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# Generation and Characterization of Temperature Resistant Mutant of Recombinant PJ156/CAV-17 Virus

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**Previous study revealed that a recombinant virus between poliovirus (isolate PJ156) and coxsackie A virus serotype 17 (CAV-17), namely PJ156/CAV-17, was temperature sensitive. It is well known that two amino acids in 3D region (His-73 and Ile-362) are determinants for temperature sensitivity of poliovirus, in particular for Sabin 1 strain. However, it is not known whether those amino acids affect the temperature sensitivity for other enteroviruses. Sequence analysis of 3D region of PJ156/CAV-17 showed that amino acid in 3D-73 and -362 were Tyr and Ile, respectively, similar with the sequence of parental CAV-17 virus. Since amino acid in 3D-73 of PJ156/CAV-17 was not His, it is suggested that the temperature sensitivity of the PJ156/CAV-17 was associated with the Ile-362. To confirm this suggestion, the temperature-sensitive escape mutants of PJ156/CAV-17 were generated by blind passaging at 39.5 °C. The escape mutants were then recovered and plaque purified, and the sequence of 3D region was determined. It was found that the amino acid in 3D-362 was Thr, instead of Ile. Consequently, Ile-362 was proved to be involved in temperature sensitivity of PJ156/CAV-17. Full sequences of both viruses were also determined and compared. Furthermore, the characteristics of the temperature resistant of PJ156/CAV-17 variant were analyzed. It is confirmed that the recovered PJ156/CAV-17 virus could grow well at 39.5 °C, and there was strong correlation between temperature sensitivity and attenuation.**

Key words: Poliovirus, CAV-17, recombinant virus, temperature-sensitive

It is well known that poliovirus (PV) can recombine with other viruses. In fact, genetic recombinations of PV have been found in excreted viruses, including viruses from vaccine-associated paralytic poliomyelitis (VAPP) cases and healthy vaccinees. The recombination does not occurr only among different serotypes of the vaccine strains, but also between vaccine strains and wild type PV (Guillot *et al*. 2000; Liu *et al*. 2000; Dahourou *et al*. 2002). It is generally assumed that natural circulation of vaccine-strain derivatives is strictly limited in time. Therefore, such derivatives are believed to be unable to survive in nature long enough to evolve into highly transmissible neurophatogenic variants. However, incidents of paralytic poliomyelitis outbreaks due to circulating vaccine-derived poliovirus (cVDPV) were reported; respectively in the Dominican Republic and Haiti (Kew *et al*. 2002), the Philippines (Shimizu *et al*. 2004), in Egypt (Yang *et al*. 2003), and in Madagascar (Rousset *et al.* 2003). A sequence analysis showed that the above cVDPVs were recombinant viruses between PV and unidentified enterovirus that underwent the recombination in the noncapsid region. Moreover, we recently demonstrated that the recombination could occur between PV (isolate PJ156) and coxsackie A virus serotype 11 (PJ156/CAV-11) (Utama and Shimizu 2005) and CAV-17 (PJ156/CAV-17) (Utama and Shimizu 2006). The characteristics of resultant recombinant viruses vary, and suggested to be determined by characteristics of both parental viruses.

The recombinant PJ156/CAV-17 virus was found to be temperature sensitive; the virus could not grow at 39.5 °C (Utama and Shimizu 2006), similar to previous report (Semler *et al.* 1986). The virus did not exhibit any virulence on the PV receptor-transgenic (TgPvr) mice (Utama and Shimizu 2006). These suggest the correlation between temperature sensitivity and attenuation of the virus. From the study of PV Sabin 1 strain, it is suggested that the temperature sensitive effect associated with 3'-terminal part of the Sabin 1 genome are results from the cumulative and/or synergistic effects of at least three genetic determinants, i.e., the His-73 and Ile-362 codons of 3D, which coding RNA polymerase, and nucleotide G-7441 of 3'-untranslated region (Bouchard *et al.* 1995; Georgescu *et al.* 1995). Since the 3'-terminal part of the PJ156/CAV-17 was originated from CAV-17, the temperature sensitivity of this recombinant virus is supposed to be due to the sequence in 3'-terminal part of CAV-17. In this study, temperature resistant mutants were generated in order to determine the sequence contributed to the temperature sensitive (Ts) phenotype of the recombinant PJ156/CAV-17. Furthermore, the temperature resistant (Tr) PJ156/CAV-17 virus was characterized, including its neurovirulence to confirm the correlation between temperature sensitivity and attenuation.

