	05 Sep 07 (04 Apr 07–01 Feb 08)	25 May 10	7	33 (28–38)
19	**2007-5**	BOS09-06	20 Apr 09	17	44	21 Oct 07 (14 Mar 07–07 June 08)	02 June 09	2	19 (12–27)
20	2007-6	KTS09-05	27 Feb 09	16	37	[18 Nov 07]	—	1	[15]
21	2008-1	KDS09-08	05 June 09	6	15	[28 Nov 08]	—	1	[6]
22	2010-1	KBS11-01	22 Feb 11	6	13	[10 Sep 10]	—	1	[5]
23	2010-2	NIS11-01	22 Nov 11	11	18	[05 Apr 11]	—	1	[7]

[a] Numbered in estimated order of emergence (Fig. 1).
[b] Emergences that expanded into well-defined cVDPV2 lineage groups (shown in boldface type) were previously described and numbered according to the date of detection of the earliest isolate (2004-1, 1; 2005-8, 2; 2005-6, 3; 2005-5, 4; 2005-10, 5; 2007-4, 6; 2007-5, 7) (15). All other emergences were previously described as "undefined."
[c] Abbreviations for state names are given in the legend to Fig. 1.
[d] P1/capsid K_T evolution rate was assumed to be constant at 1.1%/year (29 nt substitutions/year). HPD, highest posterior density.
[e] Eight additional cases (three from Kano, three from Sokoto, and two from Kebbi) associated with cVDPV2, all from lineage group 2005-8, were reported in 2012. The most recent case had an onset date of 24 November 2012, and the isolate had 65 nt substitutions in VP1. One new VDPV2 emergence was associated with a 2012 case in the southern state of Edo (onset on 22 May 2012; the isolate had 6 nt substitutions in VP1). (Data as of 31 December 2012; for updates, see the Global Polio Eradication Initiative website [http://www.polioeradication.org/].) —, single isolate.
[f] Calculated from the estimated date of the initiating tOPV dose to the date of the most recently isolated specimen (through 2011).
[g] Values in brackets are point estimates with indeterminate confidence intervals.
[h] Compared against a simulated unmutated Sabin 3/Sabin 2 recombinant with the recombination junction at the same location as in the natural isolates.

namics of all Nigerian VDPV2s from 2005 through mid-2011, comparing the Bayesian skyline plot with the total monthly case counts (Fig. 4 and 8). N_e increased ~20-fold from mid-2005 to late 2006 (as new emergences were appearing), after which it fluctuated over a narrow range up to late 2008. In 2009, N_e increased by ~10-fold for January to March, stabilized, and then declined ~10-fold after two rounds of mass tOPV campaigns on 30 May and 1 August (Fig. 8). The sharp fluctuations in virus population dynamics are reflected in the monthly case counts in 2009 (Fig. 4). N_e stabilized near 2007-2008 levels from late 2009 to the end of 2010 and gradually increased in 2011 (Fig. 8), as two new emergences appeared and as virus of lineage group 2005-8 continued to circulate in eight northern states via at least 23 separate chains of transmission (see Fig. S2 in the supplemental material). As expected from the combined epidemic curves (Fig. 4), the skyline plot for lineage group 2005-8 (not shown) was nearly congruent with that for all Nigerian VDPV2s.

Relative substitution rates in VP1 and complete P1/capsid regions. All WPV and candidate VDPV isolates are routinely characterized by VP1 sequencing by the GPLN (16, 19, 21). To assess the sensitivity of VP1 sequencing as a screening tool for divergent Sabin strain derivatives, we plotted the number of nucleotide substitutions in the VP1 region as a function of the estimated number of nucleotide substitutions in the P1/capsid region for all 2005-2011 Nigerian VDPV2 isolates (Fig. 9). A strong linear correlation (slope = 0.93; R^2 = 0.90) between the relative frequencies of VP1 and P1/capsid region nucleotide substitutions was observed. Although some early VDPV isolates had a higher density of nucleotide substitutions in the VP1 region than elsewhere in the P1/capsid region (Table 1), possibly reflecting early fixation of selected substitutions (see below), the differences tended to diminish over time (Fig. 9). Therefore, we conclude that VP1 sequencing has sufficient sensitivity to identify VDPVs, even at levels of divergence as low as 0.7% (i.e., 6 nt substitutions in VP1).

Estimated number of VDPV2 infections. The proportion of WPV infections that result in paralytic cases is lowest for PV2 (~1:1,800) among the three poliovirus serotypes (45). Under the

TABLE 2 Nucleotide differences in the P1/capsid region across independent VDPV2 emergences[a]

Emergence	Lineage group	Earliest isolate	Value for emergence																							
			S	1	2	3	4	5	6	7	8	9[b]	10	11	12	13	14	15	16	17	18	19	20	21	22	23
S		Sabin 2																								
1	**2004-1**	BAS05-01	20		0.00	0.06	0.00	0.00	0.04	0.08	0.05	0.09	0.00	0.00	0.00	0.00	0.01	0.00	0.03	0.04	0.04	0.13	0.04	0.00	0.00	0.05
2	2005-1	SOS05-01	18	38		0.00	0.00	0.06	0.00	0.06	0.00	0.00	0.06	0.05	0.00	0.00	0.03	0.00	0.00	0.00	0.00	0.03	0.02	0.00	0.00	0.00
3	2005-2	LAS05-01	12	30	30		0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.03	0.00	0.00	0.08	0.04	0.11	0.05	0.04	0.00	0.00	0.00	0.00
4	2005-3	KDS05-01	15	35	33	27		0.00	0.00	0.00	0.00	0.00	0.00	0.02	0.05	0.00	0.00	0.00	0.00	0.00	0.05	0.04	0.00	0.00	0.00	0.00
5	2005-4	KBS06-01	15	35	31	27	30		0.00	0.00	0.00	0.00	0.00	0.05	0.00	0.00	0.00	0.07	0.00	0.00	0.05	0.00	0.00	0.07	0.00	0.00
6	**2005-5**	JIS06-06	27	45	45	39	42	42		0.00	0.05	0.02	0.00	0.08	0.00	0.06	0.03	0.05	0.00	0.00	0.00	0.06	0.10	0.05	0.10	0.02
7	**2005-6**	BOS06-03	18	35	34	30	33	33	45		0.00	0.00	0.06	0.00	0.00	0.00	0.06	0.03	0.00	0.00	0.04	0.05	0.04	0.00	0.00	0.00
8	2005-7	BOS06-01	17	35	35	29	32	32	42	35		0.00	0.00	0.05	0.00	0.09	0.03	0.00	0.05	0.00	0.04	0.00	0.00	0.00	0.00	0.00
9	**2005-8**[b]	JIS06-01	14	31	32	26	29	29	40	32	31		0.00	0.10	0.00	0.00	0.03	0.00	0.00	0.10	0.05	0.04	0.00	0.00	0.00	0.06
10	2005-9	JIS06-04	17	37	33	29	32	32	44	33	34	31		0.07	0.00	0.00	0.00	0.07	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
11	**2005-10**	SOS07-01	26	46	42	38	40	39	49	44	41	36	40		0.00	0.00	0.11	0.00	0.00	0.00	0.04	0.00	0.03	0.00	0.00	0.00
12	2005-11	BAS06-01	24	44	42	35	37	39	51	42	41	38	41	50		0.00	0.03	0.00	0.06	0.06	0.00	0.05	0.08	0.00	0.00	0.00
13	2006-1	BOS06-02	6	26	24	18	21	21	31	24	21	20	23	32	30		0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
14	2006-2	SOS08-09	49	68	65	61	64	64	74	63	64	61	66	67	71	55		0.03	0.07	0.04	0.08	0.12	0.08	0.00	0.00	0.06
15	2007-1	NIS07-01	12	32	30	22	27	25	37	29	29	26	27	38	36	18	59		0.04	0.00	0.10	0.04	0.08	0.08	0.00	0.00
16	2007-2	BAS08-06	39	57	57	49	54	54	66	57	53	53	56	65	59	45	82	49		0.07	0.10	0.05	0.05	0.00	0.02	0.11
17	2007-3	ANS07-01	7	26	25	17	22	22	34	25	24	19	24	33	29	13	54	19	43		0.00	0.02	0.00	0.00	0.00	0.00
18	**2007-4**	KBS08-03	29	47	47	39	42	42	56	45	44	41	46	53	53	35	72	37	61	36		0.06	0.06	0.00	0.00	0.04
19	**2007-5**	BOS09-06	42	54	58	52	55	57	65	57	59	54	59	68	63	48	80	52	77	48	67		0.05	0.04	0.07	0.03
20	2007-6	KTS09-05	36	54	53	48	51	51	57	52	53	50	53	60	55	42	78	44	71	43	61	74		0.00	0.00	0.04
21	2008-1	KDS09-08	14	34	32	26	29	27	39	32	31	28	31	40	38	20	63	24	53	21	43	54	50		0.00	0.00
22	2010-1	KBS11-01	12	32	30	24	27	27	35	30	29	26	29	38	36	18	61	24	50	19	41	50	48	26		0.00
23	2010-2	NIS11-01	17	35	35	29	32	32	43	35	34	29	34	43	41	23	62	29	50	24	44	57	51	31	29	

[a] Values below the diagonal are numbers of pairwise P1/capsid region nucleotide differences; values above the diagonal are proportions (percent) of P1/capsid region nucleotide differences from Sabin 2 that were shared among isolates. The codon for amino acid $VP1_{143}$ was excluded. Well-defined cVDPV2 lineage groups are shown in boldface type.
[b] Values exclude the first 93 nt in the P1/capsid region of JIS06-01, which were derived from Sabin 3.

assumption that the case-to-infection ratio for VDPV2s is similar to that for WPV2, we estimate from the number of reported cases that ~700,000 cVDPV2 infections have occurred in Nigeria since 2005. The actual number of infections is likely to be higher, as the relatively high frequency of isolates with no close relatives (representing "orphan" lineages, signaled by long branches on the trees) is an indication that cases had been missed in some areas.

Genetic properties of the VDPV2 isolates. Two determinants are the main contributors to the attenuated phenotype of Sabin 2 in transgenic mice (82) and primates (58): A_{481} in the 5′-untranslated region (5′-UTR) and Ile_{143} in VP1. Both determinants were lost in all VDPV2 isolates (Table 5). For the 5′-UTR determinant, evidence of direct $A_{481} \rightarrow G$ reversion was found for only a small proportion of isolates (20 of 403; 5.0%), representing 16 of the 23 emergences, usually those not associated with lineage groups. In the remainder, A_{481} had been exchanged out by recombination with other enteroviruses. Early lineage group 2005-8 isolates were Sabin 3/Sabin 2 5′-UTR recombinants with sequences containing the $U_{472} \rightarrow C$ reversion of the major Sabin 3 attenuation determinant (83). A total of 12 distinct 5′-UTR recombinant classes were detected, some generated by serial recombination events (our unpublished results).

The second critical determinant of the attenuated phenotype, VP1-Ile_{143}, had reverted in all VDPV2 isolates. The majority of isolates (399 of 403 [99.0%]; 20 of 23 independent emergences) had the VP1-$Ile_{143} \rightarrow Thr$ replacement in the WPV2 consensus (37), which remained fixed in the population, except for one lineage group 2005-8 isolate, KNS09-022, which had a superimposed (VP1-$Thr_{143} \rightarrow Ala$) replacement. Three other isolates, each representing independent emergences, had the alternative replacements VP1-$Ile_{143} \rightarrow Asn$ (isolate KDS05-01) and VP1-$Ile_{143} \rightarrow Val$ (isolates ANS07-01 and KBS11-01) (Table 5).

