exchange factor, GBF1, and inhibits the replication of PV, but not encephalomyocarditis virus (EMCV) (Belov *et al.*, 2008; Cuconati *et al.*, 1998; Irurzun *et al.*, 1992; Maynell *et al.*, 1992). Geldanamycin targets Hsp90 and interferes with the folding of PV capsid, but not with RNA replication, probably in cooperation with Hsp70 (Geller *et al.*, 2007; Macejak & Sarnow, 1992). Hippuristanol, a natural product of the coral *Isis hippuis*, inhibits RNA binding of eIF4A and delays the appearance of virus proteins in PV replication for 2 h (Bordeleau *et al.*, 2006). Calpain inhibitors inhibit the replication of echovirus 1, possibly by targeting the activities of calpain 1 and 2 (Upla *et al.*, 2008). Pyrrolidine dithiocarbamate (PDTC) inhibits polyprotein processing of entero- and cardioviruses by transporting zinc ions into the cells with different mechanisms depending on the virus species (Krenn *et al.*, 2005; Lanke *et al.*, 2007). For PDTC, a mechanism via the ubiquitin–proteasome pathway is also proposed for the inhibitory effect on coxsackievirus B3 (CVB3) infection (Si *et al.*, 2005). The advantage of using antiviral compounds that target cellular factors is the limited emergence of resistant mutants. Mutants resistant to geldanamycin and PDTC were not isolated despite attempts (Geller *et al.*, 2007; Krenn *et al.*, 2009), although a mutant resistant to brefeldin A was isolated after five passages in cultured cells (Crotty *et al.*, 2004).

In our previous attempt to identify potent anti-enterovirus compounds that target cellular factors, we identified a Raf-1 inhibitor, GW5074, by screening pharmacologically active compounds (Arita *et al.*, 2008b). However, inhibitors for the downstream signalling pathway of Raf-1 [MEK/extracellular signal-regulated kinase (ERK) signalling] did not affect PV replication. Actually, we did not identify any potent anti-enterovirus compounds other than GW5074 in the kinase inhibitor library examined in this screening. This suggested that GW5074 has several targets for its inhibitory effect, as observed in an *in vitro* kinase assay (Bain *et al.*, 2007). Therefore, in the present study, we attempted to identify the target of GW5074 by searching for cellular kinase inhibitors that have anti-enterovirus activity similar or related to that of GW5074. We have found that combined treatment with inhibitors for MEK1/2 or Akt1/2 and a receptor tyrosine kinase inhibitor, AG1478, effectively suppresses PV and EV71 replication in the absence of GW5074. We have also found that some receptor tyrosine kinase inhibitors have anti-enterovirus activity by themselves. A single mutation in viral protein 3A was required for partial resistance to a receptor tyrosine kinase inhibitor, Flt3 inhibitor II, and to GW5074. The mutation was known previously as a determinant of resistance to enviroxime, which is a potent anti-enterovirus inhibitor that suppresses the initiation of positive-strand RNA synthesis (Heinz & Vance, 1995; Wikel *et al.*, 1980). These observations suggested that some cellular kinase inhibitors and enviroxime have a conserved target in viral protein 3A to suppress enterovirus replication.

METHODS

Cells, viruses, reagents and chemical library. RD cells (a human rhabdomyosarcoma cell line) were cultured as monolayers in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10 % fetal calf serum (FCS) and used for titration of pseudoviruses and screening of anti-PV and anti-EV71 compounds. PV and EV71 pseudoviruses (TE-PV-Fluc mc and TE-EV71-Fluc mc), which encapsidated luciferase-encoding PV and EV71 replicons with capsid proteins derived from PV (Mahoney) and EV71 (Nagoya), respectively, were prepared as reported previously (Arita *et al.*, 2006, 2008a, b). A PV pseudovirus mutant [TE-PV-Fluc mc (5318A)] that has adenine at nt 5318 was prepared with cDNA of a luciferase-encoding PV replicon by site-directed mutagenesis as described previously (Arita *et al.*, 2006, 2008a). Kinase inhibitors included in the LOPAC^{1280} drug library (Sigma-Aldrich), ZM336372 (Calbiochem), Akt inhibitor VIII (Sigma-Aldrich), STO-609 (Calbiochem), FK506 (Fermentek), SL327 (Sigma-Aldrich) and U0126 (Sigma-Aldrich) were used for screening of kinase inhibitors (see Supplementary Table S1, available in JGV Online). For screening of receptor tyrosine kinase inhibitors that can substitute for the effect of AG1478, cFMS receptor tyrosine kinase inhibitor, Flt3 inhbitor II, IGF-1R inhibitor II, TGF-β RI inhibitor III and VEGFR tyrosine kinase inhibitor III were purchased from Calbiochem. Each compound (10 mM) was prepared in DMSO and then used for screening.

Screening of kinase inhibitors. For the screening of kinase inhibitors that have a cooperative inhibitory effect with GW5074, RD cells (1.0×10^4 cells per well in 100 µl medium) were cultured at 37 °C in 96-well plates (Becton Dickinson), followed by addition of 1.6 µl kinase inhibitor solution (10, 5.0, 2.5 and 0 mM), 10 µl GW5074 (120 and 0 µM) and 200 IU PV pseudovirus in 50 µl DMEM/10 % FCS. Cells were incubated at 37 °C for 7 h (final concentrations of kinase inhibitors: 100, 50, 25 and 0 µM; final concentrations of GW5074, 7.5 and 0 µM) (Fig. 1a) and the luciferase activity of the cells was measured with the Luciferase Assay system (Promega), using a TR717 Microplate luminometer (Applied Biosystems) according to the manufacturer's instructions. For the screening of kinase inhibitors that have a synthetic inhibitory effect with SL327, U0126, AG1478 and wortmannin, RD cells (1.0×10^4 cells per well in 50 µl medium) were cultured at 37 °C in 96-well plates, followed by addition of 1.6 µl kinase inhibitor solution (5.0, 2.5 and 0 mM), 50 µl SL327 (150 µM), U0126 (150 µM), AG1478 (150 µM) and wortmannin (6 µM) solution. The cells were inoculated with 200 IU PV pseudovirus and the luciferase activity of the cells at 7 h post-infection (p.i.) was measured (Fig. 1b). The inhibition index, which is the ratio of luciferase activity of the drug-treated cells to that of mock-treated cells (1.0 for mock-treated cells in the absence of GW5074 and for GW5074-treated cells in the presence of GW5074), was determined for each drug in the presence or absence of GW5074. Cutoff values of the screening were set to select at least one compound that showed an inhibitory effect without apparent cytotoxicity in the screening. For these criteria, we set the cutoff at below the mean−2SD, or at <10 % of the mean in the case that the SD was larger than the mean. Accordingly, the cooperative inhibitory effect of the compounds was defined as a reduction of the inhibition index in the presence of GW5074 (7.5 µM) by <10-fold of that in the absence of GW5074. The synthetic inhibitory effect of the compounds was defined as reduction of the inhibition index in the presence of SL327 (25 µM), U0126 (25 µM), AG1478 (25 µM) or wortmannin (2 µM) by $<10^3$-fold of that in the absence of these kinase inhibitors.

Evaluation of the cytotoxicity of compounds. Cytotoxicity of the compounds was evaluated by two methods: one was observation of cell viability at 7 h under the same conditions as used for the screening and the other was the determination of 50 % cytotoxic concentration (CC_{50}) of drugs by the measurement of ATP

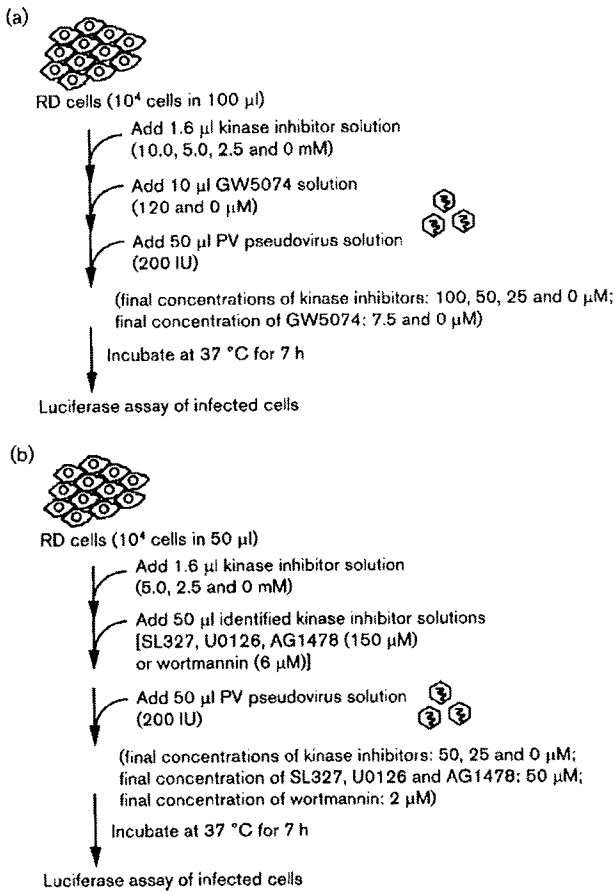


Fig. 1. Screening of kinase inhibitors. (a) Scheme for screening of kinase inhibitors that have a cooperative inhibitory effect with GW5074 on PV pseudovirus infection. (b) Scheme for screening of kinase inhibitors that have a synthetic inhibitory effect without GW5074 on PV pseudovirus infection.

concentration as a marker of metabolically active cells, as described previously (Arita *et al.*, 2008b). RD cells (1.4×10^4 cells per well in 100 µl medium) were cultured at 37 °C in 96-well plates (Becton Dickinson), followed by addition of 100 µl compound solution in a range of concentrations (7.8–500 µM) for GW5074, AG1478, IGF-1R inhibitor II and Flt3 inhibitor II. The CC_{50} value of each drug was also measured in the presence of U0126 (25 µM) and Akt inhibitor VIII (2 µM). Cells were incubated at 37 °C for 7 h and then subjected to measurement of ATP by using a Cell Titre-Glo Luminescent Cell Viability Assay kit (Promega) according to the manufacturer's instructions.

Quantification of virus RNA by real-time TaqMan PCR. Real-time TaqMan PCR was performed as described by Nijhuis *et al.* (2002). Virus RNA was reverse-transcribed by using the Reverse Transcription system (Promega) and then subjected to real-time PCR by using TaqMan Fast Universal PCR Master Mix (Applied Biosystems) with primers (forward primer and reverse primer 2) and a probe (probe 1) (Nijhuis *et al.*, 2002). The fluorescence emission of the probe was monitored and analysed by using the Applied Biosystems 7500 Fast Real-Time PCR system, as described previously (Arita *et al.*, 2008b).