## MATERIALS AND METHODS

**Generation of Temperature Resistant PJ156/CAV-17.** To generate temperature sensitive (Ts) escape mutant PJ156/CAV-17, the virus was infected to HEp-2 cells and cultured at 39.5 °C in Dulbecco's modified Eagle's medium (DMEM) supplemented with 2% bovine calf serum (maintenance medium). The cytophatic effect (CPE) was observed for 5 days. If the CPE was negative, the virus was then blind passaged until the virus develops complete CPE. Afterward, the virus was recovered and plaque purified. The temperature resistant (Tr) phenotype was finally confirmed by culturing the virus at 39.5 °C in the maintenance medium.

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**Plaque Purification of Temperature Resistant PJ156/ CAV-17.** Plaque purification was performed on an HEp-2 cell monolayer as described previously (Arita *et al*. 2005; Utama and Shimizu 2005; Utama and Shimizu 2006). A tenfold serial dilution of viruses prepared in the maintenance medium were inoculated in HEp-2 cells using 6-well plates, and incubated at 35.0 °C for 30 min. The cells were covered with 2 ml of 0.5% Agarose-ME in DMEM with 5% bovine calf serum. After incubation at 35.0 °C for 3 days, plates were stained with 2 ml of 0.5% neutral-red in maintenance medium containing 0.5% Agarose-ME. Plaque size was measured, and plaque numbers were calculated after incubation at 35.0ºC for a further day.

**Identification of Determinant Sequence Associated with the Temperature Sensitivity.** To identify the amino acids responsible for temperature sensitivity, the viral RNAs were extracted from both Ts and Tr PJ156/CAV-17 viruses, respectively, using High Pure Viral RNA Kit (Roche, Germany). The viral RNA was used as template for amplification of partial 3D region (approximately 1.5 kb) by RT-PCR using Access RT-PCR System (Promega, Madison, USA). Primer UG7 (5'-TTTGAAGGGGTGAAGGAACCAGC-3') and UC12 (5'-TCAATTAGTCTGGATTTTCCCTG-3') were used for amplification. RT-PCR was carried out in 50 µl reaction mixture containing 2 µl RNA template, 10 µl AMV/Tfl buffer, 1 µl dNTP (10 mM), 4 µl $MgCl_2$ (25 mM), 1 µl AMV Reverse Transferase, 1 µl Tfl DNA Polymerase, 2 µl each primer, and 27 µl $ddH_2O$. Reverse transcription was performed at 48 °C for 45 min, followed by thirty cycle of PCR reaction; 94 °C, 10 sec; 50 °C, 10 sec; 65 °C, 1 min. Amplified cDNA fragments were purified with Wizard SV Gel and PCR Clean-Up System (Promega, Madison, USA), and sequenced on Genetic Analyzer ABI 3100 (Applied Biosystem, USA) using primer CA-3D1S (5'-GAGCGGGCCAGTGTGGTGGAG-3'). In addition, full length sequence (7.44 kb) of both viruses was also determined.

**One-Step Growth-Curve and Temperature Sensitivity Analyses.** One-step growth-curve experiments were conducted by infecting a monolayer of HEp-2 cells with viruses at a multiplicity of infection (MOI) of 10 $CCID_{50}$ per cell (Shimizu *et al*. 2004; Utama and Shimizu 2005; Utama and Shimizu 2006). At different times post-infection, the cells and supernatant were collected, frozen and thawed three times, and then centrifuged (10,000 *x g*, 5 min) to remove cell debris. Virus titers in the supernatants were determined by the end-point dilution method in HEp-2 monolayer-cultures in 96-well plates at 35.0 °C. To test temperature sensitivity, one-step growth experiments were carried out at 35.0 °C and at 39.5 °C, respectively.

**Neurovirulence Test.** Groups of eight (four male and four female) 5-week-old PV receptor-transgenic (TgPvr) mice were inoculated intracerebrally with 30 µl virus solution (Shimizu *et al*. 2004; Utama and Shimizu 2005; Utama and Shimizu 2006). Tenfold dilutions of virus solution were made in MEM with 2% bovine calf serum so that each mouse received approximately $10^{1.3}$ to $10^{6.3}$ of $CCID_{50}$. Mice were examined over 14 days for paralysis and/or death. The amount of virus that caused 50% paralysis and/or death dose ($PD_{50}$) was calculated.