Apart from the codon for $VP1_{143}$, nucleotide substitutions accumulated approximately uniformly across the P1/capsid region (Fig. 9), with evidence of purifying selection at most amino acid positions (Table 3). The detailed pattern of nucleotide substitu-

TABLE 3 Substitution rates for P1/capsid region sequences (2,637 nt) of cVDPV2 lineage groups

Substitution parameter[a]	Bayesian rate (nt substitutions/site/yr [10^{-2}]) (95% HPD) for cVDPV2 lineage group[b]			
	2005-6	2005-8	2005-10	2007-4
K_T	1.21 (0.91–1.50)	1.12 (1.05–1.20)	1.16 (0.81–1.47)	1.09 (0.71–1.46)
K'_S	1.14 (0.86–1.43)	0.88 (0.84–0.93)	1.20 (0.85–1.54)	1.05 (0.66–1.41)
K'_A	0.08 (0.006–0.16)	0.12 (0.10–0.15)	0.10 (0.004–0.20)	0.09 (0.01–0.19)
A'_S	1.04 (0.78–1.31)	0.80 (0.75–0.84)	1.08 (0.75–1.38)	0.98 (0.64–1.33)
B'_S	0.06 (0.003–0.14)	0.08 (0.07–0.09)	0.06 (0.006–0.12)	0.11 (0.01–0.22)

[a] Substitution parameters (K_T, total substitutions; K'_S, synonymous substitutions; K'_A, nonsynonymous substitutions; A'_S, synonymous transitions; B'_S, synonymous transversions) were normalized to total sites by using the following relationships: $K'_S = K_S \times L_S/L_T$, $K'_A = K_A \times L_N/L_T$, $A'_S = A_S \times L_S/L_T$, and $B'_S = B_S \times L_S/L_T$ (L_S, number of synonymous sites; L_N, number of nonsynonymous sites; L_T, total number of sites) (47). Estimates of the number of each category of site were calculated by implementation of the program MEGA5 (64) (L_T sites = 2,637; L_S sites ≈ 865; L_N sites ≈ 1,708).

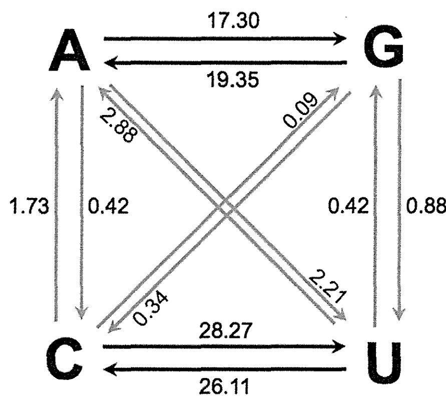

FIG 5 Maximum likelihood estimates of relative frequencies of specific base changes (10^{-2}) (black arrows, transitions; gray arrows, transversions) at all codon positions within the P1/capsid regions of the 361 2005-2011 cVDPV2 isolates from the major 2005-8 lineage group during divergence from the Sabin 2 root sequence.

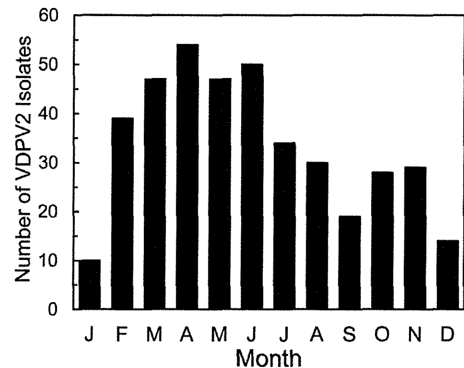

FIG 6 Cumulative seasonality of specimens containing VDPV2 in Nigeria, 2004 to 2011. Note that AFP cases appeared ~10 days earlier than specimen collection and that VDPV2 exposure likely occurred 3 to 4 weeks earlier than specimen collection.

tion (Fig. 5), predominantly (~80%) synonymous transitions (Table 3), closely matched that previously described for WPV1 lineages (47).

The codon for VP1-Thr_{24} was deleted in four isolates. Three of the isolates (BAS07-04, JIS07-03, and BAS08-01, of lineage group 2005-8) clustered closely on the tree (see Fig. S2 in the supplemental material), and their sequence relationships were consistent with localized circulation of the deletion variant for about a year. The fourth deletion variant, KTS09-22 (also of lineage group 2005-8), arose independently of the others, and there was no evidence of secondary spread (see Fig. S2 in the supplemental material). The 30 (PV3) or 32 (PV1 and PV2) amino-terminal residues of VP1 are disordered within the intact poliovirion (84); form a flexible, externalized amphipathic domain during virus uncoating (85); and can accommodate numerous amino acid substitutions within (47) and across (86) poliovirus serotypes. Variants with single-codon deletions in this domain occasionally emerge transiently within populations of circulating polioviruses of all three serotypes but constitute a very small proportion (<0.2%) of poliovirus isolates worldwide (our unpublished results).

All but seven isolates were vaccine/nonvaccine recombinants, with P2 and P3 noncapsid sequences derived from unidentified species C enteroviruses (87), a property typical of cVDPVs (19, 21, 32, 38, 40, 75) and associated with poliovirus circulation (88–90). Apart from isolates of the largest lineage group, 2005-8, in which the first 31 P1/capsid region codons were derived from Sabin 3, all other VDPV2 P1/capsid region sequences were derived exclusively from Sabin 2.

Phenotypic properties of VDPV2 isolates. cVDPVs had recovered two key properties of WPVs: the capacity to cause paralytic poliomyelitis in humans and the capacity for sustained person-to-person transmission. Nearly all Nigerian VDPV2s were isolated from patients with AFP; >75% of those patients examined 60 days after onset of paralysis had residual asymmetric paralysis typical of poliomyelitis (14, 15). Moreover, the genetic relationships among the 403 cVDPV2 isolates document their capacity for widespread circulation for more than 6 years. Experimentally measurable virus properties that are possible surrogates for neuropathogenicity for humans and transmissibility are (i) virus yields in HEK293 cells, (ii) neurovirulence for transgenic mice expressing the human CD155 PVR, and (iii) virus yields during growth at supraoptimal temperatures.

(i) Virus yields in HEK293 cells. HEK293 cells are derived from human neuronal cells (68), and neurovirulent polioviruses grow to higher yields in these cells than do attenuated strains (69). Sabin 2, MEF-1 (a neurovirulent WPV2 reference strain), and 25 Nigerian VDPV2 isolates (including the first isolates of each emergence) were grown in single-step growth experiments in HEK293 cells, and virus yields were measured by plaque assays on HeLa cells. Yields at 37.0°C were lowest for Sabin 2, ANS07-01, BOS06-02, and KDS05-01 and 3- to 5-fold higher for MEF-1, KTS09-02, BAS05-01, SOS05-01, BOS07-02, BOS09-06, and KBS11-01 (Table 5). Yields at 39.5°C were reduced by 1.5- to 15-fold for most isolates, but those of Sabin 2 dropped ~700-fold, and isolates

TABLE 4 Estimated time to first divergence of cVDPV2 lineage groups

Lineage group	Estimated date of initiating tOPV dose (day, mo, yr) (95% HPD)	Estimated date of first diverging node (day, mo, yr) (95% HPD)	Estimated mean time to first divergence (mo)[a]
2004-1	22 Sep 04 (03 Apr 04–20 Apr 05)	18 Mar 05 (12 Jan–17 May 05)	5.9
2005-5	13 Aug 05 (10 Apr–06 Dec 05)	24 Oct 05 (29 July 05–16 Jan 06)	2.6
2005-6	10 Sep 05 (21 Apr 05–08 Feb 06)	13 Jan 06 (09 Nov 05–13 Mar 06)	4.1
2005-8	26 Oct 05 (22 July 05–12 Jan 06)	27 Dec 05 (21 Nov 05–27 Jan 06)	2.0
2005-10	03 Dec 05 (11 May 05–16 July 06)	15 June 06 (18 Mar–16 Sep 06)	6.4
2007-4	05 Sep 07 (04 Apr 07–01 Feb 08)	07 Jan 08 (30 Oct 07–17 Mar 08)	4.0
2007-5	21 Oct 07 (14 Mar 07–07 June 08)	06 May 08 (01 Feb–09 Aug 08)	6.5

[a] Calculated from the estimated date of the initiating tOPV dose to the estimated date of the first diverging node. The P1/capsid K_T evolution rate was assumed to be constant at 1.1%/year (29 nt substitutions/year).

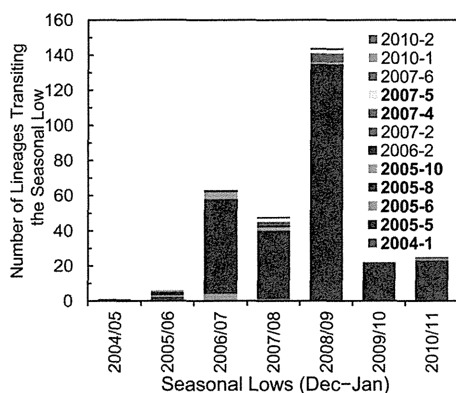

FIG 7 Estimated number of observed lineages that survived the 2004-2005 to 2010-2011 December-to-January seasonal bottlenecks. Color-coding of individual emergences and lineage groups (boldface type) does not correspond to that used in Fig. 1.

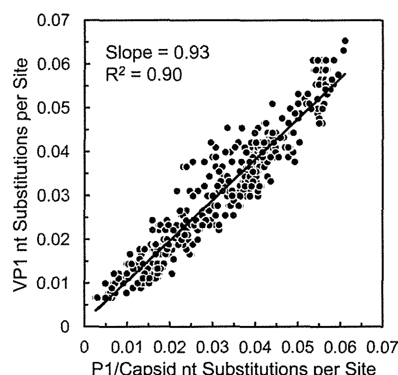

FIG 9 Frequency of nucleotide substitution into the VP1 region relative to the P1/capsid region among the 2005-2011 Nigerian VDPV2 isolates.

ANS07-01 and KDS09-08 did not grow at all (data not shown). The latter results reflect primarily the temperature-sensitive phenotype (see below), which in Sabin 2 is largely determined by A_{481} (91).

(ii) Neurovirulence for PVR-Tg21 transgenic mice. The above-described findings, together with clinical reports and the sequence properties of the Nigerian VDPV2 isolates, suggested that they would be neurovirulent when introduced into the central nervous systems of experimental animals (58, 82). We selected six isolates, representing four independent VDPV2 emergences (lineage groups 2005-6, 2005-8, and 2005-10, and 2006-1) with various degrees of genetic difference from the parental Sabin 2 strain, for neurovirulence testing by intracerebral inoculation of PVR-Tg21 transgenic mice (39) (Table 5). All but one (JIS06-01) of the six VDPV2s tested were isolated from AFP patients reported to have had residual paralysis at 60 days (15). Isolates BOS06-03, BOS07-02, and KTS09-02 were highly neurovirulent (PD_{50} = 2.0 to 2.4), similar to MEF-1 (PD_{50} = 2.5) and unlike Sabin 2 ($PD_{50} \geq$ 7.5) (Table 5). Isolates JIS06-01 and SOS07-01 were slightly less neurovirulent (PD_{50} = 3.3 to 3.8) and nonrecombinant isolate BOS06-02 was much less neurovirulent (PD_{50} = 5.9) than the other VDPV2 isolates but more neurovirulent than Sabin 2. Although the number of isolates compared was small, increased yields in HEK293 cells at 37.0°C correlated with neurovirulence in PVR-Tg21 mice (Table 5). Within lineage groups 2005-6 and 2005-8, neurovirulence appeared to increase with increased divergence from Sabin 2.

