Table 2
Comparison between cycling probe real-time PCR and VZV LAMP-RFLP assays.

Cycling probe real-time PCR	LAMP-RFLP		
	Wild-type	vOka	Negative
A. Extracted DNA was used for analysis			
Wild type	23	0	3
vOka	0	1	0
Negative	2	0	9
Total (n = 37)	25	1	12
B. Swab sample without DNA extraction was used for analysis			
Wild type	23	0	2
vOka	0	1	0
Negative	2	0	10
Total (n = 37)	25	1	12

LAMP, loop-mediated isothermal amplification; RFLP, restriction fragment length polymorphism.

PCR was compared between swab samples with and without DNA extraction. As shown in Fig. 5, the threshold cycles of samples with (DNA) and without DNA extraction (Swab) were well correlated (R^2 = 0.7309). However, the cycle numbers of the DNA samples were generally low in comparison to the swab samples. Additionally, one sample was found to be positive when extracted DNA was assayed; however, this sample was below the detection limit when assayed directly without DNA extraction (Table 2A and B).

5. Discussion

As shown in Fig. 2, both VZV wild-type strain-specific and vOka strain-specific cycling probe real-time PCRs amplified specific viral DNA. Moreover, no cross-reaction was observed with HSV-1 and HSV-2, which belong to the same herpes virus subfamily (alphaherpesvirus subfamily) as VZV and can cause vesicular skin lesions. Thus, this initial validation study demonstrates the high specificity of the cycling probe real-time PCR methods for the amplification of each VZV strain. In addition to the specificity, we demonstrated the utility of strain-specific cycling probe real-time PCRs for the quantitative analysis of viral DNA load (Fig. 3). This assay was also highly sensitive as evidenced by its lower limit of detection of 10 copies per reaction for both wild-type-specific and vOka-specific assays. Moreover, inter- and intra-assay evaluations demonstrated low coefficients of variation values (Table 1). Thus, this method is considered to be highly reliable for the detection of strain-specific viral DNA.

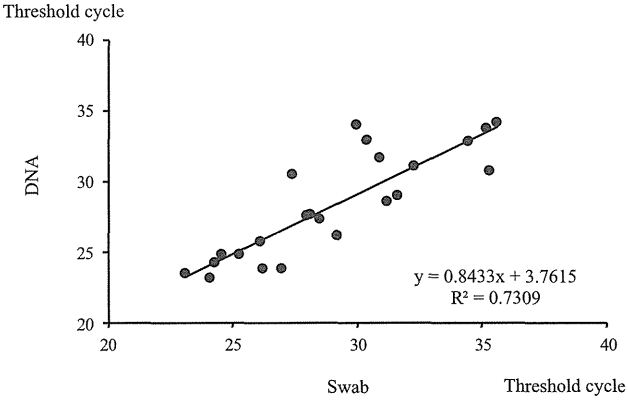

Fig. 5. Correlation of the threshold cycles for cycling probe real-time PCR using swab samples with and without DNA extraction.

Although quantitative evaluation of swab samples is considered to be difficult, a fluid sample directly collected from vesicular skin lesions, peripheral blood mononuclear cells, or dorsal root ganglia can be used for quantitative analysis of VZV DNA load (Cohrs and Gilden, 2007; Kimura et al., 2000; Pevenstein et al., 1999; Quinlivan et al., 2007). Therefore, it is suggested that the cycling probe real-time PCR methods developed in this study would be useful for both molecular epidemiological analysis and pathophysiological analysis of VZV infection by quantitative evaluation of viral DNA load in clinical specimens, particularly in vaccine recipients.