## RESULTS

**Generation of Temperature-Resistant PJ156/CAV-17.** The recombinant PJ156/CAV-17 virus showed complete CPE at 39.5 °C in day-4 after twelve time blind passage. The virus was then plaque purified. Plaque assay showed that the plaque size of PJ156/CAV-17 (Tr) mutant was slightly bigger (1-2 mm) as compared with original PJ156/CAV-17 (Ts) (<1 mm) (Fig 1). Ten purified isolates were cultured at 39.5 °C in maintenance medium, and was confirmed to be able growth at 39.5 °C.

**Identification of Amino Acids Determined Temperature Sensitivity.** Previous studies revealed that two amino acids in 3D region (His-73 and Ile-362) were determinants of temperature sensitivity (Bouchard *et al*. 1995; Georgescu *et al.* 1995). A sequence analysis of 3D region of PJ156/CAV-17 showed that amino acid in 3D-73 and -362 were Tyr and Ile, respectively, similar with the parental CAV-17 sequence. Since amino acid in 3D-73 was not His, it is suggested that the temperature sensitivity of the virus was due to the Ile-362. To confirm this suggestion, the PJ156/CAV-17 (Tr) viruses were recovered, and the sequences of partial 3D region from eight plaque-purified isolates were determined. It is found that the amino acid in 3D-362 in all isolates was Thr, instead of Ile (Fig 2). Full length sequences (7.44 kb) of PJ156/CAV-17 (Ts) and PJ156/CAV-17 (Tr) were determined and compared. It is found that 35 nucleotides were different between both viruses, and 17 of 35 nucleotide mutations were associated with 15 amino acids substitution (Table 1). In particular, there were 3 amino acids substitution in 3D region; Gly-163-Arg, Ala-197-Val, and Ile-362-Thr, respectively. Based on current knowledge, since no other mutations associated with temperature sensitivity, it is suggested that Ile-362 was determinant for temperature sensitivity of PJ156/ CAV-17.

**One-Step Growth of Recombinant Viruses.** A one-step growth experiment of the PJ156/CAV-17 (Ts) and PJ156/CAV-17 (Tr) viruses was conducted at 39.5ºC (Fig 3). PV strains such as Sabin 1, Mahoney, and PJ156 were used for comparison. As expected, Sabin 1 was temperature sensitive, while Mahoney and PJ156 were temperature resistant. On the other hand, recombinant PJ156/CAV-17 virus was temperature sensitive; it could not grow at 39.5 °C. However, two isolates of PJ156/CAV-17 resulted from blind passaging (PJ156/CAV-17 (Tr-1) and PJ156/CAV-17 (Tr-2)) could grow at 39.5 °C. Although the growth those viruses were slightly

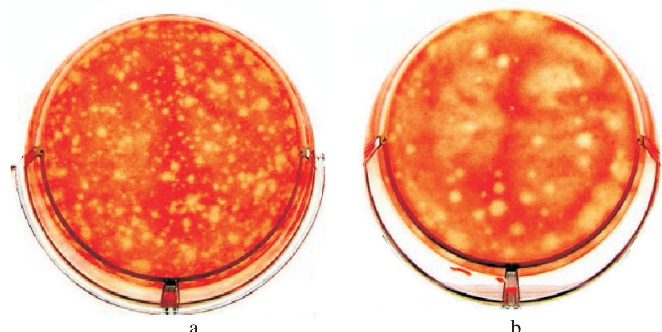


Fig 1 Plaque size of the PJ156/CAV-17 (Ts) (a) and PJ156/CAV-17 (Tr) (b).

Capsid Protein | Nonstructural Protein

VP4 | VP2 | VP3 | VP1 | 2A | 2B | 2C | 3A | 3B | 3C | 3D

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Sabin 1              61:KYVGNKITEVDEHMKEAVDHYAGQLMSLDINTEQMCLEDAMYGTDGLEALDLSTSAGYPY 120
Mahoney              61:............Y............................................... 120
PJ156                61:............Y.......................................T....... 120
CAV-17               61:............Y............................................... 120
PJ156/CAV-17(Ts)     61:............Y............................................... 120
PJ156/CAV-17(Tr)     61:............Y............................................... 120

Sabin 1             361:AIFETVTWENVTFLKRFFRADEKYPFLIHPVMPMKEIHESIRWTKDPRNTQDHVRSLCLL 420
Mahoney             361:.T.......................................................... 420
PJ156               361:.T..K......................X................................ 420
CAV-17              361:...........................V................................ 420
PJ156/CAV-17(Ts)    361:...........................V................................ 420
PJ156/CAV-17(Tr)    361:.T.........................V................................ 420
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Fig 2 Amino acid alignment of partial 3D region.