Isolation of PV mutants resistant to inhibitors. RD cells (1.4×10^4) were infected with PV (Mahoney) at an m.o.i. of 10 in the presence of inhibitors as follows: guanidine hydrochloride (GuaHCl), 0.5, 1.0 and 2.0 mM; GW5074, 12.5, 25 and 50 µM; Flt3 inhibitor II, 3.1, 6.3 and 12.5 µM. Cells were incubated at 37 °C until all cells exhibited cytopathic effect (CPE) or at day 3 p.i. Collected cell lysates for each inhibitor were mixed and then used for the next passage. Passage was repeated 12 times or until a resistant phenotype was observed by the appearance of CPE. Resistant mutants were isolated by limiting dilution, and then the non-structural protein-encoding regions of the viral genome were analysed as described previously (Arita *et al.*, 2005b).

Measurement of the interferon (IFN) response. RD cells (4.2×10^4) in 24-well plates were incubated at 37 °C for 7 h in the presence of the following compounds: GW5074, 50 µM; U0126, 25 µM; Akt inhibitor VIII, 2 µM; AG1478, 25 µM; IGF-1R inhibitor II, 25 µM; Flt3 inhibitor II, 25 µM. The relative expression levels of OAS1 and STAT1 mRNAs in the cells were determined by real-time PCR using an IFN Response Watcher kit (TaKaRa) as indicators of the IFN response. β-Actin mRNA was used as the endogenous control, and the expression level of OAS1 and STAT1 mRNAs in the treated cells was normalized by that in mock-treated cells.

RESULTS

Screening of kinase inhibitors that have a cooperative inhibitory effect with GW5074 on PV infection

To identify cellular kinase inhibitors that have anti-enterovirus activity similar or related to that of GW5074, we performed two rounds of screening with a library of cellular kinase inhibitors. In the first screening, we aimed to identify cellular kinase inhibitors that have a cooperative inhibitory effect with GW5074 (see below for definition of the inhibitory effect). In the second screening, we aimed to identify cellular kinase inhibitors that have a synthetic inhibitory effect with the kinase inhibitors identified in the first screening in the absence of GW5074. Cooperative inhibitory effect was defined as an inhibitory effect that enhances the inhibitory effect of GW5074 on PV replication. Synthetic inhibitory effect was defined as an inhibitory effect on PV replication observed in a combined treatment of compounds, each of which showed no inhibitory effect in individual treatment.

To identify cellular kinase inhibitors that have a cooperative inhibitory effect with GW5074, we performed screening with a kinase inhibitor library in the presence or absence of a suboptimal concentration of GW5074 (7.5 µM) in RD cells (Fig. 1a; Supplementary Table S1). The inhibitory effect of GW5074 has been observed in L20B (murine), HEp-2c and RD (human) cells and thus is not species-specific (Arita *et al.*, 2008b). Therefore, we used RD cells for the present screenings because this cell line is susceptible to both PV and EV71. Cells were inoculated with PV pseudoviruses that have a luciferase-encoding PV

replicon genome encapsidated in a PV capsid, as described previously (Arita *et al.*, 2006, 2008b), and then the luciferase activity in the cells was measured at 7 h p.i. The mean luciferase activity in the mock-treated cells infected with PV pseudoviruses was 1.3×10^6 relative light units (RLU), and that in GW5074-treated cells was 2.4×10^4 RLU. The mean inhibition index of kinase inhibitors (100 μM) in the presence or absence of GW5074 was 0.64 (with an SD of 0.33) for kinase inhibitors that did not cause apparent cytotoxicity. We identified MEK1/2 inhibitors (SL327 and U0126), an EGFR inhibitor (AG1478) and a phosphatidylinositol 3-kinase (PI3K) inhibitor (wortmannin) as compounds with a cooperative inhibitory effect with GW5074 (Fig. 2). SL327, U0126 and AG1478 showed the cooperative inhibitory effect with GW5074 at concentrations of $\geqslant 50$ μM. Effective concentrations of wortmannin in the cells as a PI3K inhibitor or as an enhancer of EMCV mutant viral protein synthesis were 0.1–1.0 μM (Okada *et al.*, 1994; Svitkin *et al.*, 1998), thus far lower than that examined in the screening (25–100 μM). Therefore, we also examined lower concentrations (0.78–6.3 μM) and found that wortmannin showed a cooperative inhibitory effect in this range of concentrations.

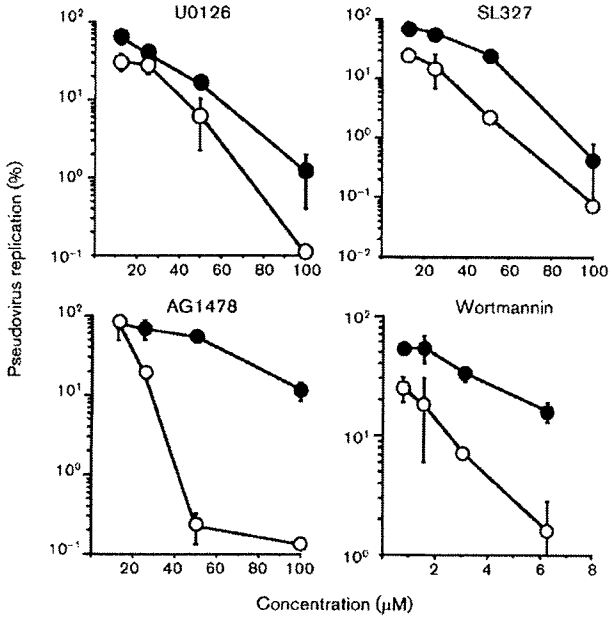


Fig. 2. Kinase inhibitors that showed a cooperative inhibitory effect with GW5074 on PV pseudovirus infection. RD cells (1.0×10^4) in a 96-well plate were inoculated with 200 IU PV pseudovirus in the presence of kinase inhibitors with (○) or without (●) GW5074 (7.5 μM). Luciferase activity at 7 h p.i. is shown. Pseudovirus replication in the absence of inhibitors (mock-treated) and in the absence of kinase inhibitors, but with GW5074 (GW5074-treated), was taken as 100%.

Screening of kinase inhibitors that have a synthetic inhibitory effect with MEK1/2 inhibitors, AG1478 and wortmannin on PV infection

Next, to identify cellular kinase inhibitors that have a synthetic inhibitory effect in the absence of GW5074, we performed screening with the identified kinase inhibitors (SL327, U0126, AG1478 and wortmannin) that showed a cooperative inhibitory effect with GW5074 in the first screening (Fig. 1b). The mean inhibition index of kinase inhibitors in the presence or absence of SL327, U0126, AG1478 and wortmannin was 1.5–2.7 (with an SD of 1.5–3.8) for kinase inhibitors that did not cause apparent cytotoxicity. For SL327, U0126 and AG1478, but not for wortmannin, an Akt1/2 inhibitor (Akt inhibitor VIII) was identified as a compound that showed a synthetic inhibitory effect on PV pseudovirus infection. GW2974 (an EGFR/ERBB2 inhibitor) and diacylglycerol kinase inhibitor I showed a synthetic inhibitory effect with AG1478, but not with other inhibitors (Fig. 3a). The inhibitory effect of individual compounds was not significant compared with the synthetic inhibitory effect of these compounds ($<10^3$-fold reduction of replication); they caused at most a 50-fold reduction of replication [in the presence of 50 μM AG1478 (Fig. 3b)]. Combined treatment with U0126 (25 μM), Akt inhibitor VIII (2 μM), AG1478 (50 μM) and wortmannin (2 μM) showed a significant inhibitory effect (inhibition index of 2.1×10^{-4}). In this combination, U0126, Akt inhibitor VIII and AG1478 were essential. The effect of wortmannin was weaker than that of Akt inhibitor VIII, and compensated only partially for the effect of Akt inhibitor VIII.

Screening of receptor tyrosine kinase inhibitors that can substitute for the synthetic inhibitory effect of AG1478 on PV infection

The effective concentration of AG1478 (12.5–50 μM) in the synthetic inhibitory effect was far higher than that reported for the inhibition of EGFR phosphorylation in cells (0.5–1.0 μM) (Isaacson *et al.*, 2007; Soeda *et al.*, 2008). Therefore, we analysed other receptor tyrosine kinase inhibitors that could substitute for the effect of AG1478 with U0126 (25 μM) and Akt inhibitor VIII (2 μM). We found that IGF-1R inhibitor II and Flt3 inhibitor II, but not GuaHCl, GW5074 or other receptor tyrosine kinase inhibitors, showed a cooperative inhibitory effect with U0126 and Akt inhibitor VIII, although these receptor tyrosine kinase inhibitors have a strong inhibitory effect by themselves, in contrast to AG1478 (Fig. 4a). CC_{50} values of these receptor tyrosine kinase inhibitors and GW5074 were determined to be reduced by 3- to 10-fold in the presence of U0126 and Akt inhibitor VIII (Fig. 4b; see Discussion).