After initial validation analysis of the specificity and sensitivity of the method, its reliability for clinical use was evaluated by the analysis of swab samples collected from patients suspected of having varicella or zoster. The results of the VZV wild-type strain cycling probe real-time PCR were almost the same as those of the VZV LAMP-RFLP. Three false positive samples were identified by the VZV wild-type strain cycling probe real-time PCR. As the sensitivity of the method is better than that of the VZV LAMP-RFLP, it is considered that the false positive samples contained small amounts of VZV DNA that were lower than the detection limit of the LAMP method. Although two false-negative samples were identified by the VZV wild-type strain cycling probe real-time PCR, the reason for the false-negative result is unknown. Additional samples should be examined to determine the precise negative predictive value in future study.

Although only one swab sample contained vOka DNA, the results of the cycling probe real-time PCR were consistent with the result of LAMP-RFLP. Thus, our current data suggest that the VZV cycling probe real-time PCR is a reliable method for discrimination between the two strains in clinical samples. Moreover, the strong correlation of threshold cycles for both the VZV wild-type strain-specific cycling probe real-time PCR and the real-time PCR (Fig. 4) suggests that this method could be useful for quantitative analysis of clinical specimens.

The detection of viral DNA in swab samples without DNA extraction has been demonstrated using LAMP methods (Enomoto et al., 2005; Ihira et al., 2010). This is a great advantage for laboratory diagnosis because it makes the assay rapid, easy, and less expensive to perform. Therefore, we attempted to detect viral DNA from swab samples without DNA extraction using cycling probe real-time PCR and real-time PCR. Although the sensitivity of cycling real-time PCR was slightly lower in samples without DNA extraction compared to samples with the DNA extracted (Table 2 and Fig. 5), the direct cycling probe real-time PCR using swab samples appears to be useful for quantitative evaluation of viral DNA because it is easy to perform (Fig. 5). Thus, the direct cycling probe real-time PCR may be an alternative strategy for the examination of large numbers of samples due to its ease of performance.

Recently, it has been demonstrated that Varivax (Merck, Whitehouse Station, NJ) and Varilrix (GlaxoSmithKline, Melbourne, Australia), which are used commonly in the United States and Europe, contain a small fraction of the wild-type alleles in gene 62 (positions 105705, 107252, and 107599) based on sequencing analysis of the TA clones from one batch of the vaccine. Additionally, both wild-type and vaccine-type alleles in gene 62 have been found in the isolate from a zoster patient (Quinlivan et al., 2012). Thus, although it has been demonstrated that the SNP at position 105705 was useful for discrimination between wild-type and vaccine-type strains (Argaw et al., 2000; Gomi et al., 2002), a large number of isolates should be analyzed by sequencing to determine whether the present SNP analysis can completely discriminate between wild-type and vaccine-type strains in Japan. Furthermore, since vOka/Biken (Biken, Osaka, Japan) is generally used in Japan, it is also necessary to conduct sequence analysis of the clones from vOka/Biken. Additionally, it is important to

develop molecular methods for discrimination between wild-type and vaccine-type strains using other SNPs located in gene 0 (Peters et al., 2012) and gene 38 (Quinlivan et al., 2012).

In summary, this study demonstrated that cycling probe real-time PCR can differentiate between the VZV wild-type strain and the vOka strain, as well as determine viral DNA load. In addition, this method can be used for the analysis of clinical swab samples with or without DNA extraction. Thus, cycling probe real-time PCR would be alternative tool for high-throughput molecular discrimination between VZV wild-type and vOka strains.

Conflict of interest declaration

The authors do not have any commercial or other associations that might pose a conflict of interest.

Ethical approval

This study was approved by the review boards of Fujita Health University (#09-045).