Table 1 Sequence different between PJ156/CAV-17 (Ts) and PJ156/CAV-17 (Tr)

| Region | | Nucleotide | | | Amino acid | | |
|---|---|---|---|---|---|---|---|
| PJ156 | CAV-17 | Position | Ts | Tr | Position | Ts | Tr |
| VP4 | | 878 | C | T | 46 | Leu | Phe |
| | | 911 | A | G | 57 | Ile | Val |
| VP2 | | 1148 | G | A | 67 | Asp | Asn |
| | | 1491 | C | T | 181 | Ser | Phe |
| | | 1562 | A | T | 205 | Ser | Cys |
| 2A | | 3491 | A | G | 36 | Asn | Asp |
| | | 3493 | T | C | | | |
| | | 3753 | T | C | 123 | Ile | Thr |
| 2B | | 4116 | C | T | 95 | Thr | Ile |
| 2C | | 4130 | A | G | 3 | Ser | Gly |
| | 2C | 4940 | T | C | 273 | Tyr | His |
| | 3B | 5396 | A | C | 9 | Lys | Arg |
| | | 5397 | A | G | | | |
| | 3C | 5738 | G | A | 101 | Val | Ile |
| | 3D | 6473 | G | A | 163 | Gly | Arg |
| | | 6576 | C | T | 197 | Ala | Val |
| | | 7071 | T | C | 362 | Ile | Thr |

Fig 3 One-step growth of parental and recombinant viruses. (—◇—) Sabin 1, (—□—) Mahoney, (—△—) PJ156, (—×—) PJ156/CAV-17, (—○—) PJ156/CAV-17 (Tr-2), (—✳—) PJ156/CAV-17 (Tr-1).

slow as compared to the Mahoney and PJ156 strains, they finally could grow well after 6 h post infection.

**Neurovirulence Test.** To analyze the neurovirulence of the PJ156/CAV-17 (Tr), groups of 8 PV receptor-expressed transgenic mice (TgPvr mice) were intracerebrally inoculated with the virus, along with Sabin 1 and PJ156 viruses. As shown in Table 2a, the PJ156/CAV-17 (Tr) exhibited 50% paralysis and/or death dose ($PD_{50}$) of 2.66, which is lower than that of parental PJ156 (3.66). On the other hand, as expected, Sabin 1 (vaccine strain) showed a high $PD_{50}$ value (>8.06). These results imply that the PJ156/CAV (Tr) was more virulence than the parental PJ156. In another experiment, it was shown that PJ156/CAV-17 (Ts) possessed a high $PD_{50}$ (>6.78), which similar with Sabin 1 (>7.53), whilst Mahoney (wild-type strain) possessed low $PD_{50}$ value (3.41) (Table 2B). These results confirmed that there was a dramatic change of neurovirulence phenotype from PJ156/CAV-17 (Ts) to PJ156/CAV-17 (Tr). These results also exhibited a strong correlation between temperature sensitivity or resistance and attenuation or neurovirulence.

## DISCUSSION

A recombinant virus between poliovirus (isolate PJ156) and coxsackie A virus serotype 17 (CAV-17) was recently constructed and characterized (Utama and Shimizu 2006). The resultant recombinant PJ156/CAV-17 virus showed a temperature sensitive phenotype. To determine the sequence contributed to the temperature sensitive (Ts) phenotype of the PJ156/CAV-17 virus, elevated temperature escape mutants were generated by performing blind passage at 39.5 °C. The PJ156/CAV-17 virus could grow well at 39.5 °C in day-4 after twelve time blind passage. The PJ156/CAV (Tr) showed a bigger plaque size (1-2 mm) as compared with original PJ156/CAV-17 (Ts) (<1 mm) (Fig 1). Ten purified isolates were cultured at 39.5 °C in maintenance medium, and was confirmed to be able growth at 39.5 °C, indicating from complete CPE. One-step growth experiment showed that PJ156/CAV-17 (Tr) could grow at 39.5 °C, although the growth was not as good as parental PJ156 or Mahoney strain.