The inhibitory effect of these kinase inhibitors was also observed for EV71 pseudovirus and PV (Mahoney) infection (Fig. 5a, b). Treatment with kinase inhibitors was effective after the uncoating process of PV (Mahoney) (Fig. 5b), suggesting that the target step of the inhibitory

(a)

Compound	Kinase inhibitors with synthetic inhibitory effect
SL327	Akt inhibitor VIII (4.3×10^{-3})
U0126	Akt inhibitor VIII (4.0×10^{-3})
AG1478	Akt inhibitor VIII (9.3×10^{-3}), GW2974 (2.7×10^{-2}), diacylglycerol kinase inhibitor I (2.8×10^{-3})
Wortmannin	Not identified

(b)

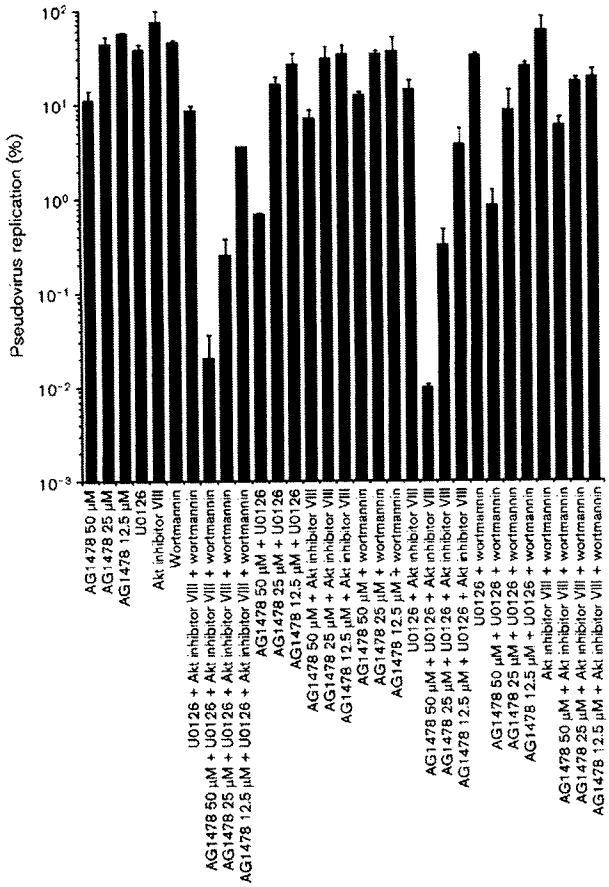
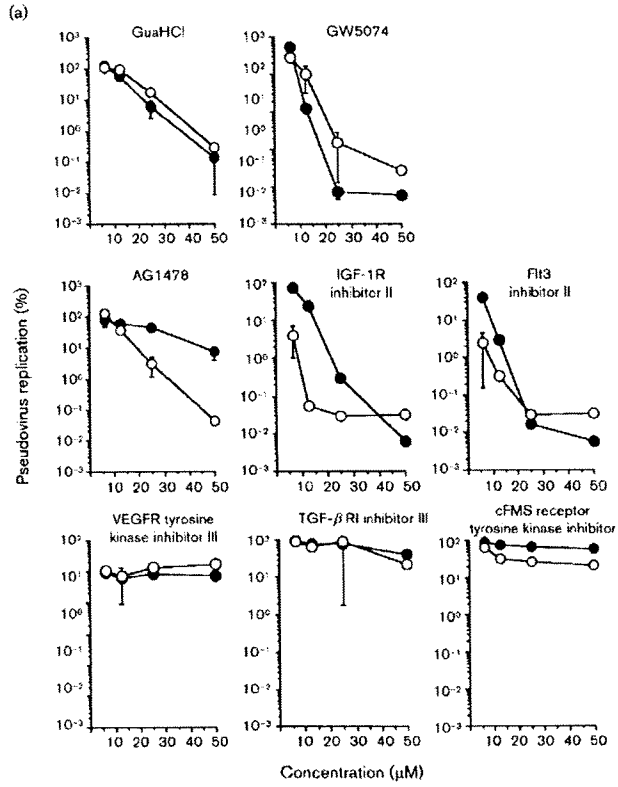


Fig. 3. Kinase inhibitors that showed a synthetic inhibitory effect on PV pseudovirus infection. (a) Identified kinase inhibitors that showed a synthetic inhibitory effect with SL327, U0126, AG1478 and wortmannin. Inhibition index of each compound in the synthetic inhibition is shown in parentheses. (b) Combined treatment with kinase inhibitors. RD cells (0.7×10^4) in a 96-well plate were inoculated with 200 IU PV pseudovirus in the presence of AG1478 (12.5, 25 and 50 μM), U0216 (25 μM), Akt inhibitor VIII (2 μM) and wortmannin (2 μM). Luciferase activity at 7 h p.i. is shown. Pseudovirus replication in mock-treated cells was taken as 100%.

(a)



(b)

Compound	CC_{50} (μM)	
	Mock-treated	+ U0126 + Akt inhibitor VIII
GW5074	250	64
AG1478	150	39
IGF-1R inhibitor II	16	1.7
Flt3 inhibitor II	58	18

Fig. 4. Synthetic inhibitory effect of receptor tyrosine kinase inhibitors on PV pseudovirus infection. (a) RD cells (1.4×10^4) in a 96-well plate were inoculated with 200 IU PV pseudovirus in the presence of tyrosine kinase inhibitors, GuaHCl and GW5074 in mock-treated cells (without U0126 and Akt inhibitor VIII, ●) and in U0126 (25 μM)- and Akt inhibitor VIII (2 μM)-treated cells (○). Luciferase activity at 7 h p.i. is shown. Pseudovirus replication in mock-treated cells and in U0126- and Akt inhibitor VIII-treated cells was taken as 100%. (b) CC_{50} values of kinase inhibitors in the absence or presence of U0126 (25 μM) and Akt inhibitor VIII (2 μM).

effect was the replication process after uncoating. Treatment with kinase inhibitors did not induce the expression of OAS1 or STAT1 mRNA (Fig. 5c), suggesting that the inhibitory effect of these kinase inhibitors was independent of the IFN response.

Isolation of mutants resistant to receptor tyrosine kinase inhibitors

We attempted to isolate mutants of PV (Mahoney) resistant to receptor tyrosine kinase inhibitors, in order to identify their targets in the viral proteins. We examined Flt3 inhibitor II because the inhibitory effect of AG1478 was weak and IGF-1R inhibitor II showed relatively high cytotoxicity (Fig. 3b). We also examined GuaHCl (0.5–2.0 mM) treatment as a positive control for the isolation of

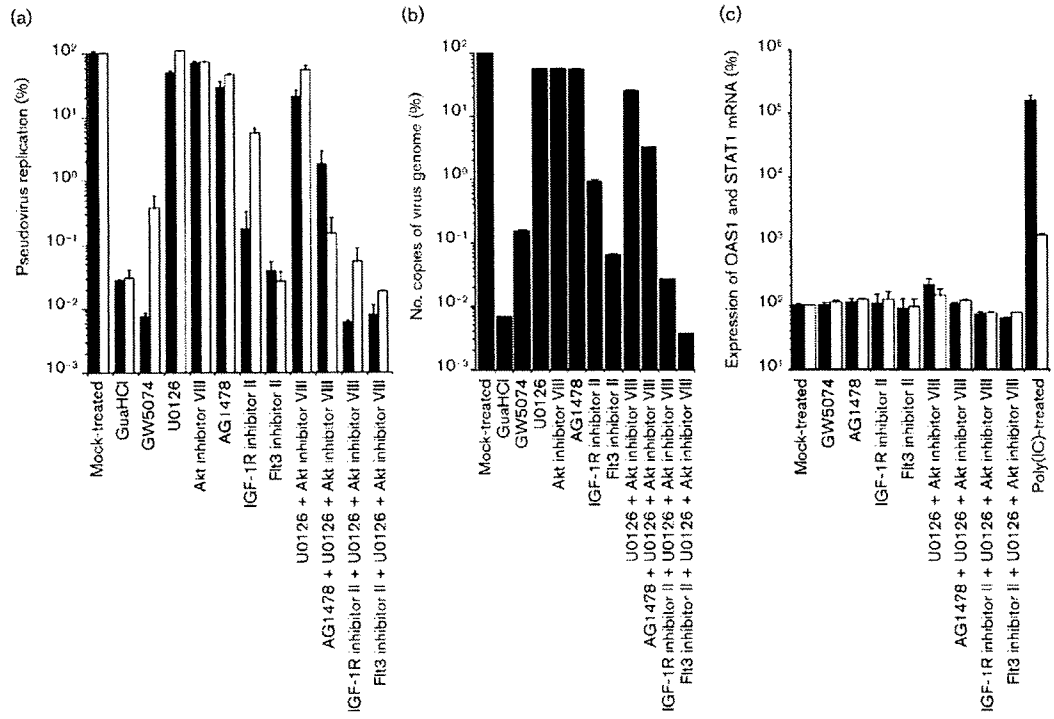


Fig. 5. Synthetic inhibitory effect of kinase inhibitors on EV71 pseudovirus and PV (Mahoney) infection. (a) Synthetic inhibitory effect of kinase inhibitors on PV and EV71 pseudovirus infection. RD cells (1.4×10^4) in a 96-well plate were inoculated with 200 IU PV and EV71 pseudoviruses in the presence of the indicated inhibitors: GuaHCl (2 mM), GW5074 (50 μM), U0126 (25 μM), Akt inhibitor VIII (2 μM), AG1478 (25 μM), IGF-1R inhibitor II (25 μM) and Flt3 inhibitor II (25 μM). Luciferase activity at 7 h p.i. (for PV pseudovirus infection, filled bars) and 10 h p.i. (for EV71 pseudovirus infection, empty bars) is shown. Pseudovirus replication in mock-treated cells was taken as 100 %. (b) Synthetic inhibitory effect of kinase inhibitors on PV (Mahoney) replication. RD cells (4.2×10^4) in a 24-well plate were infected with PV (Mahoney) at an m.o.i. of 5 and then incubated at 37 °C for 1 h in the absence of inhibitors. After washing, the cells were incubated with GuaHCl (2 mM), GW5074 (50 μM), U0126 (25 μM), Akt inhibitor VIII (2 μM), AG1478 (25 μM), IGF-1R inhibitor II (25 μM) and Flt3 inhibitor II (25 μM) at 37 °C for 7 h. Number of copies of the virus genome in infected cells at 8 h p.i. is shown. The number of copies of the virus genome in mock-treated cells (1.4×10^9 copies) was taken as 100 %. (c) IFN response in cells treated with kinase inhibitors. Relative expression levels of OAS1 (filled bars) and STAT1 (empty bars) mRNAs in the treated cells are shown. Poly(IC)-treated cells were taken as a positive control for the IFN response.

resistant mutants, and GW5074 (12.5–50 μM) treatment as a negative control for the isolation (Arita *et al.*, 2008b). After four passages of PV (Mahoney) in the presence of GuaHCl, clear CPE was observed in infected cells in the presence of GuaHCl (Fig. 6a). In contrast, CPE was not observed in infected cells during 12 passages in the presence of GW5074, and only weak CPE was observed in the presence of Flt3 inhibitor II (Fig. 6a; data not shown).

To confirm the inhibitory effect on replication, we analysed the copy number of the virus genome in infected cells. Surprisingly, substantial amounts of virus RNA were detected in infected cells inoculated with viruses after just four passages in the presence of GW5074 or Flt3 inhibitor II, as well as in those inoculated with a mutant resistant to GuaHCl (Fig. 6b). Moreover, these resistant mutants showed cross-resistance to each other, but not to GuaHCl. RD cells infected with the mutant resistant to GW5074 at an m.o.i. of 10–0.1 showed complete CPE at 24 h p.i. in the absence of GW5074 (Fig. 6c). However, the appearance of CPE in the infected cells was suppressed in the presence of GW5074 (at most 50 % of cells showed CPE when infected at an m.o.i. of 10, and no CPE was observed at m.o.i.s of 1.0 or 0.1), as observed in parental PV (Mahoney) infection. The genomic sequences of resistant mutants showed a common mutation in viral protein 3A [G (parental) to A (resistant mutants) at nt 5318] that caused an amino acid change at position 70 (Ala to Thr) (Fig. 6d). A PV pseudovirus mutant with this mutation [TE-PV-Fluc mc (5318A)] showed a phenotype resistant to GW5074 and Flt3 inhibitor II, as observed for the isolated resistant mutants, in contrast to its parental pseudovirus (TE-PV-Fluc mc) (Fig. 6e). However, TE-PV-Fluc mc (5318A) was still sensitive to GuaHCl and IGF-1R inhibitor II and to combined treatment with MEK1/2 and Akt1/2 inhibitors.

