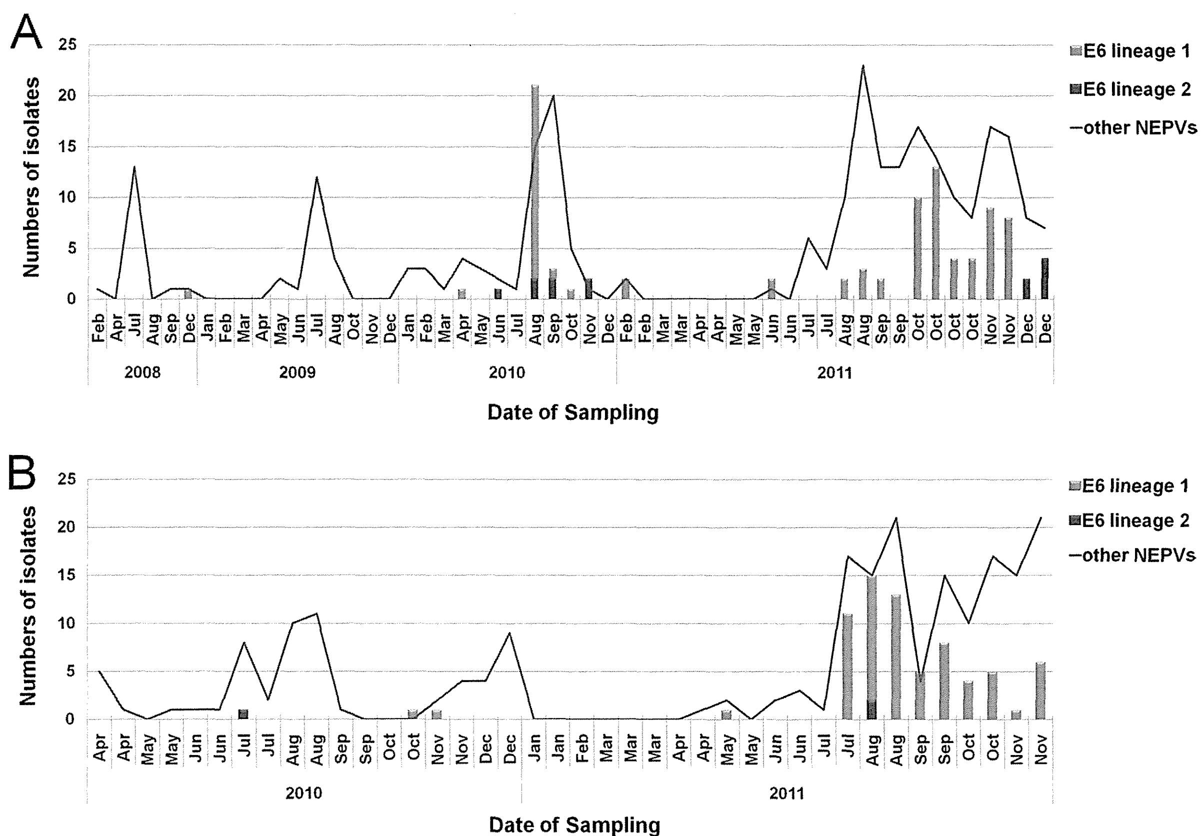

FIG 3 Monthly distribution of isolates of two E6 lineages and other NPEVs in Jinan, 2008 to 2011 (A) (25) and in Linyi, 2010 to 2011 (B). (Adapted from reference 25.)

2011. The pathway of E6 transmission is illustrated in Fig. 5. Strain JNEW100811.24 from Jinan in 2010 served as the ancestor of a majority of isolates in Linyi in 2011 and of a considerable number of strains in Jinan in 2011.

The VP1 pairwise contrast of environmental E6 was visualized in Fig. 6. In the data set comprised of 79, 142, and 167 sequences, respectively, of sublineage 1a, lineage 1, and lineage 1 plus lineage 2 ([lineages 1 plus 2] all environmental E6 isolates), the frequency of each base at each position was determined. Nucleotide changes were identified in 27 positions in the VP1 regions of sublineage 1a when the range of the substitution per site ratio was set within 0.03 to 0.97 (Fig. 6). The nucleotide with the highest frequency at each position was identical with the correlating nucleotide of strain JNEW100811.24. There was an increasing trend in the number of nucleotide changes. That is in sublineage 1a, only a small number of nucleotide changes were found; more changes occurred in lineage 1, and the largest number of nucleotide changes was found in lineages 1 plus 2.

Origin, evolutionary rate, and molecular clock phylogeny of lineage 1. The 684-nt VP1 sequences of environmental E6 isolates of lineage 1 ($n = 151$) were analyzed for divergence time and evolutionary rate using the Bayesian MCMC method (Fig. 7). The collection date of the samples served as the time information of environmental isolates. The mean rate of lineage 1 is estimated as 3.024×10^{-5} substitutions per site per day (95% HPD, 2.289×10^{-5} to 3.716×10^{-5}). The t_{MRCA} of all Linyi members of sublineage 1a was traced back to 24 December 2010 (95% HPD). So, under the assumption that it is an ancestor of a lineage—not of all the members of the lineage itself—that is spread between the two cities, the ancestor of this sublineage was likely to be imported into Linyi from between August and December 2010. The t_{MRCA} estimate for lineage 1 was dated to 22 June 2007 (Fig. 7).

DISCUSSION

In addition to contributing to the polio eradication program, environmental surveillance has shown its value in monitoring the circulating NPEVs. It is a useful approach to trace prevalent and minor circulating enteroviruses in human populations (7). Moreover, several studies had revealed a close relationship among environmental and clinical isolates, reflecting the potential power of environmental surveillance to herald epidemics in the context of the low morbidity/infection ratio of HEV infection (19). In regions where there is no specialized HEV surveillance system, such as the situation in China, environmental surveillance is of more significance in understanding HEV circulation.

The titers of HEVs in environmental specimens are lower than those in fecal specimens of infected humans. So, concentration is a necessary step before inoculation. The filter absorption method was used in this study, and this procedure is relatively more convenient and simpler than the two-phase separation method and polyethylene glycol (PEG) precipitation method (26). Although no systematic comparative studies on sensitivity have been published (6), the high HEV-positive rate and the large number of isolates detected in this study demonstrated the high efficiency of

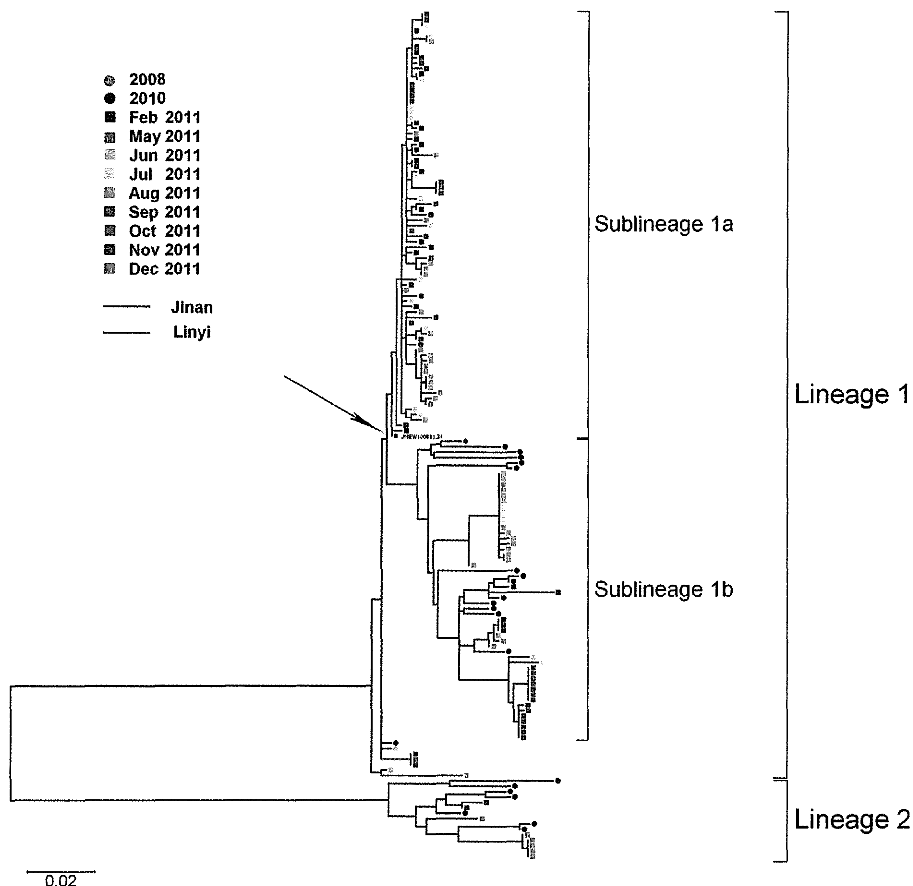

FIG 4 Phylogenetic relationships among all E6 isolates from the environmental surveillance in the two cities from 2008 to 2011. The phylogenetic tree was constructed using Mega, version 5.0, using the ML method based on 684-nt (nt 2629 to 3312 of strain D'Amori) partial VP1 sequences. Branches in red and blue indicate E6 isolates from Linyi and Jinan, respectively. An arrow indicates the location of the isolate JNEW100811.24. For clarity, the name of each isolate is not shown in this figure; instead isolates are represented and distinguished by various colors for different sampling times.

the method used. In the cell culture approach, the abundance of predominant serotypes or lineages in the sewage might have the potential to interfere with the minor circulating serotypes or lineages present in the same samples. So, increasing the number of parallel vials inoculated will improve sample sensitivity (6). In this study, 18 parallel cell vials with standard monolayer cultures were used for each cell line, and along with the abundant isolation of predominant lineage 1 of E6, a minor circulating E6 lineage and other serotypes were also obtained. Another benefit from the increased number of inoculated vials is that more VP1 sequence data will be achieved, especially for predominant serotypes or lineages. VP1 sequence is correlated with HEV evolution and widely used for molecular epidemiology investigations (7, 15). Hence, VP1 sequences of environmental isolates with records of collection