Previous studies revealed that mutations in the 5'NCR, base 2438 of VP3, bases 2741, and 2795 of VP1, base 6203 of 3Dpol, and base 7441 of the 3'NCR were associated with

Table 2 Neurovirulence of PJ156/CAV-17 (Ts) and PJ156/CAV-17 (Tr) viruses on Tg-mice

| A Virus | Dose (log $CCID_{50}$ mouse$^{-1}$) | | | | | | | $PD_{50}$[b] |
|---|---|---|---|---|---|---|---|---|
| | 8.18 | 5.78 | 4.78 | 3.78 | 2.78 | 1.78 | 0.78 | |
| Sabin 1 | 5/8[a] | ND | ND | ND | ND | ND | ND | >8.06 |
| PJ156 | ND | ND | ND | 5/8 | 0/8 | 0/8 | 0/8 | 3.66 |
| PJ156/CAV-17 (Tr) | ND | 8/8 | 8/8 | 7/7[c] | 4/8 | 1/8 | ND | 2.66 |

| B Virus | Dose (log $CCID_{50}$ mouse$^{-1}$) | | | | | | | $PD_{50}$[b] |
|---|---|---|---|---|---|---|---|---|
| | 7.28 | 6.28 | 5.28 | 4.28 | 3.28 | 2.28 | 1.28 | |
| Sabin 1 | 2/8[a] | ND | ND | ND | ND | ND | ND | >7.53 |
| Mahoney | ND | ND | ND | 7/8 | 3/8 | 1/8 | 0/8 | 3.41 |
| PJ156/CAV-17 (Ts) | ND | 0/8 | 0/8 | 0/8 | 0/8 | 0/8 | ND | >6.78 |

[a]No. of paralyzed or dead mice/no. of total mice, [b]$PD_{50}$ was calculated by the Karber formula (Karber 1931), [c]one mouse was died after injection of the virus, ND = Not determined.

phenotypic reversion of the temperature sensitivity of PV, particularly Sabin 1 strain (Bouchard *et al.* 1995). In addition, two amino acids in 3D region (His-73 and Ile-362) were determinants for temperature sensitivity of PV Sabin 1 (Georgescu *et al.* 1995). Although it is reported that a chimeric plasmid from cDNA clones of poliovirus and coxsackievirus produces a recombinant virus that is temperature-sensitive (Semler *et al.* 1986), it is not known whether those amino acids affect the temperature sensitivity of CAV, including CAV-17. A sequence analysis of partial 3D region of PJ156/CAV-17 (Ts) showed that amino acid in 3D-73 and -362 were Tyr and Ile, respectively (Fig 2). On the other hand, it was found that the amino acid in 3D-73 and 3D-362 of PJ156/CAV-17 (Tr) were Tyr and Thr, respectively. Consequently, Ile-362 was proved to be involved in temperature sensitivity of PJ156/CAV-17. To identify other determinants, full sequences of both PJ156/CAV-17 (Ts) and PJ156/CAV-17 (Tr) viruses were also determined and compared. It is found that 35 nucleotides were different between both viruses (data not shown), and 17 of 35 nucleotide mutations were associated with 15 amino acids substitution (Table 1). In particular, there were 3 amino acids substitution in 3D region; Gly-163-Arg, Ala-197-Val, and Ile-362-Thr, respectively. Although there were several mutations in capsid and other nonstructural protein-coding regions, based on current knowledge, none of these mutations are related to temperature sensitivity of PV and other enteroviruses. Hence, it is suggested that Ile-362 was a determinant for temperature sensitivity of PJ156/CAV-17.

The PJ156/CAV-17 (Tr) exhibited a $PD_{50}$ of 2.66, which is lower than that of parental PJ156 (3.66) (Table 2a). Sabin 1 was used as control for vaccine strain, and as expected, it showed a high $PD_{50}$ value (>8.06), which means very low neurovirulence. Contrary to this, PJ156/CAV (Tr) was high virulence, even more virulence than the parental PJ156. In another experiment, it was shown that PJ156/CAV-17 (Ts) possessed a high $PD_{50}$ (>6.78), which similar with Sabin 1 (>7.53), whilst Mahoney (wild-type strain) possessed low $PD_{50}$ value (3.41) (Table 2b) (Utama and Shimizu 2006). These results confirmed that there was a dramatic change of neurovirulence phenotype from PJ156/CAV-17 (Ts) to PJ156/CAV-17 (Tr). Moreover, the neurovirulence of PJ156/CAV-17 (Tr) was higher than PJ156 and wild-type Mahoney strain. In most PVs, there is a strong correlation between virulence or attenuation and temperature resistance or sensitivity (Shimizu *et al.* 2004; Yang *et al.* 2005). Our results also support the fact that there is a correlation between temperature resistant and high virulence.