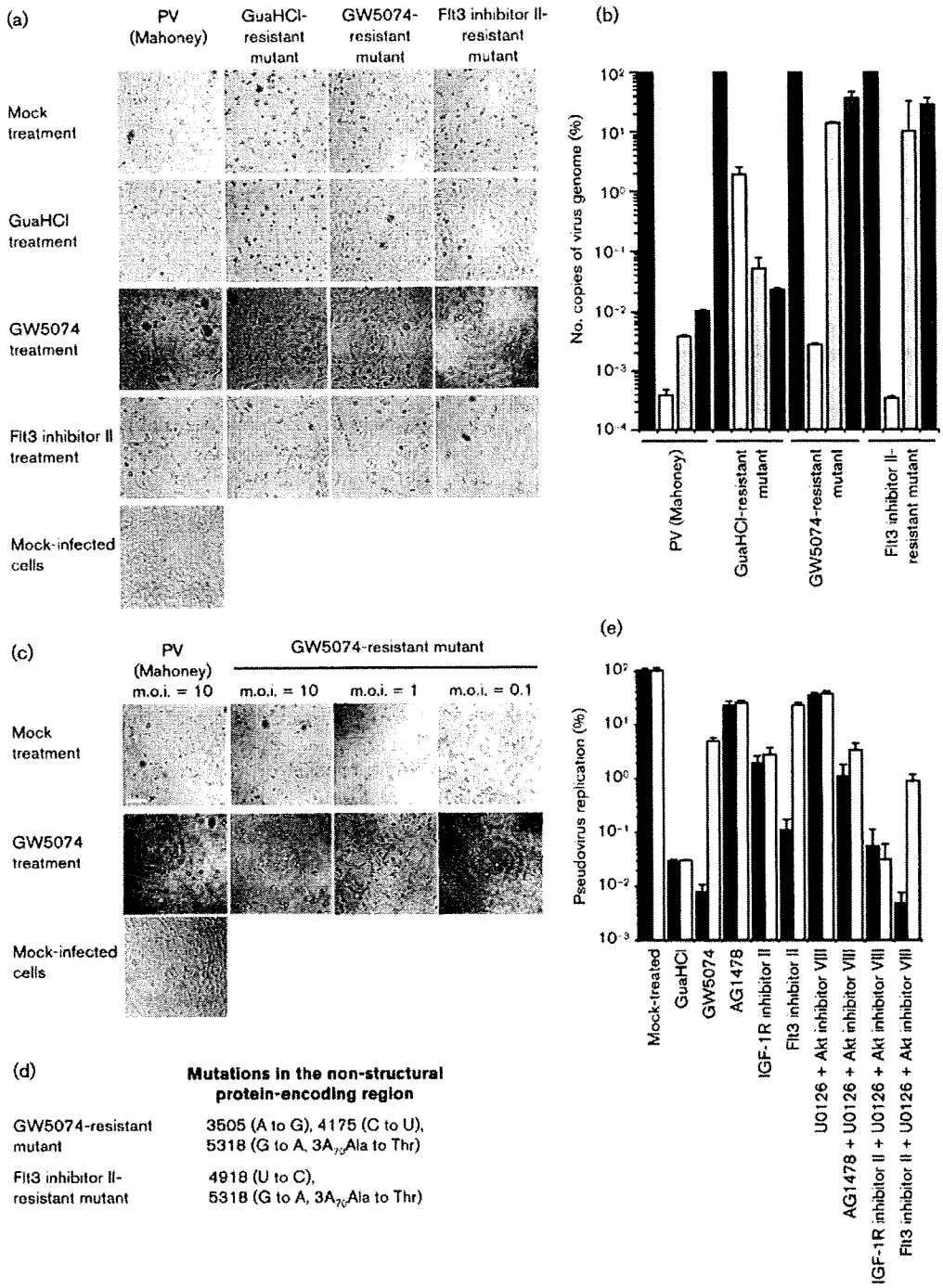
DISCUSSION

In this study, we identified kinase inhibitors that could suppress enterovirus replication either cooperatively with GW5074 or synthetically with other kinase inhibitors that did not show the inhibitory effect by individual treatment. We identified MEK1/2 inhibitors (SL327 and U0126), an EGFR inhibitor (AG1478) and a PI3K inhibitor (wortmannin) that showed a cooperative inhibitory effect with GW5074, and an Akt1/2 inhibitor (Akt inhibitor VIII) that showed a synthetic inhibitory effect with MEK1/2 inhibitors and AG1478, but not with wortmannin (Fig. 3a). We also identified GW2974, an inhibitor for EGFR and ERBB2 [which both belong to the EGFR family of receptor tyrosine kinases (Rusnak *et al.*, 2001)], and diacylglycerol kinase inhibitor I that showed a synthetic inhibitory effect with AG1478. GW2974 targets the same signalling pathway as AG1478, and thus would have an effect similar to that of AG1478. For diacylglycerol kinase inhibitors, a more active inhibitor (diacylglycerol kinase inhibitor II) did not show a synthetic inhibitory effect with AG1478 (data not shown). Therefore, we characterized the inhibitory effect of MEK1/2 inhibitors, AG1478, wortmannin and Akt1/2 inhibitor on enterovirus infection.

Combined treatment with MEK1/2 inhibitor, AG1478, wortmannin and Akt1/2 inhibitor showed a strong synthetic inhibitory effect (Fig. 3b). Individual treatment with these kinase inhibitors did not affect PV or EV71 infection, thus a synergy of these kinase inhibitors was essential for the inhibitory effect. This is consistent with a recent observation by Autret *et al.* (2008) that PI3K/Akt signalling does not affect the growth of PV. In EMCV infection, wortmannin treatment even increased viral protein synthesis and the yield of virus (Svitkin *et al.*, 1998). In contrast, treatment with an inhibitor for PI3K significantly reduced viral RNA synthesis and release of progeny viruses in CVB3 infection (Esfandiarei *et al.*, 2004). In CVB3 infection, a MEK1/2 inhibitor reduced viral protein synthesis and progeny virus release (Luo *et al.*, 2002). Therefore, the MEK/ERK and PI3K/Akt signalling pathways might affect enterovirus infection in different ways depending on the virus species, but seemed to have little effect on PV and EV71 replication, at least by themselves. The effect of wortmannin in the synthetic inhibitory effect was weaker than that of Akt inhibitor VIII, but partially compensated for the effect of Akt1/2 inhibitor (Fig. 3b), suggesting that the target of the synthetic inhibitory effect was Akt1/2. The concentrations of MEK1/2 inhibitor (25 μM for U01216) and Akt1/2 inhibitor (2 μM for Akt inhibitor VIII) tested are in physiologically relevant orders of magnitude: 20 μM for U0126 (Favata *et al.*, 1998) and 1–10 μM for Akt inhibitor VIII for its full inhibitory effect in cells (Logie *et al.*, 2007). In contrast, the specificity of AG1478 was not supported by the effective concentration needed for its synthetic inhibitory effect (25–50 μM), which is far higher than those reported for its full activity in cells (0.5–1.0 μM) (Isaacson *et al.*, 2007; Soeda *et al.*, 2008). The inhibitory effect of other receptor tyrosine kinase inhibitors, an IGF1R inhibitor (IGF-1R inhibitor II) and an Flt3 inhibitor (Flt3 inhibitor II), was enhanced in the presence of MEK1/2 inhibitor and Akt1/2 inhibitor (Fig. 4a). Therefore, MEK1/2 and Akt1/2 seemed to be involved as the targets of the synthetic inhibitory effect, but the identity of the target(s) of these receptor tyrosine kinase inhibitors remains unknown. Combined treatment with MEK1/2 inhibitor and Akt1/2 inhibitor reduced the CC_{50} of kinase inhibitors by about 3- to 10-fold (Fig. 4b), suggesting that induced cytotoxicity, as measured by the amount of ATP of metabolically active cells, might have a role in the synthetic inhibitory effect. However, the apparent viability of the cells was not impaired severely by combined treatment under the conditions tested, except for those treated with IGF-1R inhibitor II, which showed relatively high toxicity by itself (11–24 % of mock-treated cells; see Supplementary Fig. S1, available in JGV Online). Inhibition of Akt could sensitize tumour cells to apoptotic stimuli (DeFeo-Jones *et al.*, 2005) and enhance cytotoxicity with MEK inhibitor in lymphoma cells (Leleu *et al.*, 2007). Treatment with inhibitors for MEK1/2 (25 μM of U0126) and Akt1/2 (2 μM of Akt inhibitor VIII) did not affect the inhibitory effect of GuaHCl and GW5074, although the CC_{50} value of GW5074 was also reduced with these inhibitors (Fig. 4a). These observations suggested that combined treatment with inhibitors for MEK1/2 and Akt1/2 could enhance the inhibitory effect with some receptor tyrosine kinase inhibitors, but would not generally affect PV and EV71 replication. The property of individual kinase inhibitors might affect the synthetic inhibitory effect with inhibitors for MEK1/2 and Akt1/2.

We isolated mutants resistant to Flt3 inhibitor II and GW5074 (Fig. 6). Interestingly, these resistant mutants showed cross-resistance to each other, with a common resistant determinant in viral protein 3A at position 70 [Ala (parental strain) to Thr (resistant mutants)] (Fig. 6d, e). This mutation has been observed previously in a PV mutant resistant to enviroxime, which inhibits the initiation of positive-strand RNA synthesis of rhinoviruses and enteroviruses, although a direct interaction of enviroxime with the 3A protein has yet to be detected (Brown-Augsburger *et al.*, 1999; Heinz & Vance, 1995; Wikel *et al.*, 1980). A compound called TTP-8307, which has a property similar to that of enviroxime in terms of the resistance determinant in resistant mutants, was recently described (De Palma *et al.*, 2009). Interestingly, these 'enviroxime-like' compounds (enviroxime, TTP-8307, GW5074 and Flt3 inhibitor II) have little in common in their chemical structures. Our results suggest that a property of cellular kinase inhibitors would be an essential factor for an inhibitory effect similar to that of enviroxime.