(iii) Virus yields at supraoptimal temperatures. The Sabin

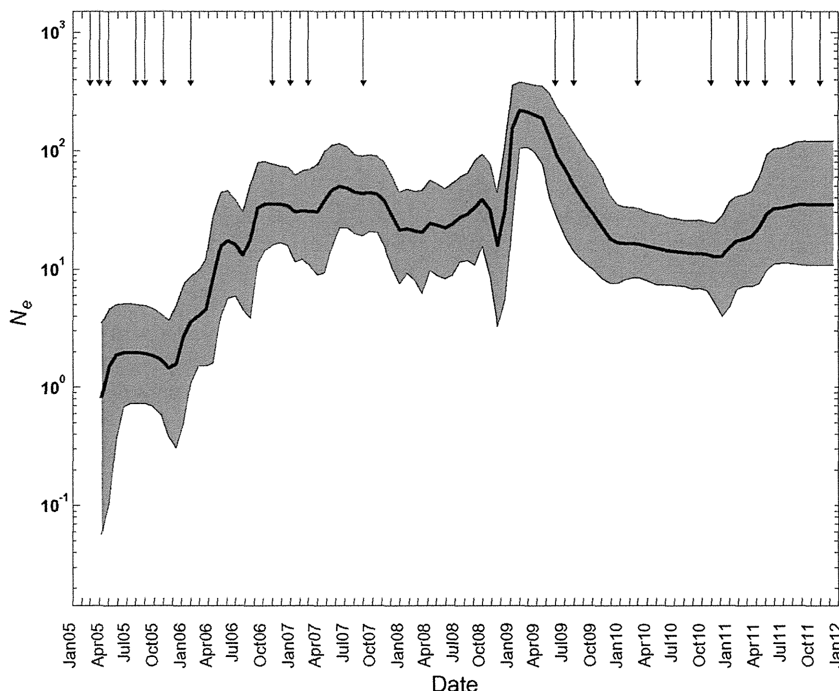

FIG 8 Bayesian skyline plot of population dynamics of all Nigerian VDPV2s, 2005 to 2011. Shaded areas represent the 95% highest posterior density (HPD) interval around the mean of the effective virus population size (N_e) estimates. Arrows indicate months of tOPV SIAs in the northern states.

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TABLE 5 Genetic properties, growth yields, and temperature sensitivity in cultured cells and neurovirulence in PVR-Tg21 mice of selected Nigerian VDPV2 isolates and reference viruses

Lineage group[a]	Virus	5′-UTR class[b]	$VP1_{143}$ residue	No. of P1/capsid region nt substitutions	$3D^{pol}$ class[c]	Virus yield in HEK293 cells (log_{10} PFU/ml) at 37.0°C[d]	Neurovirulence in PVR-Tg21 mice (PD_{50})[e]	Virus yield in RD cells (log_{10} PFU/ml) at 37.0°C[f]	Efficiency of plating in L20B cells at 39.5°C/37.0°C
Sabin 2	Sabin 2	A (Sabin 2)	I		3D:Sabin 2	8.43	≥7.5	8.72	1.4×10^{-4}
2004-1	BAS05-01	A (Sabin 2*)	T	21	3D:4-1/1	9.13	ND	8.63	0.42
2005-1	SOS05-01	A (Sabin 2*)	T	19	3D:5-1/1	9.04	ND	9.12	0.57
2005-2	LAS05-01	A (Sabin 2*)	T	12	3D:Sabin 3	8.97	ND	8.52	0.35
2005-3	KDS05-01	A (Sabin 2*)	N	16	3D:5-3/1	8.50	ND	8.23	0.42
2005-4	KBS06-01	A (Sabin 2*)	T	16	3D:5-4/1	8.96	ND	8.55	0.49
2005-5	JIS06-06	B	T	28	3D:5-5/1	8.88	ND	9.18	0.45
2005-6	BOS06-03	C	T	19[g]	3D:5-6/1	8.93	2.4	9.42	0.47
2005-6	BOS07-02	C	T	46[g]	3D:5-6/1	9.04	2.1	9.21	0.60
2005-7	BOS06-01	A (Sabin 2*)	T	18	3D:Sabin 1	8.95	ND	9.24	0.29
2005-8	JIS06-01	D1 (Sabin 3*)	T	16	3D:5-8/1	8.82	3.3	9.22	0.66
2005-8	KTS09-02	D1 (Sabin 3*)	T	70	3D:5-8/1	9.24	2.0	9.31	0.69
2005-9	JIS06-04	A (Sabin 2*)	T	18	3D:5-9/1	8.83	ND	9.27	0.35
2005-10	SOS07-01	E	T	27	3D:5-10/1	8.89	3.8	9.28	0.35
2005-11	BAS06-01	A (Sabin 2*)	T	25	3D:5-11/1	8.75	ND	8.74	0.13
2006-1	BOS06-02	A (Sabin 2*)	T	7	3D:Sabin 2	8.48	5.9	8.70	1.8×10^{-2}
2006-2	SOS08-09	A (Sabin 2*)	T	50	3D:6-2/1	8.80	ND	8.95	0.11
2007-1	NIS07-01	F	T	13	3D:7-1/1	8.79	ND	8.97	0.15
2007-2	BAS08-06	G	T	40	3D:7-2/1	8.89	ND	9.32	0.33
2007-3	ANS07-01	A (Sabin 2*)	V	8	3D:Sabin 2	8.37	ND	8.55	6.4×10^{-4}
2007-4	KBS08-03	H	T	30	3D:7-4/1	8.81	ND	8.90	0.32
2007-5	BOS09-06	A (Sabin 2*)	T	44	3D:7-5/1	9.02	ND	9.18	0.45
2007-6	KTS09-05	A (Sabin 2*)	T	37	3D:7-6/1	8.83	ND	9.29	0.57
2008-1	KDS09-08	A (Sabin 2*)	T	15	3D:Sabin 1	8.80	ND	8.96	2.5×10^{-4}
2010-1	KBS11-01	A (Sabin 2*)	V	13	3D:Sabin 1	9.01	ND	9.23	0.27
2010-2	NIS11-01	A (Sabin 2*)	T	18	3D:Sabin 2	8.74	ND	9.08	0.24
MEF-1	MEF-1	MEF-1	T		3D:MEF-1	9.16	2.5	9.20	0.92

[a] Well-defined cVDPV2 lineage groups are indicated in boldface type.
[b] 5′-UTR sequences not of Sabin 2 origin are labeled B to H.
[c] $3D^{pol}$ sequences of nonvaccine origin (see Table S1 in the supplemental material for GenBank accession numbers of complete or nearly complete genomic sequences) are numbered consecutively for each emergence (e.g., 3D:5-6/1 to 3D:5-6/3).
[d] Virus was grown in HEK293 cell monolayers (37.0°C), and yields were determined by plaque assays on HeLa cells (37.0°C).
[e] Neurovirulence was tested by intracerebral inoculation. ND, not done.
[f] Virus was grown in RD cell monolayers, and yields were determined by plaque assays on L20B cells. Asterisks indicate that all VDPV2 isolates with 5′-UTR sequences derived from Sabin 2 had a A_{481}→G reversion and that all VDPV2 isolates with 5′-UTR sequences derived from Sabin 3 had a U_{472}→C reversion.
[g] Compared against a simulated unmutated Sabin 3/Sabin 2 recombinant with the recombination junction at the same location as the in natural isolates.

OPV strains produce lower virus yields at supraoptimal temperatures than do WPVs (92). Higher virus yields at elevated temperatures may facilitate person-to-person transmission by increasing the amount of infectious virus excreted by febrile individuals. Sabin 2, all 25 VDPV2 isolates tested, and MEF-1 grew to high titers (8.23 to 9.42 log_{10} PFU/ml) in RD cells incubated at 37.0°C when measured by plaque assays on L20B cells also incubated at 37.0°C (Table 5) (73, 91). However, when cells were incubated at 39.5°C for plaque assays, the efficiency of plating of Sabin 2 on L20B cells dropped ≥6,000-fold, whereas that of MEF-1 was largely unaltered. Two VDPV2 isolates (ANS07-01 and KDS09-08) were nearly as temperature sensitive as Sabin 2, and one isolate (BOS06-02) had an intermediate temperature-sensitive phenotype (Table 5). These three isolates had retained some vaccine-like traits despite reversion of the two determinants thought to contribute most to the attenuated and temperature-sensitive phenotypes. However, these isolates also had among the fewest P1/capsid region nucleotide substitutions (7 to 15), were not vaccine/nonvaccine recombinants, and had no known progeny. The remaining 22 VDPV2 isolates were not temperature sensitive, having efficiencies of plating at 39.5°C that were only 1.3- to 8.4-fold lower than that of MEF-1. Loss of the temperature-sensitive phenotype in these viruses appeared to have occurred early during most VDPV2 emergences, did not always accompany reversion at A_{481} or $VP1_{143}$, and did not strictly correlate with the extent of P1/capsid region divergence from Sabin 2 at substitution levels above ~1%. All isolates with heterologous 5′-UTR and $3D^{pol}$ sequences had lost the temperature-sensitive phenotype, as had one nonrecombinant isolate (NIS11-01).

DISCUSSION

The large, prolonged, and widespread cVDPV2 outbreak in northern Nigeria since 2005 underscores the risks associated with the use of OPV at suboptimal rates of coverage (14, 15, 19, 21, 22, 32). Localization of the cVDPV2 outbreak almost entirely to the northern states follows the pattern of WPV1 and WPV3 transmission in Nigeria since 2005 (7, 8, 93), and the concurrent decline in the incidence of polio cases associated with all three serotypes is

likely the result of improved OPV coverage in northern communities where polio is endemic. The Nigerian cVDPV outbreak is surpassed only by the ~1983-1993 cVDPV2 outbreak in Egypt in duration and is by far the largest in total number of reported VDPV cases in a single outbreak (45). Its final magnitude and duration remain to be determined, as circulation, while apparently sharply reduced, continued into 2012 (7, 19, 22). Although the cVDPV2 outbreak in Egypt was probably of a comparable scale (49), its full extent is unknown because of the absence at that time of sensitive nationwide AFP surveillance (37); in contrast, surveillance in Nigeria was generally strong throughout the outbreak. In both countries, the cVDPVs had reestablished endemic PV2 transmission through successive low-transmission seasons.

Several key risk factors contributed to cVDPV2 emergence and spread in northern Nigeria: (i) the suspension of SIAs in the northern states in 2003 to 2004 (93), (ii) continued weakness in routine immunization with tOPV (7), (iii) insufficient tOPV coverage in previous SIAs (7), and (iv) the shift from tOPV to mOPV1, mOPV3, and bivalent OPV in SIAs conducted after March 2006 (14, 15). The particular emphasis on mOPV1 in SIAs addressed the serious threat posed by the continued circulation of WPV1, the most paralytogenic (45) and transmissible (10–12) of the three WPV serotypes. However, the background of weak routine tOPV immunization permitted a widening gap in immunity to PV2 to develop. These conditions favored concurrent independent cVDPV2 emergences at numerous locations across northern Nigeria, as the threshold of population immunity necessary to limit PV2 spread fell below critical levels (14, 15). In contrast, population immunity to PV2 in southern Nigeria remained sufficiently high to block most (if not all) local cVDPV2 emergences and limit transmission of any cVDPV2 introduced from the north to levels below detection by the AFP surveillance system. The likelihood that the immunity threshold required to block poliovirus transmission is higher in the more densely populated and humid tropical south than in the less populous and semiarid north (15, 45) offers encouraging prospects for the ultimate success of intensified SIAs in the northern states, provided that high rates of OPV coverage are attained (9) and a proper balance between vaccination with bivalent OPV (17, 18) and tOPV (14, 15) is maintained.