TABLE 1 E6 isolation from sewage in Shandong Province, 2008 to 2011

Sampling site	Year	No. of samples	No. of E6 isolates: Lineage 1	Lineage 2	Total
Jinan	2008	6	1	0	1
	2009	11	0	0	0
	2010	12	22	7	29
	2011	24	59	6	65
Linyi	2010	18	2	1	3
	2011	22	67	2	69
Total		93	151	16	167

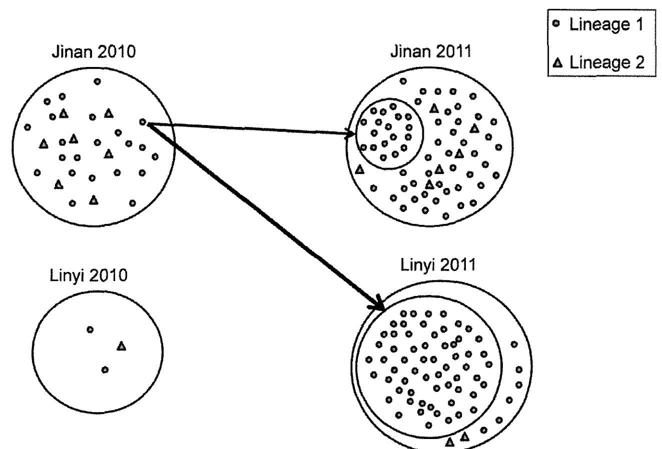

FIG 5 The distribution of the two lineages of E6 in the two cities in 2010 and 2011. An E6 virus in Jinan in 2010 (JNEW100811.24) served as an ancestor of a majority of isolates in Linyi in 2011, suggesting possible intercity transmission.

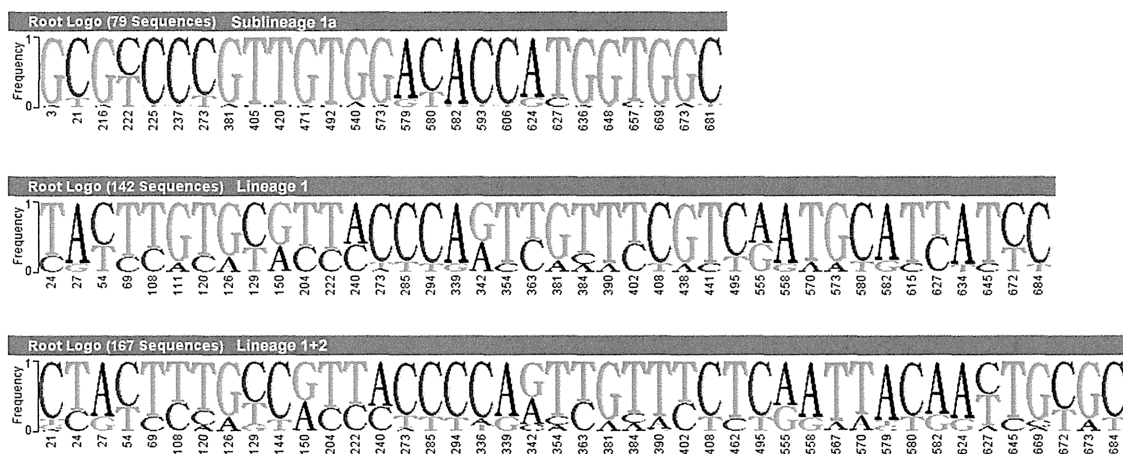

FIG 6 Sequence logo showing nucleotide diversity in the 684-nt VP1 region among the environmental E6 isolates in the two cities in 2008 to 2011. More mutated positions occurred in lineages 1 and 2 combined (all environmental E6 isolates) than in lineage 1 and sublineage 1a. The sequence logo was constructed using Phylo-mLogo. The ranges of the substitutions per site ratios are set as 0.03 to 0.97, 0.1 to 0.9, and 0.15 to 0.85 for sublineage 1a, lineage 1, and lineages 1 plus 2, respectively.

dates in the context of long-term surveillance will provide valuable information for comprehensively exploring viruses in local circulation.

HEV infection typically peaks in the summer months in temperate climates (17, 20). In this study, the monthly fluctuation of the numbers of isolates reflected similar seasonal patterns (Fig. 3). Low yield or negative results occurred in winter and spring, and peaks occurred in summer and autumn for both E6 and total NPEVs, demonstrating that environmental surveillance is useful for establishing temporal patterns of circulation. However, in interpreting the surveillance results, two factors should be considered. First, the solids in the sewage were removed in the procedure of concentration. This will result in the loss of solid-associated viruses. Second, some serotypes, such as some coxsackie A viruses of HEV-C species, cannot produce visible cytopathic effects (CPE) in these cell lines. So, these viruses cannot be recovered in the cell culture method. Considering these two factors, the actual HEV distribution in the sewage might not be completely identical with that reflected by this study.

In the environmental surveillance in 2011, the frequent E6 isolation revealed a large number of infected individuals in the two cities. According to the results of serological examination of serum specimens from AMES cases in Jinan in 2010 to 2011, HEV was demonstrated to be the predominant pathogen (positive rate,

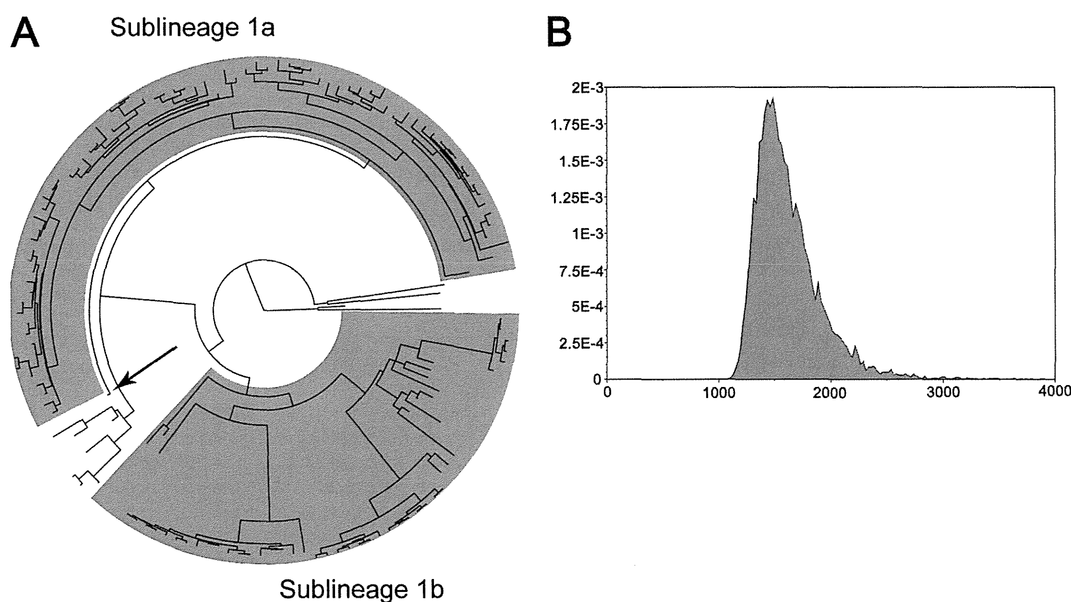

FIG 7 Origin of lineage 1 of E6 in the two cities. The 684-nt VP1 sequences of environmental E6 isolates of lineage 1 ($n = 151$) were analyzed for divergence time and evolutionary rate using the Bayesian MCMC method. (A) MCMC tree of lineage 1 viruses visualized in FigTree. The two major sublineages are highlighted. An arrow indicates the position of isolate JNEW100811.24. (B) The date of the MRCA of lineage 1 of E6 was estimated to be 22 Jun 22 2007 using a relaxed molecular clock model implemented in BEAST and visualized in Tracer. The numbers on the horizontal axis indicate the days before 25 November 2011, when the last batch of E6 viruses of lineage 1 was isolated.

37.9%), followed by HSV and MuV (13). Also, 10 enteroviruses were isolated from limited CSF specimens, including the two E6 strains which were genetically closely related to environmental viruses. Considering the high E6 activity reflected by environmental surveillance and the intimate genetic relationship between environmental and AM isolates, it is reasonable to assume that E6 was responsible for a considerable proportion of AM cases in these 2 years.

Several studies had assessed the sensitivity of PV environmental surveillance. Hovi et al. (5) reported that by analyzing a single 400-ml specimen, PV circulation could be detected if about 100 individuals were infected with PV in a population of 700,000 people. Lodder et al. (14) found that it is possible to detect 100 PV-infected individuals in a population of tens of thousands uninfected individuals. In our situation, both the JNEW and LYSC plants treated 200,000 tons of sewage daily. So, based on our procedure of concentration and inoculation and assuming (i) that the virus excreted into stools is 10^7 infectious doses/day/person, (ii) that the viruses are dispersed uniformly in the sewage, and (iii) that the recovery rate of the filter method is 50%, it can be calculated that one isolate from 0.8 liters of sewage corresponds to 140 infected individuals. Following this inference, the number of infected persons of a certain serotype can be deduced. For example, the 15 E6 isolates detected in Linyi in August 2011 suggested 2,100 infected individuals at that time. The inference is theoretical, but it might explain why a high degree of genetic diversity of NPEVs can be observed (10), even from a single sewage specimen (25, 27). The viruses from sewage are from a population, not a single person. So, when viruses with the same serotypes are simultaneously isolated from a sewage specimen, they probably derive from a population of several thousands and represent respective evolution positions in the transmission chain. Hence, the high diversity among the isolates is observed.