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原　著

MRワクチンと水痘ワクチン同時接種の効果ならびに安全性

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要　旨

【目的】MRワクチンと水痘ワクチンの同時接種の効果と安全性を評価する．

【対象と方法】MRワクチンの第1期接種時に水痘ワクチンの同時接種に同意した82名と，年齢，性別をそろえた水痘ワクチン単独接種43名，およびMRワクチン単独接種51名を対象とした．ワクチン接種前後の水痘，麻疹，風疹の各ウイルス抗体価を測定し，副反応を調査し，同時接種者には水痘抗原に対するELISPOTアッセイを実施した．さらに接種1年後に水痘罹患状況を調査し，未罹患者に水痘ワクチンを追加接種し評価した．また1歳時にMRワクチンと，水痘ワクチンが接種された28名を対象に，MRワクチンの第2期接種時に水痘ワクチンの同時接種を実施し，同様にウイルス抗体価を評価した．

【結果】水痘抗体陽転率，平均抗体価ともに単独接種群と同時接種群間で有意差はなかった．麻疹，風疹も同様に抗体陽転率，接種後平均抗体価に両群間で有意差はなかった．水痘特異的細胞性免疫能の評価では71.4％に細胞性免疫の獲得が示唆された．また，特に問題となる副反応はなかった．ワクチン接種後1年間の水痘罹患は11％であった．接種1年後に水痘ワクチンの追加接種を実施し，明確なブースター効果が確認された．MRワクチン第2期接種時の水痘ワクチン追加接種においても接種前に比べ水痘抗体価の有意な上昇を示した．

【考察】MRワクチンと水痘ワクチンの同時接種は，安全かつ有効であり，また水痘ワクチンの追加接種の有効性が示された．

キーワード：水痘ワクチン，MRワクチン，ワクチンの同時接種，定期接種

はじめに

我が国の小児予防接種を欧米先進国レベルに引き上げるため，現行の定期接種ワクチンに加え任意接種ワクチンを定期接種化し，より多くのワクチンを効率的に接種してゆく必要があり，複数ワクチンの同時接種は極めて有用な手段である．欧米ではワクチン同時接種が一般的に実施されているが，我が国ではその歴史がなく，その効果，安全性に関する基礎的なデータが求められている．

水痘ワクチンは，Takahashiら[1]により開発，実用化された唯一のヒトヘルペスウイルスワクチンである．開発当初，わが国で安全性，有効性についての数多くの知見が蓄積され[2)3)]，それを基盤として1995年米国食品医薬局の承認を受け，翌年から米国でuniversal vaccinationが開始された[4)～6)]．その目覚ましい効果については既に数多くの報告があり，水痘の季節流行パターンが消失しつつあることも明らかになっている[7)]．しかしながら，ワクチン開発国であるわが国では未だ任意接種のため，接種率は約40％程度にとどまっており，毎年春の流行期には数多くの患者が発生し，中には重篤な合併症を来たす症例がみられる．特に免疫不全宿主では極めて重症化し，中には致死的な経過をたどる例がある[8)]．費用対効果分析の結果から，定期接種化のメリットが明らかになっており[9)10)]，わが国においても早急に定期接種化がなされるべき重要なワクチンである．

そこで本研究では，水痘ワクチン定期接種化に際し，現時点で問題となる麻疹風疹混合（MR）ワクチンとの同時接種について，その効果と安全性を評価すること

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を目的とした．さらに，水痘帯状疱疹ウイルス(varicella zoster virus, VZV)感染制御に重要な役割を果たすと考えられているVZV特異的細胞性免疫反応を評価するため，被検児の末梢血単核球（peripheral blood mononuclear cells, PBMCs)を用いてinterferon-γ enzyme-linked immunospot（ELISPOT）アッセイを実施した．

対象と方法

1．対象

現在のMRワクチンの定期予防接種時期である第1期（1歳）と第2期（小学校就学前）に水痘ワクチンを同時接種し，それぞれstudy 1, study 2として実施した．