Heinz & Vance (1995) isolated mutants resistant to enviroxime by a four-step enrichment procedure with enviroxime, because resistant mutants were not obtained by a conventional method that involved picking the mutant from plaques formed in the presence of the compound. Actually, all of the resistant mutants showed impaired growth in the presence of enviroxime, despite their resistant phenotype (Heinz & Vance, 1995). During



(d)

	Mutations in the non-structural protein-encoding region
GW5074-resistant mutant	3505 (A to G), 4175 (C to U), 5318 (G to A, 3A$_{70}$Ala to Thr)
Flt3 inhibitor II-resistant mutant	4918 (U to C), 5318 (G to A, 3A$_{70}$Ala to Thr)

the passage of PV in the presence of GW5074 and Flt3 inhibitor II, we did not observe marked CPE in infected cells, in contrast to those in the selection with GuaHCl (Fig. 6a) (Arita *et al.*, 2008b). Nevertheless, a substantial population of resistant mutants was detected within four passages in the presence of GW5074 or Flt3 inhibitor II, suggesting that the fitness of these resistant mutants was not completely recovered in the presence of these inhibitors to cause complete CPE in infected cells.

In summary, we identified cellular kinase inhibitors that suppress enterovirus replication. We found that MEK1/2 and Akt1/2 inhibitors showed an inhibitory effect on PV and EV71 replication when used in a combined treatment

Fig. 6. Characterization of mutants resistant to a receptor tyrosine kinase inhibitor. (a) Appearance of CPE in cells infected with resistant mutants. RD cells (1.4×10^4) in a 96-well plate were infected with mutants resistant to GuaHCl, GW5074 and Flt3 inhibitor II, obtained after four passages at an m.o.i. of 10 in the absence (mock treatment) and the presence of each inhibitor: GuaHCl (2 mM), GW5074 (50 μM) and Flt3 inhibitor II (25 μM). Phase-contrast views of the cells at 24 h p.i. are shown. Bar, 100 μm. (b) Replication of resistant mutants in the presence of inhibitors. RD cells (1.4×10^4) in a 96-well plate were infected with PV (Mahoney) or mutants resistant to GuaHCl, GW5074 and Flt3 inhibitor II obtained after four passages at an m.o.i. of 10, and then incubated at 37 °C for 1 h in the absence of inhibitors. The cells were washed three times and then incubated in the absence (mock-treated, filled bars) or the presence of inhibitors: GuaHCl (2 mM, empty bars), GW5074 (50 μM, light-shaded bars) and Flt3 inhibitor II (25 μM, dark-shaded bars). Number of copies of the virus genome at 8 h p.i. is shown. The number of copies of the virus in mock-treated cells infected with each virus was taken as 100%. (c) Appearance of CPE in cells infected with a mutant resistant to GW5074 at different m.o.i.s. RD cells were infected with PV (Mahoney) and a mutant resistant to GW5074, obtained after four passages, at m.o.i.s of 10, 1.0 and 0.1 in the absence (mock-treated) and the presence of GW5074 (50 μM). Phase-contrast views of the cells at 24 h p.i. are shown. Bar, 100 μm. (d) Mutations observed in the non-structural protein-encoding region of the virus genome of resistant mutants. (e) Effect of combined treatment with kinase inhibitors on the replication of a PV pseudovirus mutant with the resistance mutation at nt 5318 [TE-PV-Fluc mc (5318A); empty bars] compared with its parental pseudovirus (TE-PV-Fluc mc; filled bars). RD cells (1.4×10^4) in a 96-well plate were inoculated with 200 IU PV pseudovirus in the presence of inhibitors: GuaHCl (2 mM), GW5074 (50 μM), U0126 (25 μM), Akt inhibitor VIII (2 μM), AG1478 (50 μM), IGF-1R inhibitor II (25 μM) and Flt3 inhibitor II (25 μM). Luciferase activity at 8 h p.i. is shown. Pseudovirus replication in mock-treated cells was taken as 100%.

with receptor tyrosine kinase inhibitors. We also found that GW5074, receptor tyrosine kinase inhibitors and enviroxime have a conserved target and/or mechanism of the inhibitory effect. Our results suggest that cellular kinase inhibitors could serve as a promising resource for the identification of anti-enterovirus compounds.

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Development of a reverse transcription-loop-mediated isothermal amplification (RT-LAMP) system for a highly sensitive detection of enterovirus in the stool samples of acute flaccid paralysis cases

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Abstract

Background: In the global eradication program for poliomyelitis, the laboratory diagnosis plays a critical role by isolating poliovirus (PV) from the stool samples of acute flaccid paralysis (AFP) cases. In this study, we developed a reverse transcription-loop-mediated isothermal amplification (RT-LAMP) system for a rapid and highly sensitive detection of enterovirus including PV to identify stool samples positive for enterovirus including PV.

Methods: A primer set was designed for RT-LAMP to detect enterovirus preferably those with PV-like 5'NTRs of the viral genome. The sensitivity of RT-LAMP system was evaluated with prototype strains of enterovirus. Detection of enterovirus from stool extracts was examined by using RT-LAMP system.

Results: We detected at least 400 copies of the viral genomes of PV(Sabin) strains within 90 min by RT-LAMP with the primer set. This RT-LAMP system showed a preference for *Human enterovirus species C* (HEV-C) strains including PV, but exhibited less sensitivity to the prototype strains of HEV-A and HEV-B (detection limits of 7,400 to 28,000 copies). Stool extracts, from which PV, HEV-C, or HEV-A was isolated in the cell culture system, were mostly positive by RT-LAMP method (positive rates of 15/16 (= 94%), 13/14 (= 93%), and 4/4 (= 100%), respectively). The positive rate of this RT-LAMP system for stool extracts from which HEV-B was isolated was lower than that of HEV-C (positive rate of 11/21 (= 52%)). In the stool samples, which were negative for enterovirus isolation by the cell culture system, we found that two samples were positive for RT-LAMP (positive rates of 2/38 (= 5.3%)). In these samples, enterovirus 96 was identified by sequence analysis utilizing a seminested PCR system.

Conclusions: RT-LAMP system developed in this study showed a high sensitivity comparable to that of the cell culture system for the detection of PV, HEV-A, and HEV-C, but less sensitivity to HEV-B. This RT-LAMP system would be useful for the direct detection of enterovirus from the stool extracts.

Background

In the global eradication program for poliomyelitis, the laboratory diagnosis plays a critical role by isolating poliovirus (PV) from the stool samples of acute flaccid paralysis (AFP) cases. The isolation procedure of PV have been established based on the cell culture system using a human rhabdomyosarcoma cell line (RD cells) and a mouse L cell line expressing PV receptor (L20B cells) [1,2]. The advantages of cell culture-based procedure are; 1) apparatuses for molecular diagnosis are not required, and 2) a high sensitivity (detection limit of 1 infectious dose that contains 50 to 1,000 virions in picornavirus infection) [3]. The disadvantage is that some expertise and quality control system are required for the cell culture system and for the identification of the cytopathic effect of infected cells. As for the timeliness of reporting, the cell culture-based procedure is time-consuming. It takes for 10 days to confirm the sample as PV-negative even after the introduction of the latest procedure "New Algorism" recommended by WHO [2]. Currently, detection of the circulating vaccine-derived PV (cVDPV) has a high priority in the eradication program and will be in the post-eradication era. Therefore, rapid (at the order of day) and sensitive detection of PV in laboratory diagnosis could contribute to shortening of the timeliness of reporting for mop-up vaccine campaign to control cVDPV outbreaks.

Among currently available procedures detecting RNA viruses, a reverse transcription-loop-mediated isothermal amplification (RT-LAMP) system seems to be a most promising method that meet the demands expected for the cell culture-based isolation procedure [4]. The advantages of RT-LAMP system are; 1) minimum essential equipment is an isothermal heat bath (final results can be visibly observed by the increased turbidity)[5], 2) high sensitivity (detection limits of 0.01 PFU for severe acute respiratory syndrome coronavirus, 0.1 PFU for mumps virus, 0.4 focus forming units for hepatitis A virus, 50 copies of viral genomes for swine vesicular disease virus) [6-9], 3) rapid detection (about 1 h), 4) less possibility of cross-contamination between the samples due to the one-step procedure.

In the present study, we have developed a RT-LAMP system for the detection of enterovirus, including PV. This RT-LAMP system showed a high sensitivity comparable to that of the cell culture system for the detection of PV, HEV-A, and HEV-C, but less sensitivity to HEV-B. This RT-LAMP system would be useful for the direct detection of enterovirus from the stool extracts.

Results

RT-LAMP primers for the detection of PV

To detect PV by RT-LAMP methods, we analyzed the 5'NTR for the design of the primers (Figure 1a). 5'NTR is known to be classified into two phylogenetic groups based on the primary structure, PV-like or CBV-like 5'NTR [10,11]. PV-like 5'NTR is observed for enteroviruses belonging to *Human enterovirus species* C (HEV-C) and HEV-D, and CBV-like 5'NTR is observed for those belonging to HEV-A and HEV-B, respectively [11]. Therefore, we designed the primer sets to detect PV-like 5'NTR according to conditions required for the primer in RT-LAMP reaction in terms of the location and Tm values of the primers http://loopamp.eiken.co.jp/lamp/primer.html (Figure 1b). Among the 5 primers used in the RT-LAMP reaction, 2 primers were preferable (a complete match for PV-like 5'NTR near the 3' end of the DNA fragment generated in RT-LAMP reaction, FIP primer) or specific (a complete match for PV-like 5'NTR at the 3' end of the DNA fragment generated in RT-LAMP reaction, BIP primer) to PV-like 5'NTR (Figure 2). Other 3 primers (F, B, Loop B primers) were designed with conserved sequences between PV-like and CBV-like 5'NTRs.

Sensitivity of RT-LAMP system for the detection of PV

First, the sensitivity of RT-LAMP reaction was examined by using purified viral RNA of PV(Sabin) strains (Figure 3a). In the RT-LAMP reaction, 400 copies of viral genome were detected for all the PV(Sabin) strains (4/4), and 40 copies of viral genome were detected in some samples (1/4 to 3/4). Signals of RT-LAMP were detected within 50 min of the reaction for samples with 400 copies of viral genomes (Figure 3b). For samples with 40 copies of viral genomes, the signals were detected as late as 50 to 73 min.