Environmental surveillance was initially conducted in Jinan. Linyi was selected as another surveillance site because of frequent NPEV-associated AM outbreaks in recent years in an effort to evaluate the prevalence and to monitor the circulation of HEVs. Our study showed that the predominant transmission lineage of E6 was active in Jinan in 2010 to 2011 and in Linyi in 2011. Furthermore, the results of VP1 sequence analysis revealed that the VP1 sequence of strain JNEW100811.24—an environmental isolate from Jinan in 2010—was identical with the reconstructed ancestor sequence of sublineage 1a, which mainly consisted of Linyi E6 viruses isolated in 2011, suggesting the existence of a transmission pathway from Jinan to Linyi of this lineage. It is worth noting that this is the first time that environmental surveillance has been applied to trace NPEV transmission within two human populations. Nevertheless, because environmental surveillance cannot provide information on the infected person, the transmission chains existing in the populations cannot be determined. Molecular clocks for other enteroviruses had been inferred previously (8). In this study, the estimated higher evolution rate of lineage 1 than that of global E6 (25) suggested that lineage 1 evolved faster than other E6 members.

In conclusion, this study demonstrates high E6 activities in the two cities and revealed a transmission pathway from Jinan to Linyi, demonstrating that environmental surveillance can be applied to trace HEV transmission among different regions.

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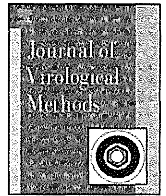

An optimized method for elution of enteroviral RNA from a cellulose-based substrate

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ABSTRACT

The Flinders Technology Australia (FTA) Elute Card is a commercial product that facilitates the collection, transport, archiving and processing of nucleic acids from a wide variety of biological samples at room temperature. While the cards have been designed so that sterile/deionized water can elute DNA easily, they are not suitable for some less stable RNAs. This study was undertaken to determine the optimal conditions such as the buffer type, buffer pH and incubation temperature for the elution of enteroviral RNA from FTA Elute Cards prior to quantitative analysis using real-time PCR (qPCR) or consensus degenerate hybrid oligonucleotide primer VP1 RT-semi nested PCR (CODEHOP VP1 RT-snPCR). TE-1 (pH 8.0), rather than sterile water, was the best buffer for high efficiency elution of enteroviral RNA at 95 °C. However, as the estimated recovery rate of viral RNA eluted from the cards averaged to be only 6.1%, enterovirus assays using FTA elution should be considered qualitative, especially at low virus titers, and therefore the results of the assay should be interpreted carefully.

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1. Introduction

Flinders Technology Australia (FTA) technology is based on a cellulose paper substrate that is treated chemically to immobilize nucleic acids from lysed cells for subsequent transport and storage. FTA cards support a wide variety of applications, including forensic tests, newborn screening, HIV detection and monitoring, therapeutic drug monitoring and, more recently, quantitative determination of experimental medicines in biological samples (Pitt, 2010; Lofgren et al., 2009; Edelbroek et al., 2009; Li and Tse, 2010). FTA cards are now a well-established method for convenient collection and storage of DNA samples, but are much less applied for RNA. However, the transportation of RNA viral genomes for diagnostic analysis is of increasing importance due to the rapid evolution and diversification of RNA viruses (Rogers and Burgoyne, 2000), which result in a growing number of severe disease outbreaks and pandemics. Appropriate methods and sample-carriers are needed urgently for safe transport and archiving nucleic acids derived from biological samples or pathogens while waiting for clinical diagnosis.

Enteroviruses (EVs) (genus *Enterovirus*, family *Picornaviridae*) are small single-stranded RNA (ssRNA) viruses with a genome of approximately 7500 nucleotides that contain a single open reading frame flanked by non-coding regions. Enteroviruses are grouped currently into four or five species of human enterovirus (HEV) based on molecular and biological characteristics: HEV-A, HEV-B, HEV-C (including or excluding polioviruses, PVs) and HEV-D (Oberste et al., 1999; Brown et al., 2003; Stanway et al., 2005). The enteroviruses are associated with several human diseases that range from asymptomatic infections to a wide spectrum of acute diseases, including mild upper respiratory illness (common cold), febrile rash (hand, foot, and mouth disease (HFMD) and herpangina), aseptic meningitis, acute hemorrhagic conjunctivitis, pleurodynia, encephalitis, acute flaccid paralysis (paralytic poliomyelitis), and neonatal sepsis-like disease (Pallansch and Roos, 2001). In addition to these acute illnesses, HEVs have also been associated with severe chronic diseases, including myocarditis, dilated cardiomyopathy and type 1 diabetes mellitus (Zhang et al., 2000; Kearney et al., 2001; Hyoty and Taylor, 2002). The prevalence and outbreaks of EV diseases have been increasing worldwide since the 1990s. During 2009–2010, wild poliovirus (WPV) was imported into polio-free countries from both polio-endemic countries and previously polio-free countries with reestablished transmission (CDC, 2010). This emphasizes the urgency of developing new diagnostic approaches, especially molecular techniques that are safer, more sensitive, more convenient, and time efficient. In addition, methods are needed for specimen collection, transport, and archiving.

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FTA Elute Cards are designed for room temperature collection, shipment, archiving, and processing of nucleic acids from a wide variety of biological samples for PCR analysis (www.whatman.com). Once immobilized on the cards, the nucleic acids are no longer infectious and do not pose a biohazard (Cortes et al., 2009). The cards have been used effectively for a variety of infectious agents, such as human papillomavirus (HPV), malaria and *Mycobacterium leprae* (Gustavasson et al., 2009; Sultan et al., 2009; Aye et al., 2011), but not yet for the detection of enteroviruses.

In this study, three parameters, elution buffer type, buffer pH and incubation temperature, were investigated to optimize the elution of enteroviral RNA from FTA Elute Cards for subsequent detection using quantitative real-time RT-PCR (qPCR) and consensus degenerate hybrid oligonucleotide primer VP1 RT-semi nested PCR (CODEHOP VP1 RT-snPCR).

2. Materials and methods

2.1. Cells and viruses titration

Representative strains from HEV species A–D were used: HEV-A (EV71, BrCr), HEV-B (CVA9, Grigg; CVB1, Conn-5; Echo14, Tow; Echo30, Bastianni), HEV-C (PV2, Sabin2), and HEV-D (EV68, Fermo). The selected prototypes were cultured in different cell lines. When complete CPE was observed, the cell lysates were obtained after the cultures were frozen and thawed three times. These viruses, with the exception of CVB1, were titrated using the RD-A cell line and the 50% cell culture infective dose ($CCID_{50}$) was calculated by the Kärber formula. HEp-2 was used for titration of CVB1.

2.2. Samples preparation

Cell lysates were applied to FTA Elute Cards (Whatman, Cat. No WB120410) in a concentric circular motion within the printed circle area according to the manufacturer's instructions. Briefly, 40 μl cell lysate was applied to each 11-mm circle. The samples were allowed to dry completely for at least 3 h at room temperature prior to punching.

2.3. RNA elution

RNA elution from cards was also performed according to the manufacturer's instructions. Two 3-mm sample disks were removed from the center of the spot using the 3-mm Harris Uni-Core device (Whatman Cat. No: WB100039) and transferred to a 1.5-ml centrifuge tube. Sterile H_2O (500 μl) was added to the tube, which was immediately pulse vortexed 15 s (5 s ×3).

Using a sterile technique, the disk was transferred immediately to a 0.5-ml tube containing 30 μl of one of the following elution buffers: deionized sterile water, TE-1 (10 mM Tris–HCl, 1 mM EDTA), 1 M Tris–HCl, Buffer EB (10 mM Tris–Cl; elution buffer from the QIAamp Viral RNA Mini Kit). The disks were completely immersed in elution buffer by centrifuging the tube briefly for 10 s before the tubes were transferred to a heat block at a fixed temperature (65 °C or 95 °C) for 30 min while shaking at 100 × *g*. The tubes were then pulse vortexed at 200 × *g* for 10 min in 1-min intervals.

After incubation, the samples were removed from the block, pulse vortexed approximately 60 times, and centrifuged at 8000 × *g* at 4 °C for 1 min. Using a sterile pipette tip, the FTA elute matrix disks were gently removed and discarded. The purified RNA was stored at −20 °C for recent detection or at −70 °C for long storage as recommended by the manufacturer.

2.4. Viral RNA

Viral RNA from representative enterovirus strains was purified with the Roche Pure Viral RNA kit (Roche Applied Science, Mannheim, Germany) and stored at −70 °C.

2.5. cDNA synthesized from viral RNA

Viral RNA (5 μl) was used to synthesize cDNA using the Prime Script RT Reagent kit, which included oligo dT primer and random 6-mers (Takara, Tokyo, Japan). Reactions were performed in a GeneAmp PCR System 2700 (Applied Biosystems, Foster City, CA): cDNA was synthesized under the following conditions: 30 min at 37 °C, 5 s at 85 °C and held at 4 °C. The cDNA was stored at −70 °C prior to real-time PCR.

2.6. Quantitative real-time PCR

Quantitative real-time PCR was performed using the protocol developed by Nujhuis et al. (2002). The following primers and probes were designed to detect all members of the *Enterovirus* genus: 5′-TCCTCCGGCCCCTGA-3′ (forward primer), 5′-AATTGTCACCATAAGCAGCCA-3′ (reverse primer), 5′-GATTGTCACCATAAGCAGCCA-3′ (reverse primer), 5′-Cy5-CGGAACCGACTACTTTGGGTGTCCGT-BHQ3-3′ (probe) and 5′-Cy5-CGGAACCGACTACTTTGGGTGACCGT-BHQ3-3′ (probe). The probes were labeled with the 5′ reporter dye indodicarbocyanine (Cy5) and black hole quencher-3 (BHQ3).

cDNA (5 μl) was assayed in duplicate in 25-μl reactions containing 12.5 μl of 2× Quantitech Master Mix (QIAGEN), 900 nM of the forward primer, 300 nM of each reverse primer, and 100 nM of each probe in an ABI Prism 7500 SDS (Applied Biosystems). The reactions were incubated at 95 °C for 15 min, followed by 45 cycles of denaturation at 95 °C for 15 s and a combined annealing-extension step at 60 °C for 1 min.