The occurrence of a large cVDPV2 outbreak in a setting of comparatively sensitive surveillance (7) offered an unprecedented view of early outbreak events, including detection of multiple independent emergences, first suggested by studies in Madagascar (40, 41). Similar dynamics may typify other cVDPV outbreaks, especially those associated with PV2 (19, 22). However, previous cVDPV outbreaks have usually occurred in settings of both low OPV coverage and weak surveillance, and the outbreaks were detected months or years after the first emergences (37, 38). For example, AFP surveillance was not yet implemented at the start of the cVDPV2 outbreak in Egypt, and the earliest available cVDPV2 outbreak isolates were from cases that occurred about 5 years after the first initiating tOPV dose (because the early outbreak isolates had been discarded). The Egypt cVDPV2 tree also had deep nodes (37), possibly signaling separate emergences, and the atypically low apparent rate of VP1 evolution observed during the Egypt cVDPV2 outbreak (37, 47) may be explained by the occurrence of separate emergences over time.

The cVDPV2 population showed exponential growth during the initial emergence and spread in 2006, stabilization, and then a second phase of exponential growth in early 2009. Four tOPV SIAs in late 2006 and in 2007, despite gaps in coverage (14, 15), may have limited further expansion of the 2005-8 lineage group. However, the absence of tOPV SIAs between 1 September 2007 and 30 May 2009 permitted the accumulation of a population of young children with no immunity to PV2, setting the stage for multiple chains of transmission transiting the 2008-2009 low-transmission season and the burst of cases in early 2009. Implementation of two tOPV SIAs in May and August 2009 was followed by a sharp decline in the number of polio cases, a decrease in the effective population size, and fewer observed lineages transiting the 2009-2010 low-transmission season. Despite the high immunogenicity of Sabin 2 in tOPV (3) and the implementation of seven SIAs using tOPV in 2010 to 2011, circulation of cVDPV2 continued (7, 19, 22) along several separate chains of transmission, indicating that critical numbers of unimmunized children continue to be missed in the tOPV SIAs in the remaining reservoirs of endemicity in northern Nigeria (9).

The observation that one lineage group predominated does not necessarily imply any fundamental difference in the potential transmissibilities among lineage groups. Random events, such as early VDPV introduction into dense populations of nonimmune people or transmission to highly mobile populations, may have shaped the observed patterns of spread. Indeed, viruses of the minor cVDPV2 lineage groups had sustained transmission for an estimated 15 to 35 months, had also diverged into separate chains of transmission within a few months of the initiating tOPV doses, and had recovered robust growth phenotypes when measured experimentally. Moreover, emergences represented by single isolates were estimated to have sustained replication for 3 to 21 months, considerably longer than the 1- to 2-month duration of vaccine virus infections in immunocompetent OPV recipients (30). Although isolates from these emergences would be classified as aVDPV2s, circulation is suspected from the frequency of their appearance in states with low levels of population immunity to PV2 (15) and known gaps in surveillance (7). Indeed, the current outbreak was foreshadowed in 2002 by the isolation of an aVDPV2 from an underimmunized child with AFP in the Plateau state (46). Like most aVDPVs described here, the Plateau isolate resembled cVDPVs, with 2.5% P1/capsid region divergence from Sabin 2 and a mosaic genome with 5′-UTR and $3D^{pol}$ region sequences derived from nonvaccine sources (46).

The genetic mechanisms for reversion of an attenuated OPV strain to a cVDPV phenotypically indistinguishable from WPV are incompletely understood and may differ in their specifics among the three Sabin strains. The Sabin 2 strain, derived from a WPV isolate with intrinsically low neurovirulence and subjected to the shortest selection pathway to attenuation (94), retained more of the biological properties of its WPV parent than did the other Sabin strains, especially the capacity for secondary spread (4–6, 25–27, 95). Because key determinants of the attenuated and temperature-sensitive phenotypes of Sabin 2 are strongly selected against during replication in the human intestine (58, 82), reversion to the WPV phenotype may occur well before the accumulation of 6 to 10 substitutions in the VP1 region. Thus, the definitions for VDPVs are not grounded in the underlying genetics of phenotypic reversion but on the poliovirus molecular clock and the need to balance sensitivity with specificity in global poliovirus surveillance. Accordingly, review of the epidemiologic settings (for cVDPVs) and clinical features (for iVDPVs) is crucial for the evaluation of surveillance data. Some early emergences, including

three with noncapsid sequences derived from OPV strains, appeared not to have recovered the full biological properties of WPVs when characterized in the laboratory, suggesting that changes in noncapsid sequences may be necessary for full phenotypic reversion (75). Nonetheless, all but one (JIS06-01) of the eight VDPV2s tested in PVR-Tg21 mice were isolated from AFP patients reported to have had residual paralysis at 60 days postonset.

The high genetic lability of Sabin 2, coupled with the lower paralytic attack rate for PV2 infections (45), highlights the need to maintain sensitive AFP and poliovirus surveillance worldwide (16). Implementation of the recently developed real-time RT-PCR screening method by the GPLN (16, 19) has resulted in the recent detection of cVDPV2 emergences in the Democratic Republic of Congo, Ethiopia, Somalia, India, Afghanistan, Yemen, Chad, and Pakistan (19, 21, 22, 45). The observation that several of these recent cVDPV2 outbreaks were also associated with multiple independent emergences has prompted a sharp upward reassessment of the risks of cVDPV2 emergence and spread in settings of low population immunity to PV2 (19, 22, 32, 37, 40, 96, 97) and consideration by the WHO of a globally synchronized switch from tOPV to bivalent OPV following control of cVDPV2 outbreaks (19, 98).

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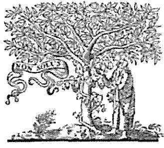

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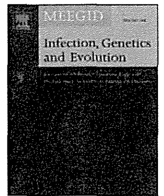

Three clusters of Saffold viruses circulating in children with diarrhea in Japan

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ABSTRACT

Saffold virus (SAFV) is a newly discovered human virus in the genus *Cardiovirus*, family *Picornaviridae*. The virus was first described from fecal specimens of a child with fever of unknown origin in 2007. A total of 454 fecal specimens were collected from children with diarrhea attended clinics in Japan, 2010–2011, 7 (1.5%) were positive for SAFV. Mixed-infections of SAFV and other enteric viruses (rotavirus, norovirus, and bocavirus) were found in four out of seven cases, while monoinfection by SAFV alone was detected in three cases. In addition to diarrhea, fever and vomiting were observed in three children and mild dehydration in one case. No particular symptoms of cough and rhinorrhea were noted. Analysis of partial VP1 nucleotide sequence of 7 Japanese SAFV strains revealed that 5 SAFV sequences were most closely related with SAFV2 reference strains, but separated into SAFV2-A (3 strains) and SAFV2-B (2 strains). In addition, the other two strains were classified as SAFV3. Our results indicated that SAFVs (SAFV2 and SAFV3) were circulated in children with acute gastroenteritis in Japan during 2010 and 2011 epidemic season.
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1. Introduction

Gastroenteritis is one of the leading causes of morbidity and mortality in infants and young children worldwide (Wiegering et al., 2011; Dennehy, 2011). The majority of acute gastroenteritis in children causes by virus infections. Among these, rotavirus, calicivirus (norovirus and sapovirus), enteric adenovirus, and astrovirus have been reported as the most important etiologic viral agents (Dennehy, 2011; Chaimongkol et al., 2012). On the other hand, there are several reports of newly discovered enteric viruses, which are potentially associated with acute gastroenteritis in human, including Saffold virus (SAFV). SAFV is a new species of *Cardiovirus* in the *Picornaviridae* family. The virus was isolated and identified recently from fecal specimens of a child with fever of unknown origin in the US (Jones et al., 2007). Several articles have confirmed recently that SAFVs have also been isolated from fecal and nasal specimens collected from patients worldwide (Abed and Boivin, 2008; Blinkova et al., 2009; Xu et al., 2009; Ren et al., 2009; Itagaki et al., 2010; Tsukagoshi et al., 2010; Dai et al., 2011; Khamrin et al., 2011; Chua et al., 2011). Most recently, the virus was also found to be associated with serious invasive infection of the CSF, myocardium, and blood specimens (Nielsen et al., 2012). On the other hand, several molecular epidemiological studies demonstrated that SAFVs have also been detected in asymptomatic controls (Blinkova et al., 2009; Xu et al., 2009). In many studies, SAFV positive subjects showed high co-infection rates with other viral pathogens (Drexler et al., 2008; Chiu et al., 2008; Xu et al., 2009; Dai et al., 2011; Khamrin et al., 2011). Seroepidemiological studies of SAFV demonstrated that most humans acquire SAFV infections in early childhood, indicating that natural infections occur in human populations (Zoll et al., 2009; Galama et al., 2011). Accordingly, it is not clear whether SAFV associates with the diseases, including gastroenteritis in humans because the clinical significance and the epidemiological data of SAFV are limited.

The genome of SAFV is a positive sense single-stranded RNA of approximately 8000 nucleotides (nt) long. The genome consists of four structural viral proteins (VP4, VP2, VP3, and VP1), and seven nonstructural proteins (2A, 2B, 2C, 3A, 3B, 3C, and 3D) (Jones et al., 2007; Chiu et al., 2008; Zoll et al., 2009; Drexler et al., 2010). SAFV comprises of at least 11 genotypes, of which the two new SAFV10 and SAFV11 sequences have recently been deposited on GenBank database (Naeen and Shimizu, unpublished data; www.picornaviridae.com).

In Japan, epidemiological surveillances of SAFV in patients with respiratory tract infection have initially been detected in the

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specimens collected in 2008 (Tsukagoshi et al., 2011), 1 year after the first report of SAFV in 2007. The SAFVs associated with respiratory tract infection in Japan were responsible for about 2% up to 24% and the virus belonged to SAFV2, 3, and 6 genotypes (Itagaki et al., 2010, 2011; Tsukagoshi, 2010, 2011; Himeda and Ohara, 2012). In addition, one SAFV3 had been found in CSF of a patient with aseptic meningitis in 2008 (Himeda et al., 2011). However, epidemiological surveillance of SAFV in children with acute gastroenteritis in Japan is limited. In order to gain the overview genetic background as well as the clinical significance of SAFV circulating in Japan, we conducted the epidemiological study of SAFV in children with diarrhea in Japan during 2010–2011. Sequence and phylogenetic analyses of SAFVs detected in the present study were further characterized for their genetic evolutionary relationships with SAFVs circulating in this area and SAFV reference strains.

2. Materials and methods

2.1. Specimen collection

A total of 454 stool specimens were collected from children with diarrhea attending several clinics, in Japan. The study period was from January 2010 to June 2011. Only the pediatric patients who had a clinical diagnosis of acute gastroenteritis with watery diarrhea have been included in this study. The ages of the patients ranged from neonate up to 6 years old. The stool specimens were also screened for other diarrheal viruses including group A, B, C rotaviruses, adenovirus, norovirus GI and GII, sapovirus, astrovirus, Aichi virus, human parechovirus, enterovirus, and bocavirus based on the protocols described previously (Yan et al., 2003, 2004; Pham et al., 2010).