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## REFERENCES

Arita M, Shimizu H, Nagata N, Ami Y, Suzaki Y, Sata T, Iwasaki T, Miyamura T. 2005. Temperature-sensitive mutants of enterovirus 71 show attenuation in cynomolgus monkeys. *J Gen Virol* 86:1391-1401.

Bouchard MJ, Lam D-H, Racaniello VR. 1995. Determinants of attenuation and temperature sensitivity in the type 1 poliovirus Sabin vaccine. *J Virol* 69:4972-4978.

Brown B, Oberste MS, Maher K, Pallansch MA. 2003. Complete genomic sequencing shows that polioviruses and members of human enterovirus species C are closely related in the noncapsid coding region. *J Virol* 77:8973-8984.

Dahourou G, Guillot S, Gall OL, Crainic R. 2002. Genetic recombination in wild-type poliovirus. *J Gen Virol* 83:3103-3110.

Georgescu M-M, Tardy-Panit M, Guillot S, Crainic R, Delpeyroux F. 1995. Mapping of mutations contributing to the temperature sensitivity of the Sabin 1 vaccine strain of poliovirus. *J Virol* 69:5278-5286.

Guillot S, Caro V, Cuervo N, Korotkova E, Combiescu M, Persu A, Aubert-Combiescu A, Delpeyroux F, Crainic R. 2000. Natural genetic exchanges between vaccine and wild poliovirus strains in humans. *J Virol* 74:8434-8443.

Karber G. 1931. Beitrag zur kollektiven Behandlung pharmakologischer Reihenversuche. *Arch Exp Pathol Pharmakol* 162:480-483.

Kew OM, Morris-Glasgow V, Landaverde M, Burns C, Shaw J, Garib Z, Andre J, Blackman E, Freeman CJ, Jorba J, Sutter R, Tambini G, Venczel L, Pedreira C, Laender F, Shimizu H, Yoneyama T, Miyamura T, van der Avoort H, Oberste MS, Kilpatrick D, Cochi S, Pallansch M, de Quadros C. 2002. Outbreak of poliomyelitis in hispaniola associated with circulating type 1 vaccine-derived poliovirus. *Science* 296:356-359.

Liu HM, Zheng DP, Zhang LB, Oberste MS, Pallansch MA, Kew OM. 2000. Molecular evolution of a type 1 wild-vaccine poliovirus recombinant during widespread circulation in China. *J Virol* 74:11153-11161.

Rousset D, Rakoto-Andrianarivelo M, Razafindratsimandresy R, Randriamanalina B, Guillot S, Balanant J, Mauclère P, Delpeyroux F. 2003. Recombinant vaccine–derived poliovirus in Madagascar. *J Infect Dis* 9:885-887.

Semler BL, Johnson VH, Tracy S. 1986. A chimeric plasmid from cDNA clones of poliovirus and coxsackievirus produces a recombinant virus that is temperature-sensitive. *Proc Natl Acad Sci USA* 83:1777-1781.

Shimizu H, Thorley B, Paladin FJ, Brussen KA, Stambos V, Yuen L, Utama A, Tano Y, Arita M, Yoshida H, Yoneyama T, Benegas A, Roesel S, Pallansch M, Kew OM, Miyamura T. 2004. Circulation of type 1 vaccine-derived poliovirus in the Philippines in 2001. *J Virol* 78:13512-13521.

Utama A, Shimizu H. 2005. Construction of a recombinant virus between poliovirus and coxsackie A virus 11. *Annal Bogorienses* 10:19-26.

Utama A, Shimizu H. 2006. Construction and characterization of chimeric virus between poliovirus and coxsackie A virus serotype 17. *J Mikrobiol Indones* 11:77-81.

Yang CF, Chen HY, Jorba J, Sun HC, Yang SJ, Lee HC, Huang YC, Lin TY, Chen PJ, Shimizu H, Nishimura Y, Utama A, Pallansch M, Kew OM, Yang JY. 2005. Intratypic recombination among lineages of type 1 vaccine-derived poliovirus emerging during chronic infection of an immunodeficient patient. *J Virol* 79:12623-12634.

Yang CF, Naguib T, Yang SJ, Nasr E, Jorba J, Ahmed N, Campagnoli R, van der Avoort H, Shimizu H, Yoneyama T, Miyamura T, Pallansch M, Kew OM. 2003. Circulation of endemic type 2 vaccine-derived poliovirus in Egypt from 1983 to 1993. *J Virol* 77:8366-8377.