The Takara company (Tokyo, Japan) customized a 180-bp fragment (position 441–620) from Coxsackievirus A16 (CVA 16, accession number: U05876) and inserted it into the pMD19-T vector for use as an EV cDNA quantitation standard. The standard used in the high concentration set consisted of 10-fold dilutions ranging from 5.00E+6 copies (particles)/μl to 5.00E+0 copies/μl.

Fluorescence threshold was set manually to generate a standard curve with optimal statistics after PCR (usually $r^2 > 0.996$ and a slope around −3.30). The concentrations of the samples were calculated based on the standard curve.

2.7. CODEHOP VP1 RT-snPCR

Viral RNA was also amplified by CODEHOP VP1 RT-snPCR (Nix et al., 2006) to compare the efficiency of amplification at different pH levels with those by real-time PCR. Briefly, after the cDNA was synthesized, the 1st step of PCR (RT-snPCR1) was carried out with 35 cycles of amplification (95 °C for 30 s, 42 °C for 30 s, 60 °C for 45 s). Two μl of PCR1 products was added for the 2nd step of PCR (RT-snPCR2) (95 °C for 30 s, 60 °C for 20 s, 72 °C for 15 s for 35 cycles). The reaction products were visualized on 2.0% agarose gels containing ethidium bromide (0.5 μg/ml), and the results of electrophoresis were scanned and digitized data were quantified as integrated optical density (IOD) using Kodak Imaging Program and Image-Pro Plus software (Eastman Kodak Company, New Haven, CT, USA). The IOD of positive control (10^3 $CCID_{50}$/40 μl Echo 30 Bastianni strain RNA) was set as 100%, and ±,+,++, and +++ signified 0–25%, 25–50%, 50–75%, and 75–100%, respectively.

Table 1
Mean Ct values derived from RNA eluted from 4 enteroviruses strains by the use of 4 different elution buffers.

Prototype	Virus titers ($CCID_{50}$/ml)	Purified RNA	Sterile water (pH 7.2)	1 M Tris–HCl (pH 8.0)	TE-1[b] (pH 8.0)	Buffer EB[b] (pH 8.5)
CVB1 (Conn-5 M16560)	$10^{7.43}$	16.74 ± 0.29 [a]	36.08 ± 1.73	40.92 ± 1.67	28.02 ± 2.49	29.37 ± 1.77
CVA9 (Grigg D00627)	$10^{7.38}$	16.21 ± 0.42	30.11 ± 2.45	41.46 ± 2.25	26.67 ± 1.98	26.14 ± 1.87
Echo14 (Tow AY302540)	$10^{7.88}$	15.67 ± 0.41	28.38 ± 1.57	41.24 ± 1.66	25.96 ± 1.52	26.82 ± 2.19
Echo30 (Bastianni AF162711)	$10^{8.00}$	15.37 ± 0.62	31.70 ± 2.41	42.09 ± 1.85	25.01 ± 1.33	26.77 ± 2.34
Totally mean Ct value	–	16.00 ± 0.37	31.57 ± 1.16	41.42 ± 0.86	26.42 ± 0.90	27.27 ± 0.97

[a] Mean threshold cycle (Ct value) ± standard deviation (SD) of 5 replicates.
[b] TE-1 buffer (10 mM Tris–HCl + 1 mM EDTA), buffer EB (10 mM Tris–Cl, pH 8.5; QIAGEN).

2.8. Elution efficiency

EV71 (HEV-A), Echo30 (HEV-B), Sabin poliovirus 2 (HEV-C) and EV68 (HEV-D) were used to compare elution efficiency from the card. The virus titers were adjusted to 10^5 $CCID_{50}$/40 μl. After serial dilution, each viral dilution was applied to an FTA Elute Card, and viral RNA was eluted from the card using TE-1 buffer (pH 10.0). Viral RNA was also extracted directly from each dilution as described in Section 2 and diluted further 20-fold as the RNA before elution to compare against the amount of RNA recovered from the card.

The recovery rate, which indicates the elution efficiency, was calculated by the cDNA copy numbers ratio between the RNA eluted from the cards and the RNA before elution.

2.9. Statistics

All data were input and archived by EXCEL 2007, the statistical analysis was calculated by SPSS, version 13.0

3. Results

3.1. Effect of the elution buffer on viral RNA yields

Four buffers (sterile water, TE-1, Tris–HCl, and Buffer EB) were used to elute the viral RNA of HEV-B prototype strains (CVB1, CVA9, Echo14 and Echo30) from the FTA Elute Cards. The average yield of viral RNA eluted from the cards was determined by real-time PCR (qPCR) (Table 1). The average threshold cycle (Ct) values were compared from at least 5 replicates included in each experiment. The following Ct values were observed: TE-1 (26.42 ± 0.90) < Buffer EB (27.27 ± 0.97) < sterile water (31.57 ± 1.16) < Tris–HCl (41.42 ± 0.86). Tris–HCl had the lowest elution capacity with highest mean Ct value (i.e., lowest number of copies). The elution capacity of sterile water fell between that of Tris–HCl and Buffer EB and TE-1, which had the highest elution capacity at 95 °C over a 30-min period. While the difference in elution capacity between TE-1 and sterile water was statistically significant ($P < 0.01$), there was hardly difference between TE-1 and Buffer EB ($P > 0.05$). However, due to convenience and cost, TE-1 is thought to be the best option for eluting enteroviral RNA from the FTA cards.

3.2. The effect of temperature and pH on the elution efficiency

The pH of the elution buffer is one of the most important parameters that must be considered during elution. The manufacturer of the FTA cards recommends a 30-min treatment at 95 °C. However, high temperatures can result in damage to RNA. Therefore, yield was assessed following treatment at 65 °C. Four viruses; EV71, Echo30, Sabin poliovirus 2 and CVB1, were adjusted to $10^{6.75}$–$10^{8.5}$ $CCID_{50}$/ml (cell culture infectious dose 50%) and were used to compare yields at different pH values. Ct values were measured by qPCR.

The RNA yield was the highest at pH 10.0 when eluted at both 95 °C and 65 °C, indicating that yield is much better in alkaline buffers than in neutral or acidic environments (Table 2). The statistical significance ($P < 0.01$) of the mean Ct values between pH 8.0 (30.24 ± 0.92) and pH 10.0 (27.35 ± 1.00) shows that the latter was much better for RNA elution when tested by qPCR.

3.3. pH of the eluting buffer for qPCR and CODEHOP VP1 RT-snPCR

pH is a key factor in a PCR. The qPCR used in this study can detect EV with a high level of sensitivity, the limit was more than 0.36 $CCID_{50}$ for CVA9 (Nujhuis et al., 2002). Molecular typing using PCR-direct sequencing will be the method used most for enterovirus genotyping. As different reagents are used for qPCR and conventional RT-PCR, the pH of both systems must be considered. The CODEHOP VP1 RT-snPCR was compared to qPCR, The detection limit for CODEHOP VP1 RT-snPCR can be as few as 10 RNA copies/reaction (Nix et al., 2006). We evaluated the effects on PCR by using sterile water and TE-1 buffer adjusted to pH 6, 7, 8 and 10. Viral RNA was extracted from Echo30 virus fluid adjusted to 10^3 $CCID_{50}$/40 μl, serially diluted 10-fold (up to 1000 times) with sterile water and TE-1 in the pH-adjusted buffers (Table 3), and subjected to qPCR and CODEHOP VP1 RT-snPCR.

Table 2
The efficiency of elution in different pH and temperature.

Virus strains	Virus titers ($CCID_{50}$/ml)	Tem	pH					
			6.0	7.0	8.0	10.0	11.0	12.0
EV71	$10^{7.75}$	95 °C	29.74 ± 0.04[a]	25.36 ± 0.45	27.60 ± 0.02	22.97 ± 0.19	34.57 ± 0.01	45.00 ± 0.00
		65 °C	33.45 ± 0.22	31.91 ± 0.07	30.20 ± 0.01	25.60 ± 0.03	27.36 ± 0.22	31.62 ± 0.01
Echo30	$10^{8.0}$	95 °C	35.93 ± 0.07	31.14 ± 0.18	28.67 ± 0.17	26.33 ± 0.20	32.49 ± 0.04	45.00 ± 0.00
		65 °C	33.64 ± 0.17	31.68 ± 0.06	28.62 ± 0.20	28.51 ± 0.02	27.71 ± 0.00	37.59 ± 0.30
Sabin 2	$10^{7.43}$	95 °C	33.40 ± 0.15	30.52 ± 0.18	27.67 ± 0.12	26.52 ± 0.19	41.48 ± 2.49	35.19 ± 0.29
		65 °C	34.10 ± 0.31	29.96 ± 0.31	27.97 ± 0.46	28.95 ± 0.06	28.34 ± 0.26	38.79 ± 1.00
CVB1	$10^{7.43}$	95 °C	34.08 ± 0.14	30.53 ± 0.03	30.48 ± 0.06	28.12 ± 0.04	31.88 ± 0.04	41.24 ± 0.10
		65 °C	35.98 ± 0.12	32.04 ± 0.23	30.51 ± 0.25	30.19 ± 0.30	37.78 ± 0.00	36.15 ± 0.33

[a] Mean threshold cycle (Ct value) ± standard deviation (SD) of 5 repetitions.

Table 3
Quantitation of enteroviral RNA by qPCR and CODEHOP VP1 RT-snPCR.

		Dilution buffer														
		Sterile water			TE-1 pH 6.0			TE-1 pH 7.0			TE-1 pH 8.0			TE-1 pH 10.0		
RNA dilution[a]	No. dilution	10^{-1}	10^{-2}	10^{-3}	10^{-1}	10^{-2}	10^{-3}	10^{-1}	10^{-2}	10^{-3}	10^{-1}	10^{-2}	10^{-3}	10^{-1}	10^{-2}	10^{-3}
Copy numbers[b]	8.E+04[b]	1.00E+04	9.00E+02	6.00E+01	4.00E+03	5.00E+02	5.00E+01	5.00E+03	7.00E+02	7.00E+01	9.00E+03	7.00E+02	6.00E+01	7.00E+03	7.00E+02	9.00E+01
Mean Ct value	24.51	27.33	31.04	35.02	29.04	31.94	35.27	28.71	31.45	34.89	27.80	31.45	35.12	28.12	31.45	34.51
RT-sn PCR1[c]	±	±	–	–	–	–	–	–	–	–	–	–	–	–	–	–
RT-sn PCR2[d]	+++	+++	++	+	++	+	±	+	++	+	+++	++	+	++	+	–

[a] The original RNA from virus Echo 30 (Bastianni) was 10^3 $CCID_{50}$/40 μl, and was then diluted in 10-fold for 3 times in Sterile Water and TE-1 at different pH.
[b] The result of qPCR for copy numbers/5 μl.
[c] The result of 1st RT-snPCR.
[d] The result of the 2nd RT-snPCR.