2.2. SAFV detection and genotype characterization

The presence of SAFV in fecal specimens was detected by RT-nested PCR which targeted the 5′-untranslated region (UTR) of the genome (Drexler et al., 2008). The SAFVs detected in our study were analyzed further by amplification of the viral protein 1 (VP1) gene (Itagaki et al., 2010) and direct sequencing of the VP1 PCR amplicon was performed using BigDye Terminator Cycle Sequencing Kit (Applied Biosystems, Foster City, CA). The sequences of partial VP1 detected in this study were compared with those of reference strains available in the NCBI GenBank database using BLAST server. A phylogenetic tree based on the partial VP1 genome sequence (348 bp, corresponding to nt position 3257–3604 of UC1 (EU376394) reference strain) was constructed by the neighbor-joining method using MEGA (v5.05) software (Tamura et al., 2011). The trees were statistically supported by boot-strapping with 500 replicates. Distances were computed using the maximum composite likelihood method and are in units of numbers of base substitutions per site. All positions containing gaps and missing data were eliminated from the dataset (Complete deletion option).

2.3. Nucleotide sequence accession numbers

The partial nucleotide sequences of Saffold virus VP1 genes described in the present study have been deposited in the GenBank database under the accession numbers JX169782–JX169788. The following SAFV VP1 sequences of reference strains obtained from the GenBank database were used in the phylogenetic analysis; SAFV1:HQ668170, HQ668173, FJ586240, EF165067, GU943516, FJ464766, FJ464777; SAFV2:AB545780, FN999911, EU681176, JN652233, JN652232, JF693617, EU681177, JF693612, EU604749, EU604747, EU604748, EU604750, FJ463601, AB54 5786, HQ668171, HQ668172, FJ374267, FR682076, GU943518, GU943517; SAFV3:FJ997531, FJ463605, EU604745, EU604746, EU681179, GU943513, AB542807, AB542806, HQ902242; SAFV4:FJ 463600, FJ463603, FJ463606; SAFV5:FJ463615, FJ463616; SAFV6:FJ 463617; SAFV7:FJ463602; SAFV8:FJ463604; SAFV9:FJ997532.

3. Results

From a total of 454 fecal specimens collected from children with diarrhea in Japan, 7 (1.5%) were positive for SAFV by RT-nested PCR screening method. The positive samples were detected sporadically along the study period in March, April, May, July–October. It was interesting to observe that among 7 SAFV positive samples, double- or triple-infections together with other enteric viruses were found in 4 (57%) pediatric patients, while monoinfection with SAFV alone was detected in 3 (43%) cases. Mixed infections of SAFV with rotavirus, norovirus, and bocavirus were observed in this population (Table 1).

Medical records of all SAFV positive patients were reviewed and clinical characteristics are shown in Table 1. Of these seven children with SAFV positive, the male to female ratio was 3:4. The age ranged from 1 to 6 years, of which SAFV infections were found frequently in children with the age of younger than 2 years. Diarrhea was observed in all patients and the number of diarrheal episodes per day was less than 4 times. Diarrhea in these patients lasted for 3 to 7 days. In seven patients who were positive for SAFV, fever and vomiting were observed in three children and mild dehydration in one case. No particular symptoms of cough and rhinorrhea were noted.

The SAFVs detected in our study were analyzed further by amplification of the partial VP1 region. All PCR products of the partial VP1 were sequenced and compared with those of 9 established SAFV reference genotypes (SAFV1–9). The phylogenetic analysis of the partial VP1 nucleotide sequences (348 nt long) of all seven SAFV strains shown in Fig.1 indicated that SAFVs detected in this study were divided into two genotypes, SAFV2 and SAFV3. Interestingly, phylogenetic evaluation among SAFV2 strains included in this analysis revealed the existence of at least two major lineages, tentatively proposed as SAFV2-A and SAFV2-B. Three strains of SAFV2 (9964/2010, 9957/2010, and 0169/2010) formed exclusively within SAFV2-A lineage. The VP1 sequences of 9964/2010 and 9957/2010 strains were more closely related with each other but less closely related to that of 0169/2010 strain. All these SAFVs showed the close genetic background with the SAFV2-A Yamagata.JPN/2069.09, Nijmegen2008, D/VI2229/2004, S19, S14, and 09T9707DK strains over 84%, while the nucleotide sequence identities with that of SAFV2-B strains were lower than 81%. In addition, it was interesting to observed that the other two SAFV2 strains (9992/2010 and 0032/2010) formed their own cluster with the SAFV Yamagata.JPN/2474.09 within SAFV2-B lineage and exhibited the nucleotide sequence identity ranging from 90.2% to 95.8%, but the nucleotide sequence identities with those of SAFV2-A strains were less than 81%. Additionally, four amino acid differences were observed among partial VP1 sequences of SAFV2-A and SAFV2-B strains found in this study (data not shown).

As shown in Fig. 1, the phylogenetic analysis revealed that the other two SAFV strains (9913/2010 and 0312/2011) detected in this study clustered with the SAFV3 reference strains. These SAFV 9913/2010 and 0312/2011 strains exhibited high level of nucleotide sequence identity greater than 99% with each other and were also closely related to the SAFV3 strains isolated previously in Japan (07-Aichi10247 and JPN08-404) with the nucleotide sequence identity ranging from 99.1% to 99.8%, but exhibited lesser nucleotide sequence identity (less than 73%) with SAFV1–2, and SAFV4–9 genotypes (data not shown).

Table 1
Clinical characteristics of SAFV-positive patients in Japan.

Lab ID	Collection date	Gender	Age (Year)	No. of diarrhea/ 24 h (time)	Duration of diarrhea (day)	Fever[a]	Vomiting (time)	Cough, rhinorrhea	Dehydration	Co-infect virus	SAFV genotype	Remark[c]
9913	10/3/2010	M	6	Unknown	Unknown	No	No	No	No	-	SAFV3	1
9957	20/7/2010	F	3	Unknown	Unknown	Yes	5–6	No	No	-	SAFV2	1
9964	30/8/2010	F	1	3	6	No	No	No	No	-	SAFV2	2
9992	10/5/2010	F	1	1–3	6	Yes	1	No	Mild[b]	Rotavirus, Bocavirus	SAFV2	3
0032	14/10/2010	M	2	Unknown	3	No	No	No	No	Bocavirus	SAFV2	1
0169	8/9/2010	M	2	2–3	7	No	No	No	No	Norovirus	SAFV2	1
0312	28/4/2011	F	1	2–3	Unknown	Yes	2	No	No	Norovirus, Bocavirus	SAFV3	1

[a] Fever refers to body temperature elevation over 37.8 °C.
[b] Body weight lose is less than 5%.
[c] 1. Patient visited clinic one time. 2. Patient visited clinic more than one time. 3. Patient visited clinic and got intravenous transfusion. 4. Patient was transferred to hospital.

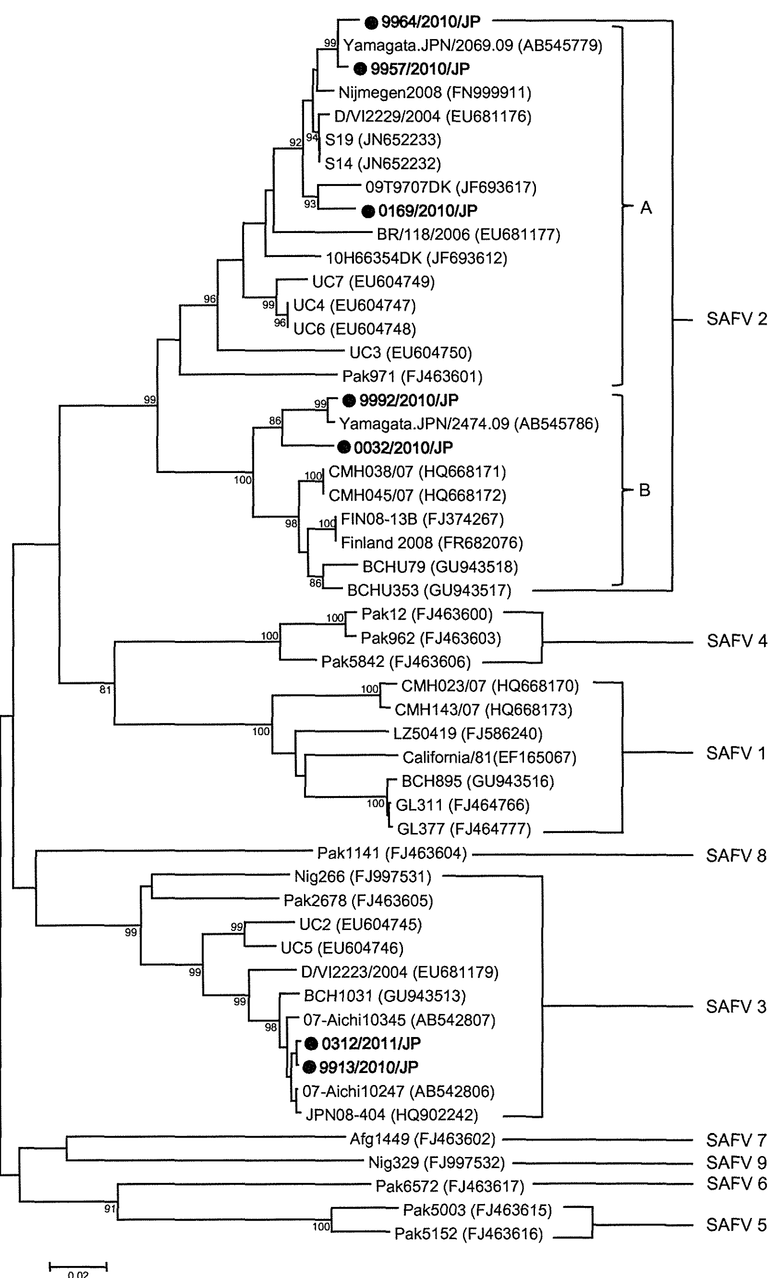

Fig. 1. Construction of the phylogenetic analysis of the partial nucleotide sequence encoding the VP1 region of SAFVs (348 bp). The tree was generated using the neighbor-joining algorithm, in MEGA v5.05. Scale bar indicates nucleotide substitutions per site and bootstrap values (>80) are indicated for the corresponding nodes. The SAFV strains detected in the present study are presented in boldface with black dot symbol.

4. Discussion

During the past few years, several new viruses related to diarrheal diseases have been discovered in human stool samples, mostly by viral metagenomic strategy and conventional virological methods (Finkbeiner et al., 2008). Since new viruses related to diarrheal diseases are discovered continuously, there is a need to constantly monitor the prevalence of these viruses in community for clarification of their genetic evolutionary and clinical significance. SAFV is one of a newly identified human *Cardiovirus* that was first reported in 2007 (Jones et al., 2007). Molecular surveillance of SAFV in Japan, 2010–2011 in the present study demonstrated that only 7 (1.5%) cases of SAFV were detected from a total of 454 specimens tested. This result indicates that SAFV infection in diarrheal pediatric patients is uncommon and sporadically identified along the study period. Previous molecular surveillance of SAFV in nasopharyngeal specimens and CSF found that SAFV2, 3, and 6 were circulated in patients in Japan (Itagaki et al., 2010, 2011; Tsukagoshi, 2010, 2011; Himeda and Ohara, 2012). Our data of SAFV genotypic characterization demonstrated that SAFVs detected in the present study are genetically diversified and the genotypes reported are SAFV2 and SAFV3 which is in good agreement with the previous reports of SAFV genotypes circulated in human populations in this country (Itagaki et al., 2010, 2011; Tsukagoshi, 2010, 2011; Himeda and Ohara, 2012).