1）第1期MRワクチン接種との同時接種（study 1）：2009年7月～2012年3月の3年間に，藤田保健衛生大学小児科，国立病院機構三重病院小児科，豊川市民病院小児科および竹内小児科医院（京都市）にて第1期MRワクチン接種を希望して来院した健康小児の中で，水痘ワクチンの同時接種に同意した82名を対象とした．同時接種者の平均年齢は16.4±4.6か月，性別は男児45例，女児37例．また，MRワクチンとの同時接種の有効性と安全性を評価するため，江南厚生病院小児医療センターにて実施したMRワクチン単独接種群51名と水痘ワクチン単独接種群42名と比較検討した．MRワクチン単独接種群の平均年齢は15.4±2.5か月，男児25例，女児26例．水痘ワクチン単独接種群の平均年齢16.9±3.0か月，男児21例，女児21例だった．MRワクチンと水痘ワクチン同時接種群と，MRワクチン単独接種群（$p=0.19$），水痘ワクチン単独接種群（$p=0.17$）間の年齢に有意差はなかった．尚，本研究については，藤田保健衛生大学倫理審査委員会の承認を受け（承認番号：09-070），説明文書に従い保護者へ説明を行い，同意を得たのちに研究へ組み入れた．

2）第2期MRワクチンとの同時接種（study 2）：国立病院機構三重病院小児科，落合小児科医院，竹内小児科医院および馬場小児科にて，1歳時にMRワクチンと水痘ワクチンの接種を受け，小学校就学前の第2期MRワクチン接種時期までに水痘の既往がなく水痘ワクチンの追加同時接種に同意した小児28名を対象とし，接種前後の各抗体価を測定した．平均年齢は5.2±0.6歳，性別は男児12例，女児16例．本研究については，国立三重病院倫理審査委員会の承認を受け，説明文書に従い保護者へ説明を行い，同意を得た後に研究へ組み入れた．

2．血清抗体価測定

ワクチン接種前と接種後（4～6週間後）のペア血清を採取し，(財）阪大微生物病研究会にて各ウイルス抗体価を測定した．測定方法は麻疹が中和法(neutralization test, NT法)，風疹は赤血球凝集抑制試験(hemagglutination inhibition, HI法)，水痘は免疫粘着赤血球凝集反応（immune adherence hemagglutination, IAHA法）とglycoprotein antigen based enzyme-linked immunosorbent assay（gp-ELISA法）の二つの方法で実施した．麻疹と風疹においては2回測定し抗体価の幾何平均値を結果とした．IAHA法とgp-ELISA法については以下の方法で実施した．

a）IAHA法：被験者血清を希釈液にて2倍希釈後，56℃60分で非働化，さらに2倍段階希釈しマイクロプレートの各wellに添加した．その後各wellに抗原液を添加し撹拌，37℃60分間静置し，補体を添加撹拌し静置，ヒトO型赤血球を添加し室温で2時間反応後ウイルス抗原添加列の凝集像を観察した．抗体価算出は赤血球凝集を抑制する検体の最大希釈倍数とした．判定基準は抗体価2倍未満を陰性，2倍以上を陽性と定義した．尚，幾何平均抗体価算出において便宜的に2倍未満を1とし，256倍以上を256とした．

b）gp-ELISA法：マイクロプレートにVZV糖蛋白抗原及びコントロールとしてMRC-5糖蛋白抗原を固相化し，その後標準血清および検体を添加，37℃60分間反応させた．その後酵素標識抗体を添加し37℃60分間反応させ，洗浄後基質・発色液を添加した後25℃40分間反応させ，最終的に吸光度（OD405nm）を測定した．検体の吸光度は，コントロールの吸光度を差し引き，標準血清による検量線を参考にして算出した値を水痘ウイルス抗体価とした．抗体価50単位以上を陽性，50単位未満を陰性と定義した．幾何平均抗体価算出においては，50単位未満は49単位として計算した．