Sterile water and TE-1 (pH 8.0) had high sensitivity in both PCR systems. While results of qPCR using cDNA from viral RNA diluted at different pH showed equivalent copy numbers, viral RNA at pH 10 were not amplified in the RT-snPCR2 when using CODEHOP VP1 RT-snPCR.

3.4. Efficiency of eluting viral RNA from the FTA elute card

The enteroviruses have a different distribution of electric charge on their surface virions. Therefore, the efficiency of elution from the FTA cards may differ depending on the species of virus.

The cDNA copy numbers before and after elution are shown in Table 4. The recovery rate was calculated as described in the Elution Efficiency (Material and methods). At higher virus titer, the rates of recovery were estimated at 5.4–7.5% in EV71, 1.0–18.2% in Echo30, 2.3–4.0% in Sabin 2 and 2.3–16.2% in EV68. The average recovery rate was 6.1% for these viruses within 10^5–10^3 $CCID_{50}$/40 μl.

4. Discussion

Clinical diagnosis and disease surveillance consider most viruses as potentially hazardous, and the response to emerging pathogens requires specialized training and a high level of containment. The FTA Elute Card is impregnated with a patented chemical formula that lyses cells and immobilizes nucleic acids, thereby rendering them safe to work within the laboratory. The nucleic acids can be easily eluted with sterile water. The cards provide high sensitivity due to the elution of DNA that is free of inhibitors, since hemoglobin, a known DNA inhibitor, is bound to the FTA matrix (Sultan et al., 2009). Conversely to DNA, RNA is less stable chemically, and is best analyzed upon receipt of the samples at the laboratory (Whatman, 2007–2009).

The elution capacity of four different buffers was compared in parallel using four strains of enteroviruses: sterile water (recommended by the Whatman protocol for DNA), TE-1, 1 M Tris–HCl and buffer EB. In this study, TE-1 proved to be a much better elution buffer for enterovirus viral RNA than sterile water by a difference of 5 mean Ct values. Buffer EB (QIAGEN, Valencia, CA, USA) had an elution capacity similar to that of TE-1, but it is expensive and not readily available. As for Tris–HCl, with a concentration of 1 M, the capability of eluting RNA was lower than that of TE-1 by a mean difference of more than 15 Ct cycles, an observation indicating that the high concentration of buffer might have inhibited the release of viral RNA from the FTA cards. Some researchers have performed field investigations or diagnoses by detecting the DNA or RNA of viruses (Abdelwhab et al., 2011), bacteria (Sultan et al., 2009; Aye et al., 2011) and fungi (Suzuki et al., 2006) that were collected on FTA cards and eluted by sterile water, and achieved the desired results. However, there are no reports of successful elution of enteroviral RNA with sterile water after collection on FTA Elute Cards. Since TE-1 (pH = 8.0) displayed a better capacity for the elution of enterovirus RNA than the other three buffers, it was used as the elution buffer in all further experiments.

Although the elution of viral RNA can be carried out at room temperature, an incubation temperature of 95 °C is recommended for removal of RNase inhibitor. However, there was no obvious difference between 95 °C and 65 °C in this study.

TE-1 (10 mM Tris–HCl, 0.1 mM EDTA), is usually used as a storage buffer for DNA and RNA. EDTA in TE chelates Mg^{2+} and other divalent metals ions and is needed to suppress most DNA and RNA degradation. Tris is a buffering agent to keep the solution at a defined pH (OpenWetWare, 2009). TE-1 is now also widely used to dissolve or resuspend nucleic acids eluted from FTA cards (Whatman, 2009). The pH of TE-1 is usually 8.0 for DNA storage and 7.5 for RNA (OpenWetWare, 2009). In this study, a much higher

Table 4
The elution efficiency of enteroviruses RNA from FTA elute card.

Virus	Elution	10^5 CCID$_{50}$/40 μl	10^4 CCID$_{50}$/40 μl	10^3 CCID$_{50}$/40 μl	10^2 CCID$_{50}$/40 μl	10^1 CCID$_{50}$/40 μl
EV71-BrCr	After	1.30E+04 ± 292.68[a]	3.90E+03 ± 83.55	1.29E+02 ± 3.68	5.38E+02 ± 53.83	4.77E+01 ± 3.23
	Before	2.29E+05 ± 14501.58	5.22E+04 ± 2053.39	2.38E+03 ± 45.77	2.03E+02 ± 25.18	1.67E+01 ± 2.24
Recovery rate (%)		5.68	7.46	5.4	265	285.07
Echo30-Bastianni	After	1.57E+04 ± 685.82	1.20E+03 ± 174.21	6.43E+01 ± 18.62	1.34E+01 ± 7.62	2.62E+01 ± 6.87
	Before	8.64E+04 ± 1815.28	4.28E+04 ± 1350.38	6.56E+03 ± 60.34	3.77E+02 ± 18.75	1.92E+01 ± 5.45
Recovery rate (%)		18.17	2.81	0.98	3.55	136.60
PV2-Sabin2	After	3.39E+02 ± 14.75	1.44E+02 ± 4.57	1.73E+01 ± 0.67	5.73E+00 ± 0.36	7.03E+00 ± 0.89
	Before	8.49E+03 ± 357.27	6.23E+03 ± 356.09	5.68E+02 ± 8.49	5.98E+01 ± 6.45	4.99E+00 ± 1.54
Recovery rate (%)		4.00	2.30	3.05	9.58	140.88
EV68-Fermo	After	1.34E+02 ± 12.84	3.95E+01 ± 4.79	7.62E+00 ± 0.74	1.40E+01 ± 3.54	2.62E+01 ± 0.21
	Before	5.85E+03 ± 59.94	8.54E+02 ± 47.66	4.69E+01 ± 2.75	8.39E+00 ± 0.62	1.00E+00 ± 0.54
Recovery rate (%)		2.28	4.63	16.23	166.39	2619.00

[a] The copy number of viral cDNAs ± standard deviation (SD) of 5 repetitions.

alkalin TE-1 harvested much more RNA by nearly 2 mean Ct values between pH 8.0 and 10.0. Apparently the high pH of the alkaline solution denatures the DNA or RNA and disentangles it from the paper matrix (Lema et al., 2006). The pH of the elution buffer should be considered and adjusted to the chosen method of detection accordingly. This study illustrated that an elution buffer with a pH between 8.0 and 10.0 did not affect the Ct values when qPCR was used as the detection method, but for CODEHOP VP1 RT-snPCR, the sensitivity is reduced if the pH is higher than 8.0.

Although the rate of recovery varied in higher titers, lower titers less than 100 CCID$_{50}$/40 μl showed more dispersion (Table 4). A large dispersion was observed as the copy number in lower titers reached to the detection limit value of qPCR (less than 100 copies/reaction).

Thus, FTA Elute Cards would be useful for transportation of clinical specimens, such as throat swabs, that contain a small amount of virus; however, the results should be considered qualitative. On the other side, PCR efficiency also differed depending on species of the virus. Therefore, the results should be interpreted carefully.

Polioviruses (PVs), members of the C genogroup of enteroviruses, are causative agents of poliomyelitis, which has been targeted for eradication by the Global Polio Eradication Initiative (GPEI) since 1988 (CDC, 2011). The Global Polio Laboratory Network (GPLN), which guides program activities through timely isolation and characterization of PVs (CDC, 2011), has evaluated the FTA and the FTA elute cards to render isolates non-infectious prior to shipment for intratypic differentiation (ITD) and sequencing (WHO, 2010). The cards can be shipped without contagion at ambient temperature, providing the possibility of substantial reductions in shipping costs and elimination of the current risk for breach of virus containment through shipment of live viruses.

5. Conclusions

A method was optimized for enteroviral RNA elution from the Whatman FTA Elute Card, and achieved a high RNA yield with qPCR or CODEHOP VP1 RT-snPCR. This method of RNA elution will be valuable not only for PVs monitored by the GPLN, but also for all non-polio enteroviruses and other single-stranded RNA viruses.