About 60% of the pediatric patients in our study were co-infected with other viral agents. This finding raises concern about an etiologic role of SAFV in gastroenteritis. In particular, a severe diarrheal disease with dehydration was observed in one patient, which had been infected with 3 viruses including rotavirus, bocavirus, and SAFV. In addition to acute gastroenteritis disease, 40% of the children presented the clinical symptoms with vomiting and fever. A preferential infection of SAFV was observed in the younger age group, which showed the similar trend with those other common acute gastroenteritis virus infections (Malasao et al., 2008; Kittigul et al., 2009; Chan-it et al., 2011). Along with the clinical analysis of SAFV, determination for clinical index of other viruses (rotavirus, norovirus, and bocavirus) may provide a better understanding of the viruses in relation to clinical symptoms. In this study, unfortunately, analysis and comparison of the clinical significances of SAFV to other viruses could not be performed due to the lack of availability of clinical data of these viruses. In order to clarify this point, further studies need to be performed. Since the detection rate of SAFV in stool specimens is relatively low and the study was not performed on healthy individuals, the relationship of this viral agent with diarrhea in human is still unclear. Although SAFV may not act as the major causative agents of diarrhea, the questions whether or not this virus might be the co-factor for other viral agents, or may require the presences of other helper viruses to establish diseases in human remain unclear. Therefore, prospective studies encompassing larger populations of patients and healthy individuals for longer periods are required. Serological testing to diagnose the primary infection, the detection of SAFV viremia, or the detection of high viral loads by quantitative RT-PCR may be the more useful tools for elucidation of SAFV-associated with the clinical disease than only a qualitative detection of SAFV RNA in fecal samples.

Sequence analysis of SAFV strains revealed that 11 genotypes of SAFVs (SAFV1–11) have been documented (www.picornaviridae.com). The accumulated epidemiological studies of SAFV reveal the detection of the virus both in respiratory secretion and stool samples imply that SAFV1–3 are more common worldwide, whereas SAFV4–11 are occasionally identified (Blinkova et al., 2009). The SAFV2 detected worldwide is itself genetically diverse, as 2 major lineages are tentatively purposed (SAFV2-A and SAFV2-B). It is interesting to point out that although SAFV2 are detected as the most predominant genotype (5 strains) in this study, 3 are clustered with SAFV2-A, while the other two are closely related to those of SAFV2-B. The data indicate that SAFV2 strains circulating in Japan seem to originate from different evolutionary ancestor. Nevertheless, full-genome sequence analysis of the SAFV2 strains may need to be conducted to elaborate their genetic backgrounds of genome diversity and recombination.

In summary, this study presents the clinical significance and genetic background information of SAFV circulating in pediatric patients with acute gastroenteritis in Japan, and reveals a genetic diversity of SAFVs circulated in this area. Therefore, the continue comprehensive screening as well as genetic molecular characterization of other genes among SAFV strains circulating in this area need to be further performed in order to monitor the incidence, genomic recombination, and genetic diversity, as well as its clinical significance in patients with diarrhea.

Acknowledgment

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Saffold Virus Type 3 (SAFV-3) Persists in HeLa Cells

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Abstract

Saffold virus (SAFV) was identified as a human cardiovirus in 2007. Although several epidemiological studies have been reported, they have failed to provide a clear picture of the relationship between SAFV and human diseases. SAFV genotype 3 has been isolated from the cerebrospinal fluid specimen of patient with aseptic meningitis. This finding is of interest since Theiler's murine encephalomyelitis virus (TMEV), which is the closely related virus, is known to cause a multiple sclerosis-like syndrome in mice. TMEV persistently infects in mouse macrophage cells *in vivo* and *in vitro*, and the viral persistence is essential in TMEV-induced demyelinating disease. The precise mechanism(s) of SAFV infection still remain unclear. In order to clarify the SAFV pathogenicity, in the present study, we studied the possibilities of the *in vitro* persistent infection of SAFV. The two distinct phenotypes of HeLa cells, HeLa-N and HeLa-R, were identified. In these cells, the type of SAFV-3 infection was clearly different. HeLa-N cells were lyticly infected with SAFV-3 and the host suitable for the efficient growth. On the other hand, HeLa-R cells were persistently infected with SAFV-3. In addition, the SAFV persistence in HeLa-R cells is independent of type I IFN response of host cells although the TMEV persistence in mouse macrophage cells depends on the response. Furthermore, it was suggested that SAFV persistence may be influenced by the expression of receptor(s) for SAFV infection on the host cells. The present findings on SAFV persistence will provide the important information to encourage the research of SAFV pathogenicity.

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Introduction

Saffold virus (SAFV) was identified from an infant with a fever of unknown origin in 2007 [1]. In the aid of phylogenetic analysis, SAFV was classified with Theiler-like rat virus, Theiler's murine encephalomyelitis virus (TMEV) and Vilyuisk human encephalomyelitis virus into the species *Theilovirus* which belongs to the genus *Cardiovirus* of the family *Picornaviridae*. Eleven genotypes of SAFV have now been identified [1–6]. SAFV was isolated from nasal and stool specimens from infants presenting with respiratory or gastrointestinal symptoms. Furthermore, the virus was isolated from the cerebrospinal fluid (CSF) specimen of patient with aseptic meningitis. Although several epidemiological studies have been reported, they have failed to provide a clear picture of the relationship between SAFV and human diseases [7]. Recently, SAFV-2 was detected by RT-PCR in 2 children: in the CSF and a fecal sample from one child with monosymptomatic ataxia caused by cerebellitis; and in the CSF, blood, and myocardium of another child who died suddenly with no history of illness [8]. Moreover, animal experiments have been carried out and two different groups reported that SAFV is neurotropic in mice [9,10]. These findings are of interest since TMEV, which is the closely related virus, is known to cause a multiple sclerosis-like syndrome in mice. TMEV persistently infects in macrophage cells *in vivo* and *in vitro*, and the viral persistence is essential in TMEV-induced demyelinating disease [11]. Therefore, the potential persistence of SAFV is an important issue. However, the precise mechanism(s) of SAFV infection still remain unclear.

It was reported that HeLa cells were most suitable cell line for the efficient growth of SAFV-3 [5]. However, we noticed that the growth of SAFV-3 in HeLa cells distinctly depends on the maintenance condition of the cells before infection. In the present study, the cell culture condition for the efficient growth of SAFV-3 and the potential persistence of SAFV-3 were studied by using two HeLa cell lines which are derived from two different laboratories.

Materials and Methods

Cells and Viruses

Two different HeLa cells were obtained from RIKEN (HeLa, RCB0007) and from Dr. Nishikawa (laboratory stock of Department of Biochemistry, Kanazawa Medical University School of Medicine) [12]. To avoid the confusion, the cells supplied from RIKEN were designated "HeLa-R" and the cells supplied from Dr. Nishikawa were designated "HeLa-N". HeLa-R was maintained in Eagle's minimum essential medium (MEM) (Nissui) supplemented with 10% newborn calf serum (CS) (Invitrogen) and 0.03% L-glutamine according to RIKEN's recommendation. On the other hand, HeLa-N was maintained in Dulbecco's modified Eagle's medium (DMEM, SIGMA) supplemented with 0.03% L-

glutamine and 10% fetal calf serum (FCS) containing 50 U/ml of penicillin and 50 μg/ml of streptomycin [12]. Additionally, in order to investigate the efficient maintenance condition for the virus growth, both cells were maintained for 2 weeks in several combinations of the above serum and medium. The detailed culture conditions for the corresponding cells are shown in Fig. S1. The appearance of cytopathic effect (CPE) was observed up to 48 hours post-infection (p.i.) and the CPE severity was graded from + to ++++ (+: <10% CPE, ++: 10~50% CPE, +++: 50~80% CPE, ++++: >80% CPE). BHK-21 cells were obtained from Cell Resource Centerfor Biomedical Research, Institute of Development, Aging and Cancer Tohoku University. BHK-21 cells were maintained in MEM supplemented with 5% CS and 0.03% L-glutamine.

The virus was prepared from infectious cDNA clone derived from JPN08-404 strain of SAFV-3, pSAF404 [13]. Briefly, pSAF404 were linearized with *Not* I, and RNA transcripts were synthesized with *CUGA* 7 RNA polymerase (Nippon gene). Then, HeLa-N cells were transfected with the transcripts derived from pSAF404 using Lipofectin (Invitrogen) according to the manufacturer's instructions. The cultured cells and supernatants were collected after 48 hours, and the virus was prepared by three freezing/thawing cycles to release virions. Furthermore, the virus was propagated by two passages on HeLa-N cells. The virus titers were determined by a standard plaque assay on HeLa-N cells. The seed virus of DA strain of TMEV was propagated in BHK-21 cells. The culture cells and supernatants were collected after complete CPE was observed, and virus lysates were prepared by three freezing/thawing cycles to release virions. The virus titers were determined by a standard plaque assay on BHK-21 cells.

Kinetics of Virus Growth in Cells

The kinetics of virus growth in HeLa-N and HeLa-R cells was analyzed. The cells were seeded at a density of 5×10^5 cells in 35-mm dishes. After 24 h, the cells were infected with virus at a multiplicity of infection (MOI) of 10 plaque forming unit (pfu) per cell. After virus adsorption at 37°C for 60 min, the cells were washed twice with Dulbecco's phosphate buffered saline (PBS), and incubated at 37°C in each medium with 1% serum. The cells and supernatants were collected at 0, 3, 6, 12, 24 and 48 h after infection and the viruses were prepared by three freezing/thawing cycles from the cells. SAFV-3 and DA viruses were titrated by a standard plaque assay on HeLa-N and BHK-21 cells, respectively.

Analysis of Short Tandem Repeat (STR) for Identification of Two Different HeLa Cells

In order to investigate whether HeLa-N and HeLa-R cells are genomically identical, the STR on genome was analyzed [14]. Analysis of STR was outsourced by BEX co. ltd. (Tokyo, Japan) using Cell ID System (Promega).

Neutralization Test

In order to generate an anti-SAFV-3 antibody for control, rabbits were immunized with SAFV-3 (JPN08-404) propagated in LLC-MK2 cells in TiterMax Gold (TiterMax USA) a few times at 1-week intervals, followed by two booster injections 1 month after the last immunization.

The titer of the challenge virus was determined on HeLa-N cells before the neutralization test was carried out. Two-fold dilutions of CS, FCS and anti-SAFV-3 antiserum were prepared by serum-free DMEM. Each sample serum (100 μl) was incubated with the challenge virus (100 TCID50/100 μl) at room temperature for 60 min. The virus-serum mixtures were inoculated into 96 well-plate containing HeLa-N cells. The cells were observed for CPE daily for 4–5 days.

Establishment of HeLa-R Cells Persistently Infected with SAFV-3

HeLa-R cells (maintained with CS) and HeLa-N cells (maintained with FSC or CS) were seeded at a density of 5.0×10^6 cells in a T75 flask. After 24 hours, the cells were infected with SAFV-3 at an MOI of 10 pfu per cell. After virus adsorption at 37°C for 60 min, the cells were washed twice with PBS, and incubated at 37°C in fresh medium with 1% serum. After 72 hours, surviving cells were washed twice by PBS and continuously cultured in fresh medium with 10% serum.