3．水痘細胞性免疫能評価

VZV特異的細胞性免疫反応を評価するため，第1期MRワクチン・水痘ワクチン同時接種群においてワクチン接種前と接種後（4週間）にヘパリン加血を採取しPBMCsを凍結保存（液体窒素）し，後日医薬基盤研究所でELISPOTアッセイを実施した[11]．96 wellプレートに抗ヒトIFN-γモノクローナル抗体を加え，4℃でover nightインキュベートし，その後，PBSにて洗浄した．PBMCsを4×10^6cells/mlに調整した浮遊液を100μlずつ各wellに加え，次にVZV抗原を含む同量のメディウムを添加した．VZV抗原は水痘ワクチンをUV処理（5,000J/m^2）して使用した．陽性コントロールとして，phytohemagglutinin-L4（Wako）を2μg/mL含むメディウムを使用した．陰性コントロールはVZV抗原を含まないメディウムとした．5%CO_2インキュベータ内で40時間反応後，PBSで洗浄し，0.4μg/mlビオチン化抗ヒトIFN-γを各wellに添加した．1時間

室温放置し，洗浄後ストレプトアビシン標識ペルオキシダーゼ添加，45分間室温放置したのち，テトラメチルベンジンを添加して3分間静置後にスポット数測定に使用した．スポットの検出はES ELISPOT system (Carl Zeiss) で行った．

4. 副反応調査

調査項目(37.5℃ 以上の発熱，発疹，咳嗽，鼻汁，接種部腫脹発赤) が記された調査票に被検児の保護者が記載する方法でワクチン接種日から接種後28日間までの症状を前方視的に調査した．

5. 水痘ワクチン接種後罹患に関する調査

第1期MRワクチンと水痘ワクチン同時接種例82名のうち藤田保健衛生大学病院ならびに豊川市民病院で接種を受けた50名を対象として，ワクチン接種後罹患のアンケート調査を行った．平成23年12月に往復ハガキによる調査を行った．調査項目は水痘罹患の有無，罹患した場合は罹患時期，発疹数，発熱の有無，感染源，抗ウイルス剤治療の有無について確認した．

6. 水痘ワクチン追加接種

上記アンケート調査の中で，水痘未罹患の場合には希望者に水痘ワクチン追加接種することを記載し，水痘ワクチン追加接種の希望者を募集した．応募者9名に水痘ワクチンを接種し，ワクチン接種前と接種後1か月の2回血清を採取し，水痘抗体価を測定した．

7. 統計学的解析

統計解析には「Microsoft エクセル統計 2008 (SSRI, Tokyo, Japan)」を使用した．ウイルス抗体価の比較はMann Whitney U-testを用い，対象者の年齢，性別，副反応発生頻度の比較はχ^2検定およびt検定で解析した．

結　果

第1期MRワクチンとの同時接種 (study 1)

1. 水痘抗体価の推移

MRワクチンと水痘ワクチン同時接種群82例の接種前水痘抗体はIAHA法で判定不能であった1例を除き全例が陰性，接種後水痘平均抗体価は，IAHA法で3.81±2.17(log2で表記)，gp-ELISA法で2.38±0.40 (log10で表記) であった (表1A)．各抗体の陽転者数はそれぞれ66例 (81.5%)，74例 (90.2%) であった．また，水痘ワクチン単独接種群42例の接種前水痘抗体も全例で陰性，接種後抗体価は3.31±2.38(IAHA法)，2.34±0.46 (gp-ELISA法) であり，それぞれの抗体陽転例は30例 (71.4%) と36例 (85.7%) だった．同時接種群と単独接種群間の各抗体価を比較すると，IAHA ($p=0.33$)，gp-ELISA ($p=0.57$) ともに2群間で有意差はなかった．

2. 水痘ELISPOTアッセイ

凍結保存細胞の解凍後にアッセイに必要なPBMCs数 (4×10^6cells/mlに調整した浮遊液を1.5ml) が得られた7名について水痘ワクチン接種前後のELISPOTアッセイの結果を解析した(表2)．水痘抗原刺激，PHA刺激および対象の数値は2回実施した平均値を記載した．VZV抗原刺激による反応が陰性コントロールに対し2倍以上の場合を陽性と定義すると，7名中5名 (71.4%) が接種後陽性となった．ワクチン接種後もIAHA法とgp-ELISA法の両方法で抗体陽転を認めなかった5名の中で，3名でELISPOTアッセイを実施し，うち2名 (No.7, 8) が陽性を示した．