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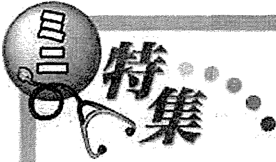

序

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OPV
IPV
DPT-IPV
ワクチン予防可能疾患

Ⅰ．ポリオという病魔

古代エジプトの都市から出土した石版画にも，片方の下肢が麻痺して細くなった人物が描かれており，ポリオは有史以来私たちを苦しめてきた感染症である。四肢の麻痺が最も特徴的な症状であるが，ポリオウイルスは運動神経細胞を侵す病原体で，呼吸をつかさどる筋肉や呼吸中枢に病変が及ぶこともある。

“鉄の肺（Iron Lung）”は，患者の首から下を気密タンクに入れ，タンク内を間歇的に陰圧にすることにより患者の胸郭を広げて吸気を起こすかつての人工呼吸器であるが，1920年代に米国でポリオによる呼吸不全を治療するために開発された。ポリオ流行時に，Iron Lungに収容された患者で病棟が埋め尽くされている記録写真も残っている。

Ⅱ．ワクチンの登場

多くの研究者たちが，ポリオを予防するワクチンの開発に挑んだ。そして，1950年代中盤に，2種類のポリオワクチンが予防に期待を抱かせる効果を示した。最初に華々しく登場したのは，ソーク博士（Jonas E. Salk）らが開発した不活化ポリオワクチン（inactivated poliovirus vaccine；IPV）であった。当時のアイゼンハワー米大統領はソークらの研究報告を支持し，全世界的にIPVを提供する用意があることを宣言した。もうひとつのワクチンはセービン博士（Albert B. Sabin）らによる経口生ポリオワクチン（oral poliovirus vaccine；OPV）であった。そのころ世界は，米国とソ連（ソビエト連邦（当時））の東西冷戦の時代であった。セービンは米国人であるが，ソ連でOPVの大規模な野外投与が行われ，素晴らしい有効性の報告がまとめられた。

Ⅲ．日本におけるポリオワクチン導入

日本は，1960年に未曽有のポリオ大流行を

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経験した。北海道で最初に検知された患者の多発は，エンテロウイルス流行期の夏に向かって全国に拡大し，年間の報告患者数は5,000人を超えた。ポリオによる麻痺は多くの場合非可逆性であり，著明な筋萎縮と相まって後遺症につながる。患者好発年齢は小児であり，彼らの人生を大きく変えてしまうこの病魔に国民は怖れ慄いた。最流行期の夏を過ぎ，発生はいったん沈静化したが，次シーズンに備えるための手段，すなわち予防のためのワクチン導入が，医学界のみならず国全体を巻き込んで議論され社会問題となった。

年が明けて1961年1～3月には，「行政指導」という形で生後6カ月から1歳半までの児を対象に，ソークIPVワクチンの「希望接種」が実施された。全国から多数の申し込みがあり，輸入されたIPVの本数では希望者全員に行き渡らなかった。やがて春を迎え，この年は九州で流行が始まった。NHKテレビは，4月から毎日「ポリオ患者発生数即日集計」を全国放送した。流行は国内各地で発生し，国産IPVが検定不合格になったこともあり，IPVの供給は全く不十分であった。また，いつの時代にもある予防接種にはつきもののワクチン不信を煽るニュースであるが，IPVの「希望接種」を済ませた者からの発症も報告された。

日本の母たちは，わが子をポリオから守りたい一心で，OPVの早期導入を求めて厚生省（当時）に押し寄せた。前年の勢いに劣らないポリオ患者増加のなかで，世論は海外で開発されて間もないOPVの緊急導入に積極的であった。そして，1961年6月10日羽田空港にOPVの原液がまず5万人分到着した。6月21日夕方，古井厚生大臣による「（OPV導入に関する）責任はすべて私にある」という談話とともに，1,300万人分のOPV緊急輸入決定が発表された。6月26日，最も大きな流行が認められていた九州でOPVの「実験投与」が始まった。押し寄せるポリオ流行の波に押されるように，その後OPVは各地で使われ，7月21日には全国の子どもたちを対象とした国内一斉投与が開始された。そして，ポリオ患者の発生が減少傾向に転じたのは，500万人程度がOPV内服を済ませたと推計される7月末のことであった。

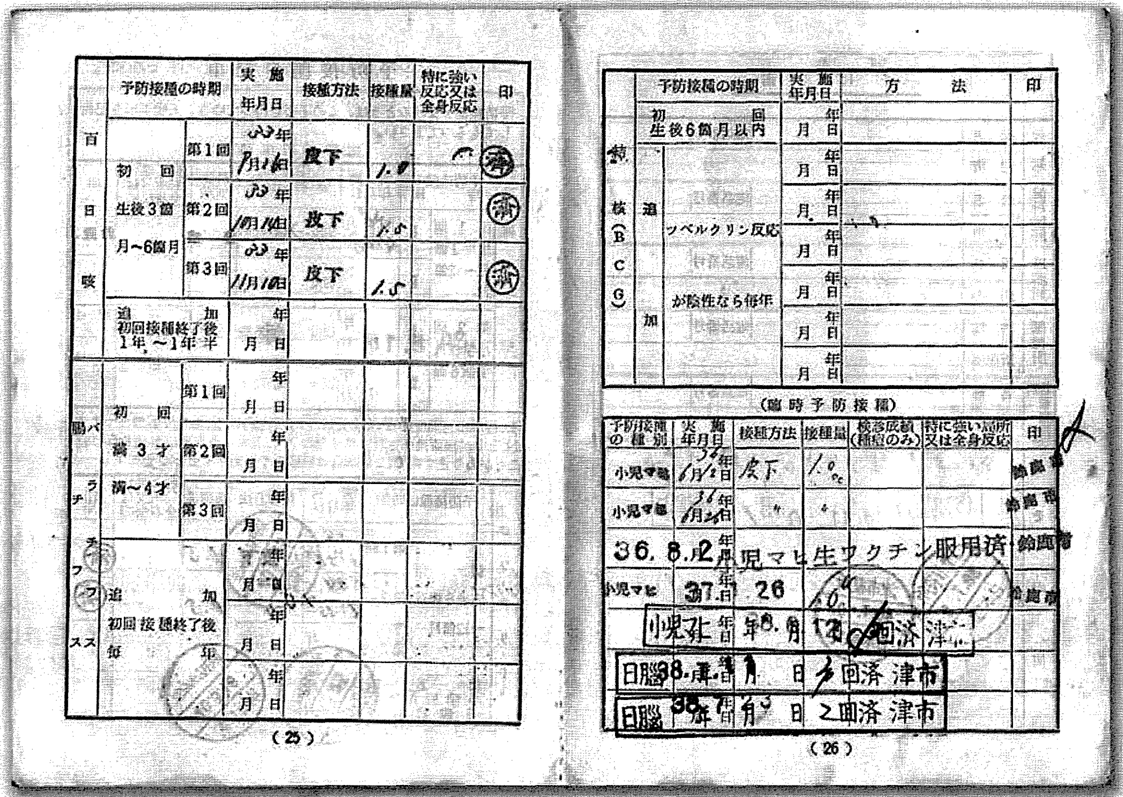
Ⅳ．私の予防接種歴

私はOPVの一斉投与を受けた世代であり，母子手帳の記録も残っている（**写真1**）。第1回目のOPV接種が昭和36年（1961年）8月2日で，間違いなくそのころ始まったばかりの一斉投与である。その後，昭和37年（1962年）1月26日，昭和38年（1963年）6月12日，写真には載ってないが母子手帳の次頁に昭和38年（1963年）8月1日にもOPV接種歴があった。

驚いたことに，昭和36年（1961年）6月12日と6月26日の2回，私はIPVを接種していた（**写真1**）。ソークIPVワクチンの「希望接種」が実施された1961年1～3月よりは遅い時期であり，私は当時3歳で，「希望接種」対象年齢でもない。おそらくは，親が予防の大切さを思って何らかの機会に接種を希望したのであろう。私の両親は医師でも医療従事者でもないが，わが子の健康を思いポリオワクチンを漏らさず接種してくれたことに今あらためて感謝したいと思う。

Ⅴ．OPVからIPVへの転換

OPVは，ポリオの予防にきわめて有効で，世界各地でポリオを駆逐してきたワクチンである。しかし，頻度は低いながらも後遺症につながるワクチン関連性麻痺（vaccine-associated paralytic poliomyelitis；VAPP）という副反応のリスクがある。野生株ウイルスが駆逐され，長年ポリオの流行がないわが国の現状においては，OPVと同等の予防効果が



予防接種の時期
実施年月日
接種方法
接種量
特に強い反応又は全身反応
印
百日咳
初回 生後3箇月～6箇月
第1回
第2回
第3回
皮下
追加 初回接種終了後1年～1年半
年 月 日
腸パラチフス
初回 満3才満～4才
追加 初回接種終了後毎年
(25)
予防接種の時期
実施年月日
方法
印
結核（BCG）
初回 生後6箇月以内
追加 ツベルクリン反応が陰性なら毎年
(臨時予防接種)
予防接種の種別
実施年月日
接種方法
接種量
検診成績（種痘のみ）
特に強い局所又は全身反応
印
小児マヒ
皮下
36. 8. 2 小児マヒ生ワクチン服用済
小児マヒ 37. 1. 26
日脳 回済 津市
(26)

写真1　母子手帳のポリオワクチン接種記録（著者提供）
①昭和36年（1961年）８月２日，②昭和37年（1962年）１月26日，③昭和38年（1963年）６月12日にOPVを接種した記録があり，1961年のOPV一斉投与を受けていた．驚いたことには，昭和36年（1961年）６月12日と６月26日に２回のIPV接種歴もあった．

期待され，より安全と考えられるワクチン，すなわちIPVへの切替えの必要性については多くの者の判断が一致する。

海外諸国に約10年の遅れをとってしまったが，IPVが導入された。本特集では，各関連項目の専門家に執筆をお願いした。ポリオとそのワクチンの効果を論じるためには，まずポリオの病態を理解することが必要と考える。ポリオウイルス血清型や，不活化ワクチンのD抗原定量法についても私たちは知っておくべきである。世界ですでに30年にわたって広く使われている豊富な実績の単独不活化ポリオワクチンが，わが国でも９月から接種が可能となった。11月からは，国内２社で開発された弱毒Sabin株由来の不活化ポリオワクチン（Sabin-strain derived IPV；sIPV）（**写真2**）を含有する４種混合ワクチン（DPT-sIPV）も導入される。sIPVは，世界に先駆けて日本で承認された。世界で長年の経験があるconventional IPV（cIPV）とわが国で開発されたsIPV，これら両ワクチンの有効性と安全性については，本特集の中で詳しく説明されているが，それぞれが今後果たしていく役割にも注目が集まる。また，IPV導入によりすべてが完了したわけではない。幼児期以降の追加接種の必要性の有無，わが国で導入が遅れている多価混合ワクチンについて，海外の状況を紹介し，ポリオワクチンや予防接種制度全体の今後を考える機会にしたい。