Western Blotting

The HeLa-R, PSAF/HeLa-R (5 passages, 30 days p.i.) and HeLa-N infected with SAFV-3 (18 hours p.i.) cells were lysed in sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis (PAGE) sample buffer (10 mM Tris-HCl (pH8.0), 1 mM EDTA (pH8.0), 2.5% SDS, 5% 2-Mercaptoethanol, 10% glycerol and 0.005% bromophenol blue). Samples were separated by SDS-15% PAGE, and transferred onto a polyvinylidene difluoride membrane (Millipore). The membrane was blocked with 5% skim milk in PBS-T (PBS containing 0.05% Tween 20) for 60 min and incubated for 60 min with rabbit anti-SAFV-3 antiserum (1:5,000) followed by incubation with horseradish peroxidase-conjugated anti-rabbit IgG (Bio-Rad Laboratories) for 60 min. Signals were detected using ECL plus Western blotting detection reagents (GE Healthcare) according to the manufacturer's instructions.

Determination of the Titer of the Virus Produced from PSAF/HeLa Cells

PSAF/HeLa-R cells (7 passages, 42 days p.i.) were seeded at a density of 1×10^6 cells in 35-mm dishes. Next day, the cells were washed twice with PBS and then incubated in fresh MEM with 10% CS. After 24 hours, the supernatants or the mixtures of the supernatants and cells were harvested. The viruses were prepared by three freezing/thawing cycles. The titers were determined by a standard plaque assay on HeLa-N cells.

Treatment with Anti IFN-α and IFN-β Antibody

PSAF/HeLa-R cells (9 passages, 50 days p.i.) were seeded at a density of 50% confluent in 35 mm dishes. Cells were incubated in MEM supplemented with 10% CS containing mouse monoclonal anti-human IFN-α antibody (1 μg/ml, PBL Biomedical Laboratories) or rabbit polyclonal anti-human IFN-β antibody (120 U/ml, PBL Biomedical Laboratories). The medium was changed every 2 days.

Immunofluorescene Staining

In order to use the anti-SAFV-3 antiserum for immunocytochemistry, 500 μl of the anti-SAFV-3 antiserum was absorbed at 4°C for 24 hours by the homogenate of 1.2×10^8 cells of HeLa-R cells. After centrifugation, the supernatant was used for the immunocytochemistry.

PSAF/HeLa-R cells (13 passages, 62 days p.i.) cultured with CS and PSAF/HeLa-R cells (9 passages, 62 days p.i.) cultured with FCS for 12 days were seeded onto cover glasses coated by poly-L-lysine. After 24 hours, cells were washed with PBS and then fixed in 10% formalin for 20 min at 4°C. After three washes with PBS, cells were permeabilized with 0.25% triton X-100 in PBS for 20 min at room temperature and blocked with 5% bovine serum

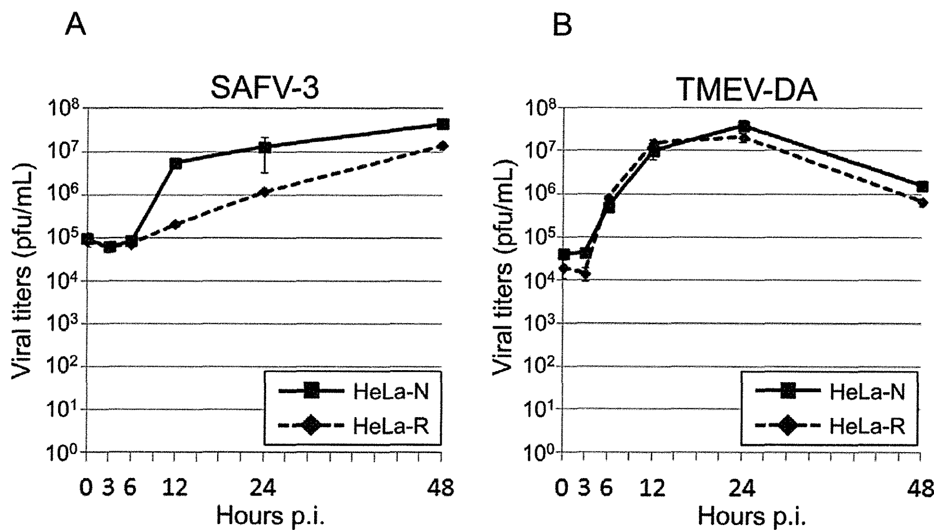

Figure 1. Growth kinetics of SAFV-3 and TMEV-DA on HeLa-N and HeLa-R cells. A: Growth kinetics of SAFV-3. Solid and broken lines indicate the growth curves of SAFV-3 on HeLa-N and HeLa-R cells, respectively. The viruses (as a mixture of cell-free and cell-associated viruses) were harvested at several time points indicated and assayed for titers by a standard plaque assay on HeLa-N cells. Titers shown are the means ± S.D. in three independent experiments. **B: Growth kinetics of TMEV-DA.** Solid and broken lines indicate the growth curves of TMEV-DA on HeLa-N and HeLa-R cells, respectively. The viruses (as a mixture of cell-free and cell-associated viruses) were harvested at several time points indicated and assayed for titers by a standard plaque assay on BHK-21 cells. Titers shown are the means ± S.D. in three independent experiments.
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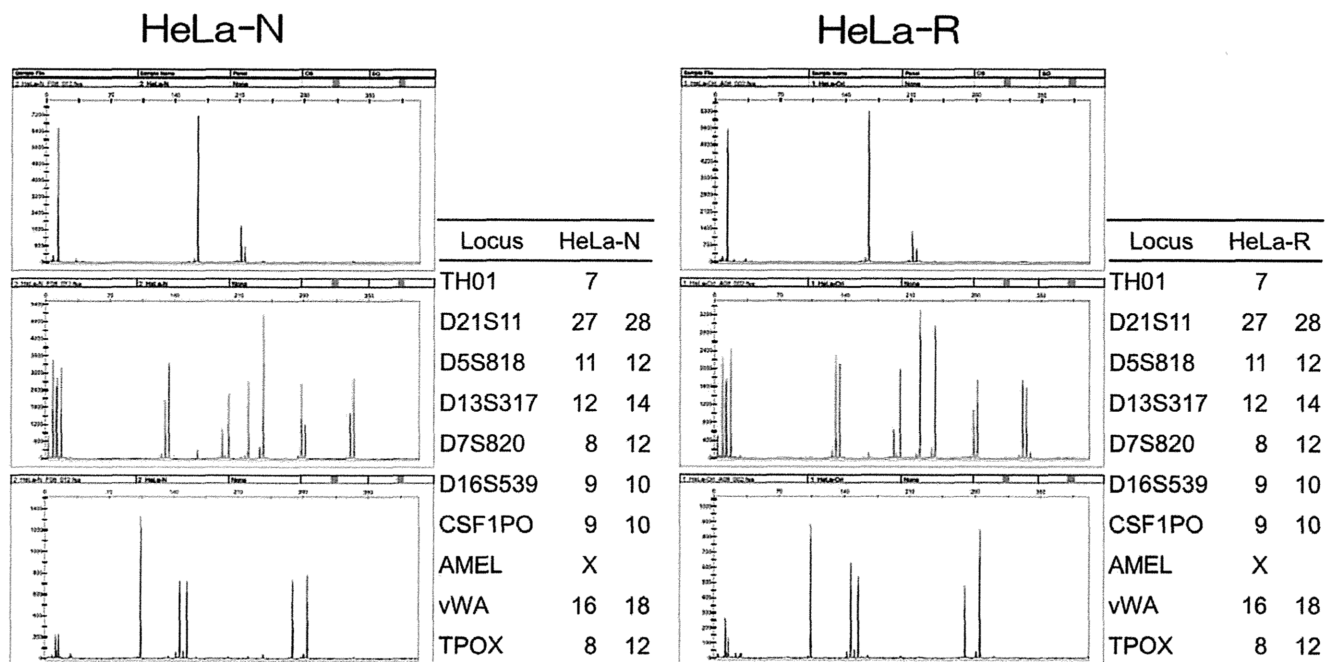
albumin (BSA) in PBS for 60 min at room temperature. Cells were incubated with the anti-SAFV-3 antiserum in PBS supplemented with 5% BSA for 60 min at room temperature. After five washes with PBS, cells were incubated with Alexa Fluor 594-conjugated anti-rabbit IgG (Molecular Probes) for 60 min at room temperature. Photomicrographs were obtained at room temperature with a microscope equipped with a digital camera (Axiovision, Carl Zeiss).

Virus Binding Assay

HeLa-N and HeLa-R cells were seeded onto cover glasses. After 24 hours, cells were washed with PBS and then fixed in 10%



Locus	HeLa-N	
TH01	7	
D21S11	27	28
D5S818	11	12
D13S317	12	14
D7S820	8	12
D16S539	9	10
CSF1PO	9	10
AMEL	X	
vWA	16	18
TPOX	8	12

Locus	HeLa-R	
TH01	7	
D21S11	27	28
D5S818	11	12
D13S317	12	14
D7S820	8	12
D16S539	9	10
CSF1PO	9	10
AMEL	X	
vWA	16	18
TPOX	8	12

Figure 2. The STR analysis to confirm the identity of HeLa cells. Left and right panels show the data on HeLa-N and HeLa-R cells, respectively. Graphs were images generated by GeneMapper ver. 3.5 (Applied Biosystems). The allele data were presented as a table on the right side of each image. Locus indicates the name of gene analyzed. It is indicated that the gene presented by one datum was homo and the gene presented by two data was hetero.
doi:10.1371/journal.pone.0053194.g002

formalin for 20 min at 4°C. After three washes with PBS, cells were blocked with 5% skim milk in PBS-T (PBS containing 0.05% Tween 20) for 60 min at room temperature without the permeabilization. Cells were incubated with SAFV-3 at an MOI of 100 pfu per cell for 60 min at 4°C. After five washes with PBS-T, cells were incubated with the anti-SAFV-3 antiserum in PBS-T supplemented with 5% skim milk for 60 min at room temperature. After five washes with PBS-T, cells reacted with virus and antiserum were detected with Alexa Fluor 594-conjugated anti-rabbit IgG as described in the section of "Immunofluorescene staining".

Viral Titration after the Transfection of SAFV Recombinant Transcripts

HeLa-N and HeLa-R cells were seeded at a density of 5×10^5 cells in 35-mm dishes. After 24 h, the cells were transfected with the SAFV recombinant transcripts (5 μg) derived from pSAF404 using DMRIE-C (Invitrogen) according to the manufacturer's instructions. The cells and supernatants were collected at 16 h after transfection and the viruses were prepared by three freezing/thawing cycles from the cells. Viruses were titrated by a standard plaque assay on HeLa-N cells.