3. 麻疹・風疹抗体価

MRワクチンと水痘ワクチンの同時接種群82名中2名が接種前麻疹抗体陽性であったため，接種前抗体陰性の80名について評価した(表1B)．その結果，接種後麻疹抗体価 (log2で表記) は5.41±1.57 (NT法)，風疹抗体価 (log2で表記) は5.54±1.74 (HI法) であった．麻疹NT抗体≧2，風疹HI抗体≧8を陽性と定義すると，各抗体のワクチン接種後陽転者はそれぞれ79例 (98.8%)，77例 (96.3%) であった．また，MRワクチン単独接種群51名の接種後抗体陽転者は，麻疹51例 (100%)，風疹49例 (96.1%) であった．接種後平均抗体価は，麻疹5.34±1.29(NT法)，風疹5.63±1.62 (HI法) であり，それぞれMRワクチンと水痘ワクチン同時接種群との間に有意差はなかった (麻疹NT抗体；$p=0.40$，風疹HI抗体；$p=0.52$)．

4. 副反応

水痘ワクチン・MRワクチン (第1期) 同時接種群 (n=50) で副反応について調査した結果，重篤な副反応は認めず，今回の調査対象とした発熱，発疹，咳嗽，鼻汁，接種部発赤や腫脹のみであった．MR単独接種群 (n=50) と比較した結果，発熱 (同時接種群：26.0%，MR単独接種群：28.0%，$p=0.82$)，発疹 (同時接種群：10.0%，MR単独接種群：12.0%，$p=0.75$)，咳嗽 (同時接種群：12.0%，MR単独接種群：12.0%，$p=1.00$)，鼻汁 (同時接種群：16.0%，MR単独接種群：20.0%，$p=0.60$)，接種部発赤 (同時接種群：4.0%，MR単独接種群：6.0%，$p=0.65$)，接種部腫脹 (同時接種群：2.0%，MR単独接種群：2.0%，$p=1.00$) と両群間に有意差は認めなかった．

5. 水痘ワクチン接種後1年間の水痘罹患状況

50名中36名 (回収率72%) から返信があり，ワクチン接種後罹患者は4例 (11.1%) であった．罹患時期は全例接種後7か月以降であり，いずれも発熱はなく，発疹数100個未満と軽症だった．感染源は同胞3例，保育所の友人1例であった．

表1　MR1期と水痘ワクチン同時接種における水痘抗体反応（A）と麻疹・風疹の抗体反応（B）

A

接種方法	測定法	接種前抗体陰性者	陽転率(%)	接種後平均抗体価(平均値±標準偏差)	p値
VZV単独	IAHA法	43	72.1	3.35±2.37*	0.368
MR+VZV		81	81.5	3.81±2.17*	
VZV単独	gp-ELISA法	42	85.7	2.36±050**	0.529
MR+VZV		82	90.2	2.38±0.40**	

*log2を表記．**log10を表記.
IAHA抗体価≧2を陽性（+），gp-ELISA抗体価≧50を陽性（+）とした.

B

ウイルス	測定法	接種前抗体陰性者	陽転率(%)	接種後平均抗体価*	接種前抗体陰性者	陽転率(%)	接種後平均抗体価*	p値
		MR+VZV			MR単独			
麻疹	NT法	80	98.6	5.41±1.57	51	100.0	5.34±1.29	0.400
風疹	HI法	80	96.3	5.54±1.74	51	96.1	5.63±1.62	0.520

*log2を表記：(平均値±標準偏差)
麻疹NT抗体価≧2を陽性（+），風疹HI抗体価≧8を陽性（+）とした.