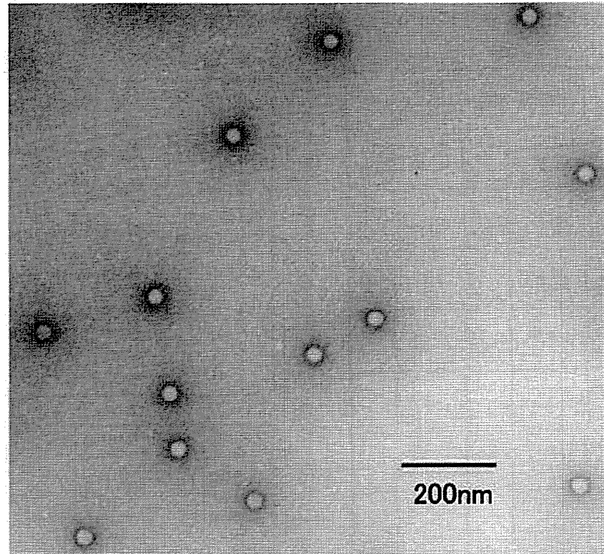


写真2　sIPV 原液（2型単価バルク）の電子顕微鏡観察像（ポリオ研，微研会，化血研提供）
sIPV は OPV の製造に用いられる弱毒ポリオウイルス Sabin 株の1型，2型および3型をそれぞれ Vero 細胞で培養し，濃縮・精製し，ホルマリン不活化後，3つの型を混合して日本ポリオ研究所で製造される．写真は2型不活化ウイルス液中の直径約30nm のウイルス粒子（酢酸ウランでのネガティブ染色）であり，ポリオウイルス粒子以外の夾雑物はほとんど認められない．1型および3型も2型と同様の写真像である．

Ⅵ．ポリオはワクチンで予防する！

OPV から IPV への定期接種の移行は，副反応の確率論でも費用対効果の判断でもなく，より安全なワクチンへの転換の潮流である。常に忘れてならないことは，ポリオは代表的なワクチン予防可能疾患（vaccine-preventable disease：VPD）であり，ワクチンの普及に勝る制御方法は存在しない。高い接種率を維持して，子どもたちを，そして人類をポリオから守りたいと思う。

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特集　今だから知っておきたいワクチンの話題

Ⅱ．各ワクチンの現状と課題

ポリオワクチン－生と不活化どちらがよいか

中野貴司（なかの たかし）　川崎医科大学小児科

要旨

経口生ポリオワクチン（OPV）は，血中中和抗体とともに腸管局所免疫も付与し，ポリオ予防のための優れた手段である．しかし頻度は低いながらも，副反応であるワクチン関連麻痺（VAPP）をはじめ，弱毒ワクチン株の神経病原性復帰という問題点がある．不活化ポリオワクチン（IPV）は良好な免疫原性を有し，安全性の点でも評価が高い．わが国においてもIPVおよびIPVとDPTの混合製剤の導入が待たれる．

Key words　経口生ポリオワクチン（OPV），ワクチン関連麻痺（VAPP），不活化ポリオワクチン（IPV），中和抗体，腸管免疫

はじめに

ポリオはもっとも代表的なワクチン予防可能疾患（vaccine-preventable disease，以下VPDと略す）であることを，まず強調したい．麻疹やHib感染症などワクチンによる予防がきわめて有効な疾患は数多く存在するが，予防接種の普及が流行の制圧に多大な役割をはたしたという点において，ポリオはトップクラスに位置する．ポリオを予防するワクチンが登場して約半世紀が経過したが，開発当初に世界中で顕著な成果をあげたワクチンは，経口生ポリオワクチン（oral polio vaccine，以下OPVと略す）であった．わが国でも，1961年の緊急導入により大流行に終止符を打っためざましい成果が歴史に残る．しかしOPVは，その普及により野生株ポリオウイルスが消滅した現代においては，頻度は低いながらも発生するワクチン関連麻痺（vaccine-associated paralytic poliomyelitis，以下VAPPと略す）が問題視される．現状のわが国において定期接種のOPVを，VAPP発症の危険性がない不活化ポリオワクチン（inactivated polio vaccine，以下IPVと略す）へと転換すべきであることは，大多数が同意するところである．承認された製剤として，IPVの使用が可能となることを一刻も早く実現したい．本稿の刊行時に，あと何カ月後にIPVが承認される見込みかは定かでないが，再度冒頭に述べたことをくり返す．ポリオはVPDである．そして，他のVPDと同様に接種可能月齢となったらなるべく早期に，具体的には乳児期前半から接種するべきワクチンであるということを忘れないでいたい．IPV導入を待つためのOPV接種差し控えによる接種率低下は，ポリオに免疫をもたない疾患流行のハイリスク集団を生みだすことになり，ポリオウイルスが侵入する隙を与えることになる．わが国で長らくポリオ流行がなかったのは，高いOPV接種率が維持されてきたからこそである．接種率の大幅な低下が危惧される際には，OPV接種の再喚起か，IPVの早期緊急導入かという議論が展開されるであろうが，ポリオワクチン接種をすませていない集団をさら

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に増大させることは，何としても避けなければならない．

ポリオワクチンの免疫原性評価に用いる抗体測定法

ワクチンの免疫原性評価のためには，ポリオウイルスに対する中和抗体価を測定する．ポリオウイルスは1型，2型，3型の3種類の血清型が存在するので，それぞれの型別に測定し評価する．ほとんどのコマーシャルラボラトリーで，検査の依頼が可能である．中和試験は，細胞変性効果（cytopathic effect，以下CPEと略す）を指標として，一般にマイクロ中和法で実施される[1]．

CPEを指標としたマイクロ中和法による抗体価の測定では，患者血清を4倍から2倍ずつ段階希釈し，100$TCID_{50}$の攻撃ウイルスを等量加え，36℃で2時間中和し細胞に接種したあと7日間観察して，50%中和を示す血清希釈の逆数を中和抗体価とする[1]．4倍以上が抗体陽性と判断されるが，疾患の発症を予防するために必要な抗体価の基準は明示されていない．ワクチン効果については，陰性（4倍未満）から8倍以上への陽転化，あるいは4倍以上の抗体価上昇を有意とする場合が多い．

経口生ポリオワクチン（OPV）

定期接種に用いられるOPVは，1型，2型，3型の弱毒ワクチン株を含有する3価ワクチンで，自然感染と同じ経路で体内に投与され，腸管局所免疫を付与するとともに，血清中和抗体も良好に上昇する．1950年代後半のわが国は，未曾有のポリオ大流行を経験していた．1960年の届出患者数は5,000名を超え，患者の多くは子どもたちであった．主症状である麻痺は後遺症や時に生命にもかかわり，国民は恐怖に慄いた．この惨状を救ったのが，Sabinが開発して間もないOPVであった．ソビエト連邦（当時）やカナダから緊急輸入され，1961年に全国一斉投与が行われた結果，流行は瞬く間に終息した．その後，国産OPVの生産も始まり，1964年からは定期予防接種として実施されるようになった．

接種により獲得される血中の中和抗体は，約4週間でピークに達し長年にわたって持続する．2回投与後の血中中和抗体獲得率は，1型と2型で90%以上，3型で80%以上とされる．また，腸管局所免疫が獲得できることは，OPVの大きな利点である．ポリオウイルス増殖の場である腸管の抵抗力が備わるため，ウイルスはそこに感染定着できない．OPVの一斉投与により短期間で流行の制御を達成できる理由は，この点に負うところが大きく，OPVは集団免疫効果に優れたワクチンである．また，経口で簡便に投与できるということも長所である（**表1**）[2]．しかし，OPVには短所もある．それらは，ワクチン株の神経毒性復帰と糞便中に排泄され伝播する性質に起因したものである．

1．ワクチン関連麻痺（VAPP）

弱毒ワクチン株による麻痺，すなわちOPVの副反応である．被投与者が発症する以外に，糞便中に排泄されたウイルスが周囲の者に感染し麻痺をおこす場合（接触例，contact case）もある．VAPP患者では2型と3型ポリオワクチン株の分離頻度が高く，1型はまれである．

OPV初回投与，男性，B細胞免疫不全者などは，VAPP発症のリスク因子である．また，OPV内服後1カ月以内の筋肉や組織への損傷はVAPP発生頻度を増加させる（provocation poliomyelitis）という報告がある[3]．乳児期に肛門周囲膿瘍を有する児への注意も指摘されている[4]．

米国では1990～1999年の免疫不全宿主や接触例も合せての解析があり，VAPP発症はOPV使用量290万接種あたり1例，OPV初回投与では90万接種あたり1例，2回目以降の投与では590万接種あたり1例という結果であった[3]．わが国の報告では1981～2006年の間に免疫異常のない被接種者から麻痺患者が出た割合は約486万接種あたり1人，接触者の場合には約789万接種あたり1人と

表1　OPVとIPVの比較（文献2）より引用）

	OPV	IPV
接種後の腸管局所免疫	強力に獲得される（○）	獲得の期待は薄い（▲）
接種後の血清中和抗体	良好に上昇（○）	非常に良好に上昇（○）
便からのワクチン株ウイルス排泄	あり（▲）	なし（○）
ワクチン関連性麻痺（VAPP）の発生	数百万接種に1例あり（▲）	なし（○）
ワクチン由来株の伝播（cVDPV）	あり（▲）	なし（○）
集団免疫効果（herd immunity）	あり（○）	期待は薄い（▲）
高温曝露によるワクチンのダメージ	失活著明（▲）	失活する（▲）
投与法	経口で簡便（○）	注射が必要（▲）
他のワクチンとの混合製剤製造	期待は薄い（▲）	可能（○）
価格	安価（○）	高価（▲）

長所に○，短所に▲を付した
OPV: oral polio vaccine, IPV: inactivated polio vaccine, VAPP: vaccine-associated paralytic poliomyelitis, cVDPV: circulating vaccine-derived poliovirus

される[5]．また，予防接種健康被害救済制度に申請し，OPVによる麻痺と認定された人数は，2001（平成13）～2010（平成22）年度の10年間で15人である．日本では，年間おおむね110万人がOPVを受けており，この結果からVAPPの頻度は100万人の接種あたり約1.4人とも計算されている[6]．頻度としては決して高くないが，麻痺は不可逆性で後遺症を残すため，放置できない副反応である．