Next, we examined the sensitivity of RT-LAMP system for enterovirus species by using cell culture supernatant of the virus without viral RNA extraction (Figure 3c). Cell culture supernatant of cells infected with PV(Sabin) strains were RT-LAMP positive at dilution of 1:10,000,000, which contains at least 0.73 to 2.4 $CCID_{50}$ of viruses (about 100 copies of the viral genomes). Echovirus 11 (belonging to HEV-B) and enterovirus 71 (EV71) strains (belonging to HEV-A) showed lower sensitivity in the RT-LAMP reaction compared to PV(Sabin) strains. The detection limit of echovirus 11 and EV71 strains were 17 $CCID_{50}$ (28,000 copies of the viral genome) and 7.8 to 53 $CCID_{50}$ (7,400 to 13,000 copies of the viral genome), respectively.

Finally, we examined to detect PV from the stool samples of AFP cases (Figure 3d). Stool samples that were positive for PV (16 samples), HEV-A (4 samples), -B (21 samples), and -C (14 samples), or negative for enterovirus (38 samples) by cell culture-based isolation were examined. PV and HEV-C were detected with high positive rates in the stool samples by RT-LAMP (94 and 93%, respectively). Unexpectedly, HEV-A was also detected with a high positive rate (100%), and HEV-B was also detected with a relatively high positive rate (52%). For stool samples

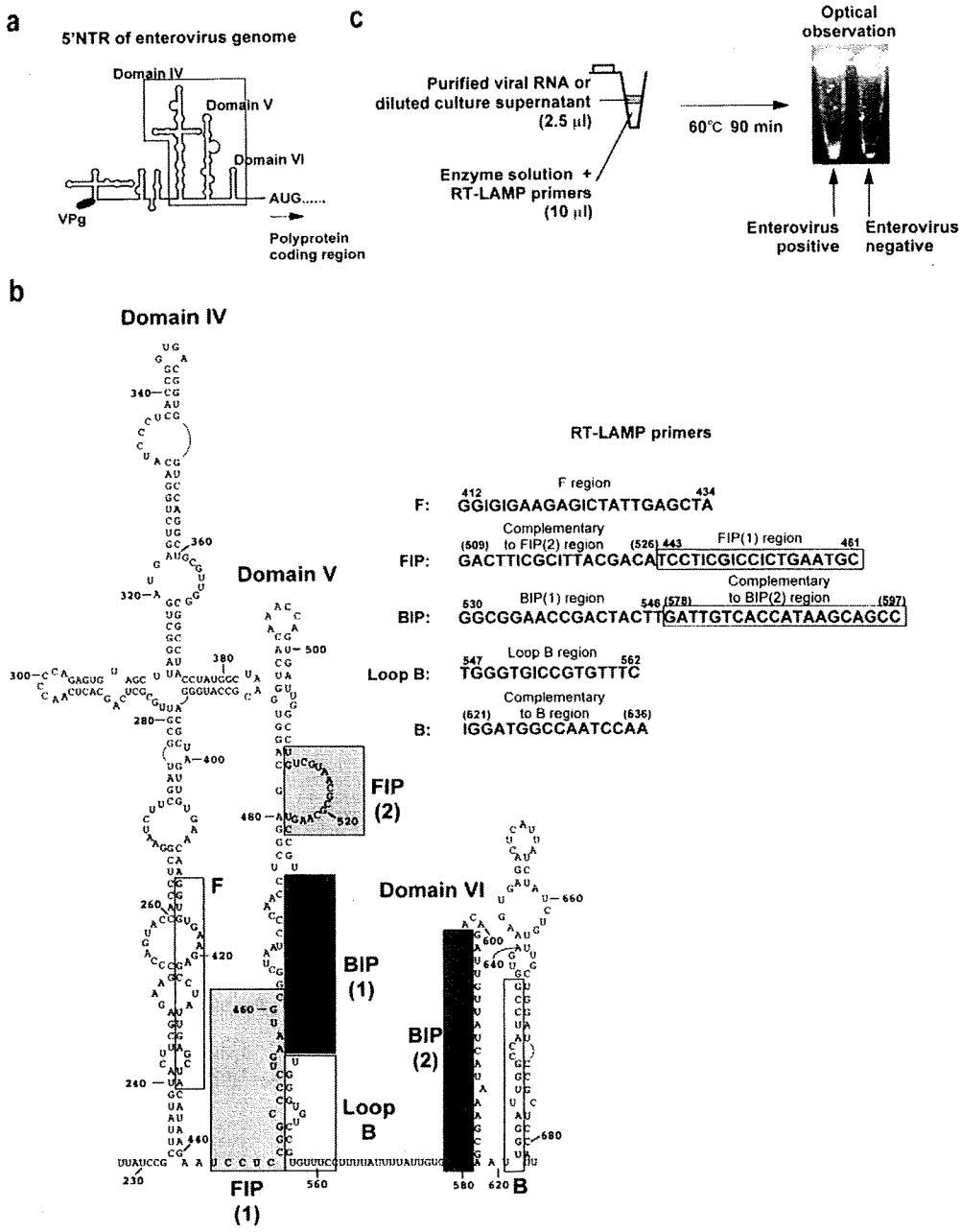


Figure 1

Regions in the 5'NTR of enterovirus genome examined for the design of RT-LAMP primers. a Schematic view of a model of the secondary structure of 5'NTR of enterovirus genome [22-24]. The region examined for the design of RT-LAMP primers is shown in a box. **b** Primary and secondary structure of 5'NTR of PV1(Mahoney) genome and RT-LAMP primers used in this study. The structure is based on the model proposed by Pilipenko et al. [22]. The region examined for RT-LAMP primers is shown in boxes on the secondary structure. The numbers on the RT-LAMP primers represent corresponding nucleotide positions on the 5'NTR. For primers that have complimentary sequence to the 5'NTR, the numbers are shown in parenthesis. **c** Scheme of RT-LAMP procedure examined in this study.

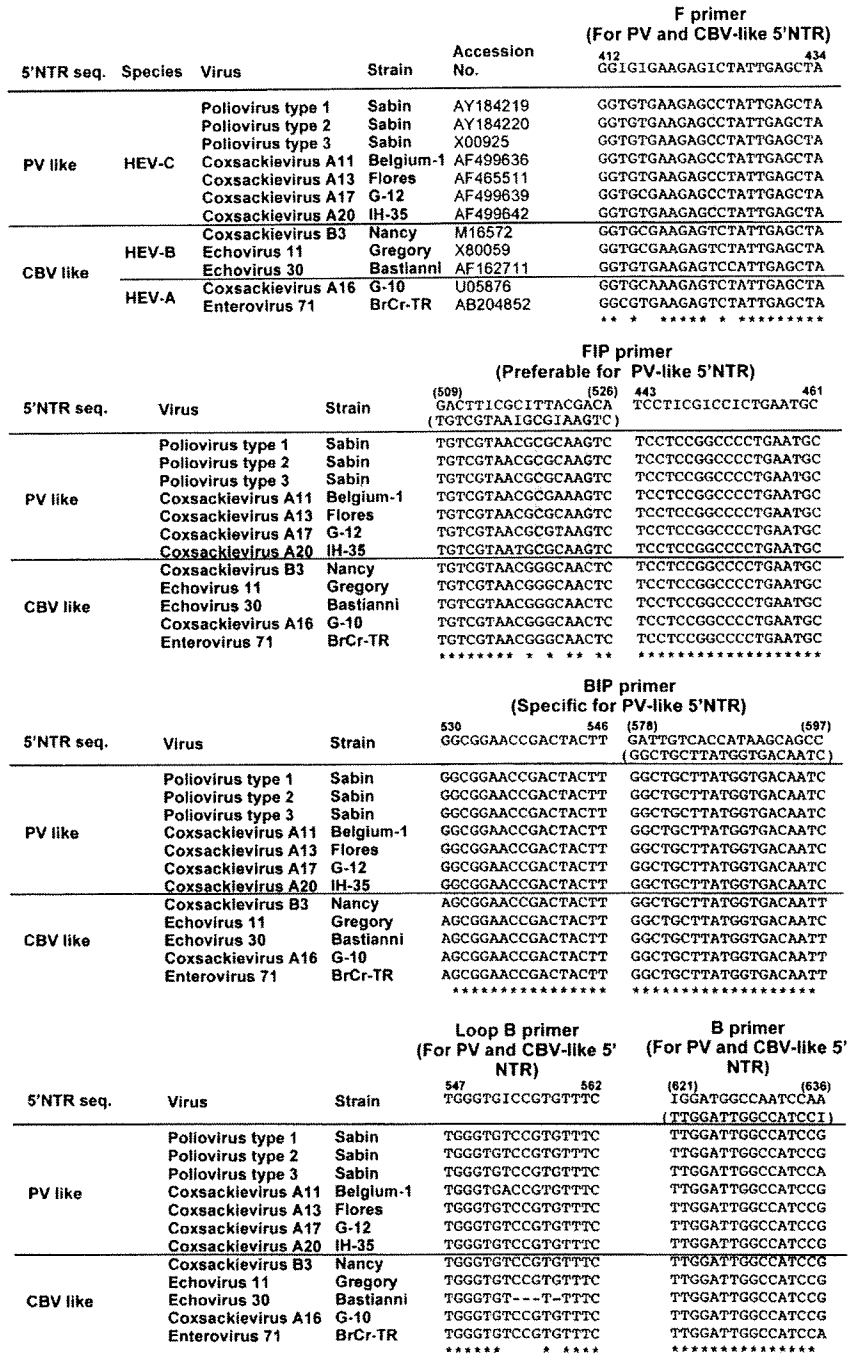


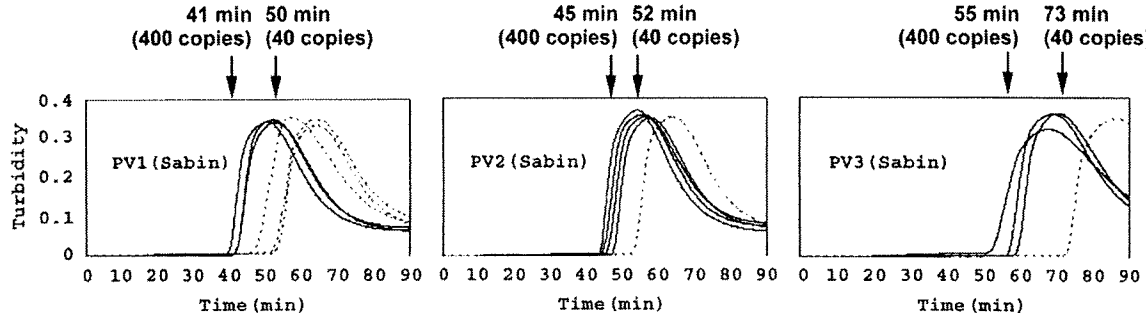
Figure 2

Comparison of the nucleotide sequences of enterovirus genomes examined for RT-LAMP primers. Enterovirus genomes are classified into PV-like and CBV-like 5'NTR [10,11]. The nucleotides characteristic to PV-like 5'NTR are highlighted in boxes colored by gray. Primers that have complete match for PV-like 5'NTR near and at the 3' end are presented as preferable and specific primers to PV-like 5'NTR, respectively.