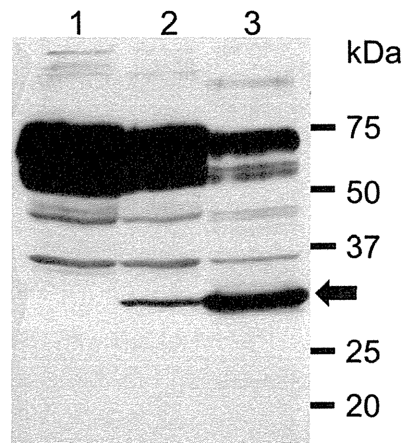


Figure 3. The detection of virus antigen in PSAF/HeLa-R cells by Western blotting. The anti-SAFV-3 antiserum detected the viral antigen of about 28~30 kDa (arrow) in the lysates of PSAF/HeLa-R cells cultured for 30 days (5 passages) (lane 2) and HeLa-N cells infected with SAFV-3 (18 hours p.i.) used for a positive control (lane 3). The band of viral antigen was not detected in the lysate of HeLa-R cells used for a negative control (lane 1).
doi:10.1371/journal.pone.0053194.g003

Results

Growth Kinetics of SAFV-3 on Two Different HeLa Cell Lines

At first, the growth kinetics of SAFV-3 on two HeLa cell lines, which are derived from different laboratories, was analyzed. The titer of SAFV-3 produced from HeLa-N increased sharply at 12 hours p.i. (5.6×10^6 pfu/ml) and gradually increased until 48 hours p.i. (4.4×10^7 pfu/ml) (Fig. 1A, solid line). The virus produced from HeLa-R increased slowly at 12 hours p.i. (2.1×10^5 pfu/ml) and gradually increased until 48 hours p.i. (1.4×10^7 pfu/ml) (Fig. 1A, broken line). The virus growth on two cell lines was clearly different each other. At 12 hours p.i., the virus titer on HeLa-N was 1 log higher than that on HeLa-R. On the other hand, the titers of TMEV-DA on HeLa-N and HeLa-R cells were almost similar. Those peaked at 24 hours p.i. (4.0×10^7 pfu/ml and 2.2×10^7 pfu/ml, respectively) and decreased gradually (Fig. 1B). In addition, the plaques were not formed on HeLa-R cells by SAFV-3 infection (data not shown).

Identification of Two Different HeLa Cell Lines by the STR Analysis

The phenotypes of HeLa cells are varied in a number of laboratories. Furthermore, interspecies cross-contamination has been reported with HeLa cells [14]. Therefore, we performed the STR analysis to identify the two cell lines, HeLa-N and HeLa-R. Fig. 2 indicates that the STRs of HeLa-N and HeLa-R are identical, indicating that those are derived from the same origin, HeLa cell line (ATCC CCL2).

Analysis of CPE on HeLa Cells Infected with SAFV-3 in Different Culture Conditions

Though it was demonstrated that HeLa-N and HeLa-R cells are identical genomically based on analysis of STR, the recommended maintenance conditions were different. Therefore, we next examined the effects of culture conditions on the growth of SAFV-3. HeLa-N and HeLa-R cells maintained with several conditions were infected with SAFV-3 at an MOI of 10 pfu per cell. The severity of CPE presented on each cell maintained with each condition was significantly different (Table.1). CPE on HeLa-N cells maintained in 10% FCS was the severest and that on HeLa-R cells maintained in 10% CS was the mildest. CPE on HeLa-N or HeLa-R cells maintained in 10% FCS was severer than that on HeLa-N or HeLa-R cells maintained in 10% CS. Interestingly, CPE on HeLa-N cells was severer than that on HeLa-R in the same conditions. No difference was observed between DMEM and MEM.

Analysis of Anti-SAFV-3 Antibody and Other Inhibitors in CS

As shown in Table 1, whichever cells (HeLa-N or HeLa-R) are used, the virus growth (or the appearance of CPE) is suppressed in

Table 1. CPE presented by SAFV-3 infection on HeLa cells which were maintained in several culture conditions.

	HeLa-N				HeLa-R			
	FCS		CS		FCS		CS	
Hours p.i.	DMEM	MEM	DMEM	MEM	DMEM	MEM	DMEM	MEM
15 h	+++	+++	++	++	++	++	+	+
48 h	++++	++++	++++	++++	+++	+++	++	++

+: <10% CPE, ++: 10~50% CPE, +++: 50~80% CPE, ++++: >80% CPE.
doi:10.1371/journal.pone.0053194.t001

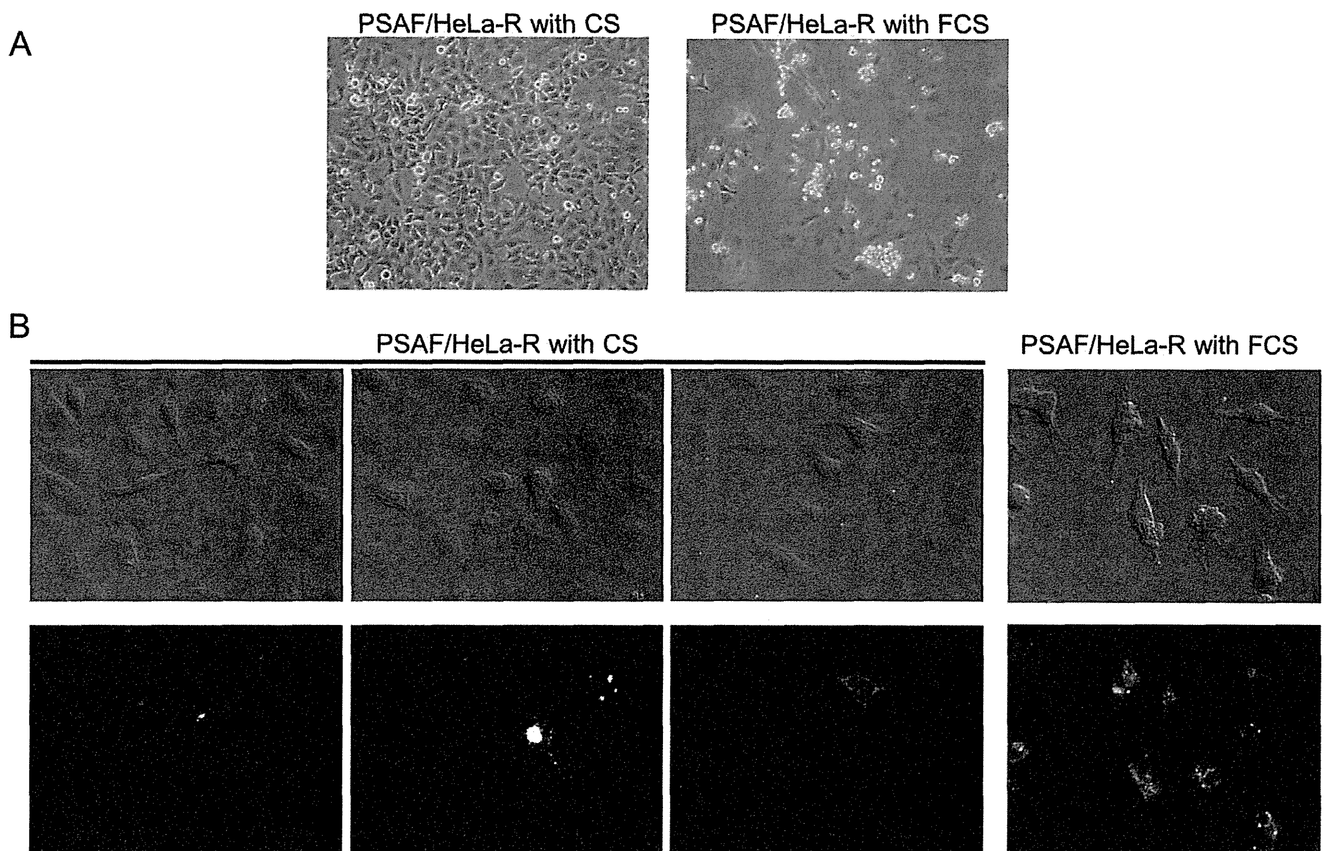


Figure 4. Effects of culture with FCS on PSAF/HeLa-R cells. A: Representative microphotographs of the CPE on PSAF/HeLa-R cells Left panel shows the PSAF/HeLa-R cells maintained in MEM with 10% CS (62 days p.i.). Right panel shows the PSAF/HeLa-R cells maintained in DMEM with 10% FCS for 12 days (from 50 days to 62 days p.i.). CPE on the PSAF/HeLa-R cells significantly increased by the culture with FCS for 12 days. Magnification: ×100. **B: Representative microphotographs of the immunocytochemistry** Left 6 panels show the PSAF/HeLa-R cells maintained in MEM with 10% CS. Right 2 panels show the PSAF/HeLa-R cells maintained in DMEM with 10% FCS. Upper and lower panels show Nomarski and fluorescent images, respectively. Virus antigen was detected with anti-SAFV-3 antiserum pre-absorbed by the homogenates of HeLa-R cells and Alexa Fluor 594-conjugated anti-rabbit IgG antibody. Viral antigen positive cells were shown in a part of PSAF/HeLa-R cells cultured with CS. After cultivation with FCS for 12 days, however, viral antigen positive cells clearly increased. Magnification: x400.
doi:10.1371/journal.pone.0053194.g004

the culture of CS. Therefore, in order to investigate whether the antibody and/or the inhibitor(s) against SAFV are contained in CS, the neutralization test was performed. Even the 2-fold dilutions of FCS and CS did not inhibit the appearance of CPE on HeLa-N maintained with FCS which is the most susceptible to SAFV-3 infection, though the anti-SAFV-3 antiserum inhibits the appearance of CPE at 3,072-fold dilution. These results indicate that the antibody and/or inhibitor(s) against SAFV are not contained in CS.

Establishment of HeLa-R Cells Persistently Infected with SAFV-3

Since the CPE caused by SAFV-3 infection on HeLa-R cells maintained with CS was extremely mild, we attempted to establish the cells persistently infected with SAFV-3. At 72 hours p.i., surviving cells were harvested and sub-cultured in fresh MEM with 10% CS. The cells growing continuously were designated PSAF/HeLa-R cells. On the other hand, HeLa-N cells maintained with FCS or with CS did not survive. Even in the case of a low MOI of 0.1 pfu per cell, HeLa-N cells did not survive. In order to confirm the persistence of SAFV-3 on HeLa-R cells, Western blotting using the anti-SAFV-3 antiserum was performed. Viral antigen was detected in PSAF/HeLa-R cells cultured for 30 days (5 passages) (Fig. 3, lane 2). PSAF/HeLa-R cells cultured for 42 days (7 passages) produced infectious (cell-free) virus at 4×10^5 pfu per 1×10^6 cells within 24 hours after the medium was changed. The titer of cell-free virus from one PSAF/HeLa-R cell is calculated to be 0.4 pfu. On the other hand, total (cell-free and cell-associated) viruses were generated at 6×10^6 pfu per 1×10^6 cells within 24 hours. The titer of total viruses from one PSAF/HeLa-R cell is calculated to be 6 pfu. In addition, the direct sequencing of viruses produced from PSAF/HeLa-R cells demonstrated that the sequence of VP1 coding region is identical to that of SAFV-3 (JPN08-404). These data demonstrate that SAFV-3 persistently infects PSAF/HeLa-R cells *in vitro*. The persistent infection of SAFV-3 in PSAF/HeLa-R was maintained for 70 days (16 passages) at least. In these culture periods, clear CPE was observed in only a part of cells. Furthermore, the cell line which is resistant to SAFV infection was not established.

Effects of Anti-IFN-α Antibody, Anti-IFN-β Antibody and FCS on PSAF/HeLa-R Cells

Although PSAF/HeLa-R cells (9 passages, 50 days p.i.) were treated by anti-IFN-α antibody (1 μg/ml/48 h) or anti-IFN-β antibody (120 U/ml/48 h) in order to investigate the involvement of IFN in the persistent infection, CPE on PSAF/