2．生ワクチン由来株の伝播（cVDPV）

生ワクチン由来株の伝播（circulating vaccine-derived poliovirus，以下cVDPVと略す）とは変異した生ワクチン由来株がヒトの間で伝播流行し，一定地域で麻痺患者が集積することがある．cVDPVであると確定するためには，流行伝播しているウイルスの分子疫学的解析が必要である．主要なウイルス表面抗原蛋白VP1において，対応するOPV株から1%以上の塩基置換が存在する場合がVDPVであり，長期のウイルス増殖や伝播を反映している[3,7]．

3．免疫不全宿主における生ワクチン由来株の長期排泄（iVDPV）

免疫不全宿主では，OPV株の糞便中への排泄が数年以上の長期にわたって持続することがある．そして，ワクチン株は体内で長期に代を重ねるうちに，変異して神経毒性を復帰することがある．これを免疫不全宿主における生ワクチン由来株の長期排泄（immunodeficient excretor of vaccine-derived poliovirus: iVDPV）といい，1960年代以降30例以上の報告がある[3]．

わが国におけるVAPPの報告

最近，約10年間にわが国でVAPPとして文献や学会報告された症例を，**表2**に示した[8,9]．男児でOPV初回内服者が，VAPP症例の大多数を占めた．OPV内服2～3週間後に発熱し，その数日～5日後に麻痺をきたした．MRI所見や神経生理検査は診断に有用であるが，病期や検査のタイミングも関係するためか，必ずしも陽性所見が得られるとはかぎらない．

ポリオウイルスワクチン株が分離された例は多くはなかったが，麻痺発症早期の適切な時期に検体が採取されていない例も目立った．12例中7例で糞便中のウイルス陽性が確認され，2型と3型ウイルスであった．髄液からポリオウイルスが検出された例はなかった．ウイルス学的診断のためには，陽性と判定できる期間が長期に持続する糞便が，もっとも適切である．糞便からのウイルス分離の重要性をくり返し喚起する必要がある．

免疫学的検査により明らかな免疫不全症が見つ

表2 VAPPが疑われた症例－わが国における最近10年間の文献報告より（文献8）9）より引用，一部症例追加）

症例	月齢	性別	OPV内服	基礎疾患	OPV－発熱	OPV－麻痺	麻痺部位	脊髄MRI所見	神経伝導速度	後遺症	糞便ウイルス	髄液ウイルス	文献など
1	10	男	1回目	肛門周囲膿瘍	26日	28日	右下肢	脊髄神経根造影効果	活動電位低下	あり	Sabin3型分離陽性	記載なし	菅谷，他．脳と発達43:5207，2011（抄録）
2	7	男	未接種	なし	―	―	両下肢	腹側馬尾造影	複合筋活動電位導出不可	あり	Sabin2型分離陽性	記載なし	宇宿，他．日児誌115:800-803，2011
3	7	男	1回目	肛門周囲膿瘍	19日	23日	右下肢	異常なし	異常なし	あり	Sabin3型分離陽性	分離&PCR陰性	Higashigawa, et al. J Infect Chemother 16:356-359, 2010
4	11	男	1回目	なし	20日	26日	右下肢	T2強調像で病変	伝導速度低下	あり	記載なし	記載なし	文，他．大阪小児科学会雑誌25:5，2008（抄録）
5	10	男	1回目	なし	15日	18日	左下肢	異常なし	活動電位低下	あり	Sabin2，3型分離陽性	分離&PCR陰性	佐久間，他．小児感染免疫21:213-217，2009
6	4	記載なし	1回目	なし	18日	21日	左下肢	記載なし	活動電位低下	あり	記載なし	記載なし	梶本，他．脳と発達40:343，2008（抄録）
7	6	男	1回目	HBsキャリア	17日	21日	両下肢	T2強調像で脊髄前角に高信号	異常なし	あり	Sabin株陽性	陰性	石羽澤，他．小児感染免疫20:S135，2008（抄録）
8	9	男	1回目	なし	1カ月以内	(発熱5日後に項部硬直)	左下肢	T2強調像で高信号	記載なし	あり	Sabin株陽性	陰性	清益，他．小児科49:1271-1275，2008
9	6	男	1回目	肛門周囲膿瘍	15日	21日	両下肢	脊髄前角に異常信号	記載なし	あり	Sabin3型分離陽性	分離陰性	朝比奈，他．脳と発達39:S343，2007（抄録）
10	18	男	未接種	なし	―	―	右下肢	未実施	未実施	あり	未実施（2型血清中和抗体上昇）	未実施	長澤，他．Neuroinfection 11:73，2006（抄録）
11	8	男	1回目	なし	14日	18日	両下肢	記載なし	活動電位低下	あり	記載なし	エンテロウイルスPCR陰性	松藤，他．日児誌108:437，2004（抄録）
12	13	男	2回目	なし	22日	22日	右下肢	T2強調像で脊髄前角に高信号	活動電位，伝導速度とも低下	あり	分離&PCR陰性	分離&PCR陰性	石井，他．日児誌107:1631-1634，2003

かった例はなかったが，12例中3例ではOPV内服前に肛門周囲膿瘍を有していた．海外の文献でも，肛門周囲膿瘍がVAPP発症の危険因子であることを指摘している報告[4]がある．現状のわが国のOPV添付文書[5]には，OPV内服1カ月以内の抜歯，扁桃摘出，緊急を要しない手術，頻回の筋肉注射はVAPP発症の危険因子であり避けることが望ましいと記載されているが，肛門周囲膿瘍については触れられていない．

予後については，全例で運動機能障害や変形萎縮など後遺症を残していた．VAPPも野生株ポリオウイルスによる麻痺と同様に，いったん麻痺をきたせばその予後は不良である．

不活化ポリオワクチン（IPV）

IPVもOPVと同様に，1型，2型，3型のポリオウイルスが混合されている．Salkが初めてIPVを開発した頃から，1型はMahoney，2型はMEF1，3型はSaukett株が使われており，スウェーデンとデンマークは1型にBrunenders株を用いる[10]．これらはすべて，野生強毒ポリオウイルス由来の株である．

IPVはこの半世紀で，濃縮精製工程やD抗原定量法の導入により免疫原性の高いワクチンに改良された．改良当初は強化不活化ポリオワクチン(enhanced potency IPV，以下eIPVと略す）とよばれたが，今ではすべてのIPVがeIPVである．含有されるD抗原の量は，かつては1型20単位・2型2

表3　海外におけるIPVの免疫原性－接種後1カ月の時点における中和抗体価（文献10）11）より引用）

IPV接種回数	接種時期	1型		2型		3型		実施研究数	接種対象者数
		陽性者の割合	中和抗体GMT	陽性者の割合	中和抗体GMT	陽性者の割合	中和抗体GMT		
2	2カ月，4カ月	89～100%	17～355	92～100%	17～709	70～100%	50～1,200	30	4,500
3	2カ月，4カ月，12～18カ月	94～100%	495～2,629	98～100%	1,518～6,637	97～100%	1,256～4,332	10	2,000
3	2カ月，4カ月，6カ月	96～100%	143～2,459	96～100%	78～2,597	95～100%	187～3,010	48	6,000
3	3カ月，4カ月，5カ月	85～100%	110～475	98～100%	92～944	86～100%	89～1,244	8	500
3	2カ月，3カ月，4カ月	93～100%	143～595	89～100%	91～561	95～100%	221～1,493	18	2,200

単位・3型4単位であったが，現在は1型40単位・2型8単位・3型32単位という組成である[10]．

IPVがポリオを予防できる理論的根拠は，①ポリオウイルスに感染すると麻痺に先んじてウイルス血症が認められること，②血中に中和抗体が存在すれば麻痺発症を予防できること，である[10]．現在のIPVは開発当初のものより免疫原性が高まり，より予防効果を期待できると考えられる．規定回数を接種した場合，麻痺性ポリオを予防できる有効率はSalkワクチンで80～90%，現在のIPVでは90%以上とされる[10]．ただしIPVは，OPVと比較して腸管局所免疫を付与できる期待が薄い（表1）．

1．免疫原性

表3は，IPV（DPTなどとの混合ワクチンを用いた成績を含む）の免疫原性検討の結果をまとめて一覧表にしたものである[10][11]．2回接種で高い抗体陽転率が得られ，3回接種後は抗体がさらに上昇する．それぞれ異なる研究結果をまとめたものであるから一概に比較はできないが，2，4，6カ月に接種した場合が，抗体陽転率・獲得された中和抗体の幾何平均抗体価（geometic mean titers，以下GMTと略す）ともにもっとも良好であった．また米国では，2，4カ月に2回接種した後に，3回目接種を6カ月時と1歳過ぎに接種して比較した研究があるが，1歳過ぎで3回目を接種したほうが3回接種後の中和抗体GMTは高値であった[10]．

米国では，1997～2000年まではIPVとOPVを併用する定期接種スケジュール（sequential schedule of IPV and OPV）を採用していた．これは，2，4カ月にIPV，その後6～18カ月と小学校就学前にOPVを投与するというものであった．OPVの副反応であるVAPPがOPV初回投与後におこることが多いため，このようなスケジュールが採用された．本スケジュールで免疫原性を検討したところ，野生強毒株由来IPVを2回接種した後にOPVを投与した結果，ブースター効果は良好であった[3][10]．現在でもsequential scheduleを用いている国がある．

長期的な予防効果については，欧米の定期接種スケジュール（表4）でIPVを4～5回接種した後は，中和抗体価で判定すると初回免疫から5～10年以上は免疫が保持されていると考えられる[10]．

2．副反応

IPVは海外で広く用いられ，安全なワクチンと位置づけられている．接種部位の発赤；2%以内，硬結；数～10%，圧痛；10～30%などが報告されているが，重篤な副反応は認められていない[10]．DPT，さらにはB型肝炎やインフルエンザ菌b型（Hib）との混合ワクチンを用いた場合でも，IPVの副反応が増強することはない．

免疫不全宿主に対しては，前述のVAPPやiVDPVのことを考えるとOPVは使用し難い．ウ