a

Purified viral RNA	Number of copies of viral genome (in 2.5 μl) 400	40	0
PV 1 (Sabin)	4 / 4	3 / 4	-
PV 2 (Sabin)	4 / 4	1 / 4	-
PV 3 (Sabin)	4 / 4	1 / 4	-
Distilled water	-	-	0 / 8

b



c

Cell culture supernatant	Dilution of culture supernatant 10^{-3}	10^{-4}	10^{-5}	10^{-6}	10^{-7}	10^{-8}	Number of copies of viral genome per $CCID_{50}$
PV 1 (Sabin)	NT	NT	NT	NT	4 / 4 (2.4)	1 / 4 (0.24)	56
PV 2 (Sabin)	NT	NT	NT	NT	3 / 4 (1.7)	0 / 4 (0.17)	110
PV 3 (Sabin)	NT	NT	NT	NT	3 / 4 (0.73)	0 / 4 (0.073)	153
Echovirus 11 (Gregory)	NT	4 / 4(170)	2 / 4(17)	0 / 2(1.7)	NT	NT	1,650
EV71(BrCr-TR)	NT	3 / 4(7.8)	0 / 4(0.78)	0 / 2(0.078)	NT	NT	1,700
EV71(Nagoya)	4 / 4(530)	3 / 4(53)	0 / 2(5.3)	NT	NT	NT	140

d

Stool extract	Number of RT-LAMP-positive sample / Total number of sample	% of positive sample
PV isolated	15 / 16	94
HEV-A isolated	4 / 4	100
HEV-B isolated	11 / 21	52
HEV-C isolated	13 / 14	93
Negative for NPEV isolation	2 / 38	5.3

Figure 3

Sensitivity and specificity of RT-LAMP system. **a** Sensitivity of RT-LAMP system for purified viral RNA of PV(Sabin) strains. **b** Kinetics of the detection in RT-LAMP system. The average time required for the detection of the signals is shown for each numbers of the copies. **c** Sensitivity and specificity of RT-LAMP system for enterovirus. Cell culture supernatants of the cells infected with enteroviruses were used for the detection of the viral RNA by RT-LAMP system. The numbers in the parenthesis show the titre of virus ($CCID_{50}$) included in the RT-LAMP reactions. The numbers of copies of the viral genome per $CCID_{50}$ are also shown for each virus. NT, not tested. **d** Sensitivity and specificity of RT-LAMP system for the viral RNA purified from stool extracts of AFP cases.

negative for enterovirus, 2 samples (derived from one AFP case) were positive by RT-LAMP. For these samples, enterovirus 96 was identified by sequence analysis of VP1 coding region utilizing a seminested PCR [12] (data not shown). Therefore, this RT-LAMP system showed a good correlation with the cell culture-based isolation especially for PV, HEV-C, and HEV-A.

We analyzed the sequence of HEV-B isolates (CAM2515 and CAM2549) that were positive for RT-LAMP, and for a HEV-C (CAM2730) and a PV2 (CAM2553) strains that were negative for RT-LAMP (Figure 4). The 5'NTR of these HEV-B isolates showed mixed genetic properties of PV-like 5'NTR and CBV-like 5'NTR. The 5'NTR of CAM2515 showed CBV-like sequence in the FIP primer-binding region, but has a PV-like sequence in the region for BIP primer. The 5'NTR of another HEV-B isolate CAM2549 and a HEV-C isolate CAM2730 showed similar sequence to PV-like 5'NTR in these regions. The sequence of the 5'NTR of PV2 isolate CAM2553 was similar to its parental PV2(Sabin).

Discussion

In this study, we have developed a RT-LAMP system for a rapid and highly sensitive detection of enterovirus including PV directly from stool samples of AFP cases without cell culture-based procedures. 5'NTR of enteroviruses is classified into two groups based on its primary structure, PV-like or CBV-like 5'NTR [10,11]. Actually, a RFLP assay utilizing *Bst*OI have been developed to differentiate these genogroups [13]. We designed RT-LAMP primers preferably to detect PV-like 5'NTR rather than PV-specific primers, because we could not find nucleotide sequences specific to PV strains but not to other HEV-C strains in the 5'NTR. Actually, cVDPVs with unknown nucleotide sequences in the 5'NTR, which was probably derived from the viral genome of other non-polio enterovirus, were isolated [14].

In the primers examined in this study, the specificity to PV-like 5'NTR was defined by 2 primers (FIP and BIP primers, Figure 2). The sequences of primers we used might detect most of the cVDPVs or immunodeficient

FIP primer (Preferable for PV-like 5'NTR)

5'NTR seq.	Virus	Strain	(509) GACTTICGCITTACGACA (526) (TGTCGTAAIGCGIAAGTC)	443 TCCTICGICCICTGAATGC 461
CBV like	HEV-B isolate	CAM2515	TGTCGTAATGGGCAACTC	TCCTCCGGCCCCTGAATGC
PV like	HEV-B isolate	CAM2549	TGTCGTGATGCGTAAGTC	TCCTCCGGCCCCTGAATGC
PV like	HEV-C isolate	CAM2730	TGTCGTAACGCGTGAGTC	CCCTCCGGCCCCTGAATGC
PV like	Poliovirus type 2	CAM2553	TGTCGTAACGCGCAAGTC	TCCTCCGGCCCCTGAATGC
PV like	Poliovirus type 1	Sabin	TGTCGTAACGCGCAAGTC	TCCTCCGGCCCCTGAATGC
CBV like	Echovirus 11	Gregory	TGTCGTAACGGGCAACTC	TCCTCCGGCCCCTGAATGC
			******** * * ** **	******************

BIP primer (Specific forPV-like 5'NTR)

5'NTR seq.	Virus	Strain	530 GGCGGAACCGACTACTT 546	(578) GATTGTCACCATAAGCAGCC (597) (GGCTGCTTATGGTGACAATC)
PV like	HEV-B isolate	CAM2515	GGCGGAACCGACTACTT	GGCTGCTTATGGTGACAATT
PV like	HEV-B isolate	CAM2549	GGCGGAACCGACTACTT	GGCTGCTTATGGTGACAATC
PV like	HEV-C isolate	CAM2730	GGCGGAACCGACTACTT	GGCTGCTTATGGTGACAATC
PV like	Poliovirus type 2	CAM2553	GGCGGAACCGACTACTT	GGCTGCTTATGGTGACAATC
PV like	Poliovirus type 1	Sabin	GGCGGAACCGACTACTT	GGCTGCTTATGGTGACAATC
CBV like	Echovirus 11	Gregory	AGCGGAACCGACTACTT	GGCTGCTTATGGTGACAATC
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Figure 4
Comparison of the nucleotide sequences of the 5'NTR in the viral genomes of enterovirus isolates. The nucleotides characteristic to PV-like 5'NTR are highlighted in boxes colored by gray.

VDPVs, which were circulating or infecting for about 2 to 10 years [14-16] (Figure 5). However, one cVDPV strain (EGY88-074), which was isolated in an early stage of the circulation in Egypt with its 5'NTR that was probably derived from other enterovirus genomes by recombination [14], contained a different nucleotide at the 3' end of F primer. This nucleotide change was not observed for another cVDPV strain (EGY93-034), which was isolated in the late stage of the circulation, suggesting that this nucleotide change was not stable during the circulation. Therefore, some cVDPV isolates with this rare mutation might not be detected with the primer set examined in this study.

We observed the specificity to PV-like 5'NTR to some extent compared to CBV-like 5'NTR in the RT-LAMP reaction, where about 100-fold difference was observed in the sensitivity (Figure 3c). However, detection of enteroviruses from stool samples showed only slightly lower positive rates for HEV-A and HEV-B (100 and 52%, respectively) compared to those for PV and other HEV-C (94 and 93%, respectively) (Figure 3d). The relatively low positive rates of HEV-B among these virus species might depend on the designed specificity of RT-LAMP to PV-like 5'NTR. Sequence analysis of 5'NTR of HEV-B isolates indicated that these field isolates could have some genetic features similar to PV-like 5'NTR (Figure 4). Therefore, with relatively low specificity of RT-LAMP system (100-fold difference between PV-like and CBV-like 5'NTR), the mixed genetic features of the 5'NTR of HEV-B field isolates might have affected the specificity of the RT-LAMP system.

An essential factor of the sensitivity of RT-LAMP system for the detection of PV from stool extracts seems to be the amount of viral RNA available for the reaction in addition to the quality of the RNA. We found one stool sample (CAM2553) was negative by the RT-LAMP among the 16 stool extract that was positive for PV by cell culture (Figure 3d). The 5' NTR of the PV2(CAM2553) did not have any nucleotide changes from that of its parental PV2(Sabin) strain (Figure 4). It is plausible that the low amount of viral RNA in the sample caused this false-negative result under the detection limit of RT-LAMP. The amounts of PV in the stool extracts were not generally high (< $10^{0.5}$ to $10^{2.5}$ $CCID_{50}$/50 μl, Table 1). In the RT-LAMP reaction examined in this study, we purified viral RNA from 200 μl of stool extract and collected in 50 μl of elusion buffer (4-fold concentration by this procedure), and then 2.5 μl of this purified viral RNA solution was used for RT-LAMP reaction. Therefore, the net amount of viral RNA used in RT-LAMP reaction corresponds to that contained in 10 μl of stool extract. For the isolation of PV, 200 μl of stool extract is inoculated into the cells. Therefore, there is 20-fold difference in the available viral RNA or the infectious units between the RT-LAMP system and the cell culture system because of the intrinsic difference of the scale of the assay (12.5 μl vs. 1.0 ml). Because of a high particle-to-infective-unit ratio of PV, which was estimated as 56 to 153 copies of viral genome per $CCID_{50}$ in this study (Figure 3c), relatively high sensitivity was attained in RT-LAMP system almost comparable to that of cell culture-based isolation. It should be noted that the sensitivity of this RT-LAMP system (< 400 copies) was not high compared to those of optimized RT-LAMP systems (1-100 copies), and was lower than that of a conventional real-time PCR system [17] (< 10 copies). Additional procedures to increase the concentration of viral RNA and to improve the quality of RNA would be helpful to improve the sensitivity of the RT-LAMP system for the detection of PV from stool extracts.

Conclusions

In summary, we developed a highly sensitive RT-LAMP system for the detection of enterovirus, including PV, from the stool extracts. The cell culture-based isolation will be needed for genetic characterization of PV isolates, particularly differentiation of wild, VDPV, and mixtures of PV and enterovirus. The RT-LAMP system would be useful for a triage of overwhelming number of clinical samples to reduce the workload and to minimize the timeliness of the report by identifying the samples negative for PV within a day.

Methods

Cells, viruses, and clinical samples

RD cells (human rhabdomyosarcoma cell line) were cultured as monolayers in Dulbecco's modified Eagle medium (DMEM) supplemented with 10% fetal calf serum (FCS) and used for titration of viruses. Virus titre was determined by measuring 50% cell culture infectious dose ($CCID_{50}$) at 35°C by a microtitration assay [18]. Stool extracts from AFP cases were used for isolation of enterovirus and for RT-LAMP reaction. The species and serotypes of enterovirus isolates were determined by sequencing of the viral genome with a primer set for 2BC coding region (2A2+ and 2C-primers) [19] and with those for VP1 coding region (292 and 222 primers) [20]. All the clinical samples and virus isolates used in this study are appropriately anonymized. Therefore, they are exempt from the regulation under the Committee for Ethical Regulation of the National Institute of Infectious Diseases.

RNA purification

Viral genomic RNA was purified from the stool extracts of AFP cases by using a High Pure viral RNA purification kit (Roche). In this purification procedure, viral genomic RNA was collected in 50 μl of distilled water purified from 200 μl of stool extracts.

RT-LAMP reaction

Primers used in this study are shown in Figure 1 (Figure 1b). Stocks of the primers were prepared in distilled water

Virus	Strain	Accession No.	F primer (For PV and CBV-like 5'NTR) 412 GGIGIGAAGAGICTATTGAGCTA 434
Poliovirus type 1	Sabin	AY184219	GGTGTGAAGAGCCTATTGAGCTA
Poliovirus type 2	Sabin	AY184220	GGTGTGAAGAGCCTATTGAGCTA
Poliovirus type 3	Sabin	X00925	GGTGTGAAGAGCCTATTGAGCTA
Poliovirus type 1	DOR00013	AF405690	GGTGTGAAGAGCCTATTGAGCTA
Poliovirus type 1	DOR00041C1	AF405682	GGTGTGAAGAGCCTATTGAGCTA
Poliovirus type 1	HAI00003	AF405669	GGTGTGAAGAGCCTATTGAGCTA
Poliovirus type 1	HAI01007	AF405666	GGTGTGAAGAGCCTATTGAGCTA
Poliovirus type 2	EGY88-074	AF448782	GGTGTGAAGAGCCTATTGAGCTG
Poliovirus type 2	EGY93-034	AF448783	GGTGTGAAGAGCCTATTGAGCTA
Poliovirus type 1	A21	EU794953	GGTGTGAAGAGCCTATTGAGCTA
Poliovirus type 1	A649	EU794964	GGTGTGAAGAGCCTATTGAGCTA
			**********

Virus	Strain	FIP primer (Preferable for PV-like 5'NTR) (509) GACTTICGCITTACGACA (526) (TGTCGTAAIGCGIAAGTC)	443 TCCTICGICCICTGAATGC 461
Poliovirus type 1	Sabin	TGTCGTAACGCGCAAGTC	TCCTCCGGCCCCTGAATGC
Poliovirus type 2	Sabin	TGTCGTAACGCGCAAGTC	TCCTCCGGCCCCTGAATGC
Poliovirus type 3	Sabin	TGTCGTAACGCGCAAGTC	TCCTCCGGCCCCTGAATGC
Poliovirus type 1	DOR00013	TGTCGTAACGCGCAAGTC	TCCTCCGGCCCCTGAATGC
Poliovirus type 1	DOR00041C1	TGTCGTAACGCGCAAGTC	TCCTCCGGCCCCTGAATGC
Poliovirus type 1	HAI00003	TGTCGTAACGCGCAAGTC	TCCTCCGGCCCCTGAATGC
Poliovirus type 1	HAI01007	TGTCGTAACGCGCAAGTC	TCCTCCGGCCCCTGAATGC
Poliovirus type 2	EGY88-074	TGTCGTAACGCGTAAGTC	TCCTCCGGCCCCTGAATGC
Poliovirus type 2	EGY93-034	TGTCGTAACGCGTAAGTC	TCCTCCGGCCCCTGAATGC
Poliovirus type 1	A21	TGTCGTAACGCGCAAGTC	TCCTCCGGCCCCTGAATGC
Poliovirus type 1	A649	TGTCGTAACGCGCAAGTC	TCCTCCGGCCCCTGAATGC
		********** *****	**********

Virus	Strain	BIP primer (Specific for PV-like 5'NTR) 530 GGCGGAACCGACTACTT 546	(578) GATTGTCACCATAAGCAGCC (597) (GGCTGCTTATGGTGACAATC)
Poliovirus type 1	Sabin	GGCGGAACCGACTACTT	GGCTGCTTATGGTGACAATC
Poliovirus type 2	Sabin	GGCGGAACCGACTACTT	GGCTGCTTATGGTGACAATC
Poliovirus type 3	Sabin	GGCGGAACCGACTACTT	GGCTGCTTATGGTGACAATC
Poliovirus type 1	DOR00013	GGCGGAACCGACTACTT	GGCTGCTTATGGTGACAATC
Poliovirus type 1	DOR00041C1	GGCGGAACCGACTACTT	GGCTGCTTATGGTGACAATC
Poliovirus type 1	HAI00003	GGCGGAACCGACTACTT	GGCTGCTTATGGTGACAATC
Poliovirus type 1	HAI01007	GGCGGAACCGACTACTT	GGCTGCTTATGGTGACAATC
Poliovirus type 2	EGY88-074	GGCGGAACCGACTACTT	GGCTGCTTATGGTGACAATC
Poliovirus type 2	EGY93-034	GGCGGAACCGACTACTT	GGCTGCTTATGGTGACAATC
Poliovirus type 1	A21	GGCGGAACCGACTACTT	GGCTGCTTATGGTGACAATC
Poliovirus type 1	A649	GGCGGAACCGACTACTT	GGCTGCTTATGGTGACAATC
		**********	**********

Virus	Strain	Loop B primer (For PV and CBV-like 5' NTR) 547 TGGGTGICCGTGTTTC 562	B primer (For PV and CBV-like 5' NTR) (621) IGGATGGCCAATCCAA (636) (TTGGATTGGCCATCCI)
Poliovirus type 1	Sabin	TGGGTGTCCGTGTTTC	TTGGATTGGCCATCCG
Poliovirus type 2	Sabin	TGGGTGTCCGTGTTTC	TTGGATTGGCCATCCG
Poliovirus type 3	Sabin	TGGGTGTCCGTGTTTC	TTGGATTGGCCATCCA
Poliovirus type 1	DOR00013	TGGGTGACCGTGTTTC	TTGGATTGGCCATCCG
Poliovirus type 1	DOR00041C1	TGGGTGTCCGTGTTTC	TTGGATTGGCCATCCG
Poliovirus type 1	HAI00003	TGGGTGTCCGTGTTTC	TTGGATTAGCCATCCG
Poliovirus type 1	HAI01007	TGGGTGTCCGTGTTTC	TTGGATTGGCCATCCG
Poliovirus type 2	EGY88-074	TGGGTGTCCGTGTTTC	TTGGATTGGCCATCCG
Poliovirus type 2	EGY93-034	TGGGTGTCCGTGTTTC	TTGGATTGGCCATCCG
Poliovirus type 1	A21	TGGGTGTCCGTGTTTC	TTGGATTGGCCATCCG
Poliovirus type 1	A649	TGGGTGTCCGTGTTTC	TTGGATTGGCCATCCG
		**********	******* *******

Figure 5
Comparison of the nucleotide sequences of the regions in the viral genomes of cVDPV and iVDPV strains examined for RT-LAMP primers. The nucleotides characteristic to PV-like 5'NTR are highlighted in boxes colored by gray.

Table 1: Titre of PV in stool samples

Stool sample	Isolated PV	Virus titre ($CCID_{50}$/50 μl)
CAM2553	PV2	$< 10^{0.5}$
CAM2554	PV1+2	$< 10^{0.5}$
CAM2885	PV3+NPEV	$< 10^{0.5}$
CAM2896	PV2	$10^{1.5}$
CAM2897	PV2	$10^{1.75}$
CAM2906	PV3	$10^{0.75}$
CAM2907	PV3	$10^{0.75}$
CAM2936	PV3+NPEV	$< 10^{0.5}$
CAM2937	PV3+NPEV	$< 10^{0.5}$
CAM2970	PV1+PV3	$10^{0.75}$
CAM2995	PV3+NPEV	$< 10^{0.5}$
CAM2996	PV3	$10^{2.5}$
CAM3017	PV2	$< 10^{0.5}$
CAM3018	PV2	$10^{1.5}$
CAM3044	PV2	$< 10^{0.5}$
CAM3045	PV2	$10^{1.5}$

in concentrations as follows; 40 μM for FIP and BIP primers, 5 μM for F and B primers, and 20 μM for Loop B primers. RT-LAMP reaction was performed by using a RNA Amplification Kit (RT-LAMP) (Eiken Chemical Co. Ltd., Tokyo, Japan). RT-LAMP reaction was prepared according to the manufacturer's instruction but in a total 12.5 μl reaction. The final concentrations of the primers were as follows; 1.6 μM for FIP and BIP primers, 0.2 μM for F and B primers, and 0.8 μM for Loop B primers. In the total 12.5 μl reaction, 2.5 μl of purified viral RNA solution or diluted cell culture supernatant were included (Figure 1c). RT-LAMP reaction was performed at 60°C for 90 min and optical density at 650 nm was measured as the turbidity by a Loopamp Realtime Turbidimeter LA-320C (Teramecs, Kyoto, Japan). The threshold of the turbidity for RT-LAMP positive sample was defined at 0.1 in the measurement [5]. The numbers of copies of the viral RNA of PV Sabin strains and prototype enterovirus strains were determined by real-time TaqMan PCR system developed by Nijhuis et al. [17], as described previously [21].

Competing interests

The authors declare that they have no competing interests.

Authors' contributions

MA carried out the development of RT-LAMP system for enterovirus. HL and DY carried out the molecular genetic analysis of enterovirus isolates. MA, YN, HY, HS carried out the isolation of enteroviruses. MA planned the project and designed experiments. MA and HS wrote the manuscript. TW and HS supervised the laboratory works. All authors read and approved the final manuscript.

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