表2　水痘ワクチン接種前後の水痘ELISPOTアッセイ結果（第1期MRワクチンと同時接種群）

Case	接種前					接種4週間後				
	IAHA	gp-ELISA	ELISPOT			IAHA	gp-ELISA	ELISPOT		
			VZV Ag	PHA	Control			VZV Ag	PHA	Control
1	<2	<50	5	168	2.5	32	466	42	200	4
2	<2	<50	1	103	0.5	16	317	n.d.	n.d.	n.d.
3	<2	<50	18	200	20	16	310	0	124	1
4	<2	<50	3.5	107	1	16	147	13	200	2
5	<2	<50	0	170	2	8	114	n.d.	n.d.	n.d.
6	<2	<50	0.7	200	0.5	8	93	n.d.	n.d.	n.d.
7	<2	<50	1	92	3	<2	<50	2.5	72	0.5
8	<2	<50	n.d.	n.d.	n.d.	<2	<50	9.7	200	3
9	<2	<50	3	194	4	<2	<50	0.5	200	1
10	<2	56	3	200	1.5	16	355	10	200	2
陽性率						70% (7/10)	70% (7/10)	71.40% (5/7)		

n.d.：未実施

6. 水痘ワクチン追加接種の効果

MRワクチンと水痘ワクチンを同時接種した1年後に9例に対して水痘ワクチンを追加接種したが，その前後の抗体価を表3に示した．初回接種時月齢は中央値12か月で，追加接種までの間隔は平均15±1.1か月（13か月～17か月）であった．IAHA法では初回接種後66.7%が抗体陽転したが，追加接種前には陽性率は33.3%に低下した．陰性化した中には初回接種後抗体価が比較的高値であった例も含まれた（64倍を示した児が1例，32倍を示した児が2例）．一方，gp-ELISA法では初回接種後陽転率は77.8%で，追加接種前には100%の陽性率であった．追加接種後の抗体陽性率はIAHA法，gp-ELISA法とも100%で，平均抗体価はIAHA法6.9±1.4, gp-ELISA法4.0±0.4と著明に上昇した.

表3　水痘ワクチン追加接種の効果

MRワクチン第1期と水痘ワクチンの同時接種者への水痘ワクチン追加接種効果.

	初回接種				追加接種				
	接種前		接種後		接種前		接種後		
Case	IAHA	gp-ELISA	IAHA	gp-ELISA	IAHA	gp-ELISA	IAHA	gp-ELISA	接種間隔（月）
1	<2	<50	32	218	<2	211	128	30,954	15
2	<2	<50	<2	<50	<2	250	32	3,182	14
3	<2	<50	8	204	<2	198	64	5,012	16
4	8	<50	32	180	<2	208	128	12,813	13
5	<2	<50	<2	<50	<2	150	32	2,102	17
6	<2	60	64	364	<2	334	128	7,654	15
7	<2	73	<2	414	2	388	≧256	20,948	15
8	<2	<50	64	770	64	5,496	≧256	12,694	15
9	<2	<50	32	148	4	371	128	15,348	15
陽性率（%）			66.7	77.8	33.3	100	100	100	
平均抗体価（Ave. ±SD）			3.3±2.6	2.3±0.4	1.0±2.0	2.5±0.5	6.9±1.4	4.0±0.4	15.0±1.1*

（平均抗体価はIAHA法：log2，gp-ELISA法：log10で表記.）
*平均接種間隔を示す

第2期MRワクチンとの同時接種（study 2）

1. 水痘抗体価の推移（表4A）

第2期MRワクチンと水痘ワクチンの同時接種では，接種前水痘平均抗体価および陽性率はIAHA法で2.96±3.10, 55.6%，gp-ELISA法で3.02±0.92, 89.3%であったが，ワクチン接種後は，IAHA法で6.07±1.38, 100%，gp-ELISA法で3.97±0.56, 100%であり，接種前後で平均抗体価の有意な上昇（それぞれ$p=0.0017$, $p=0.0003$）が認められた.

2. 麻疹・風疹抗体価（表4B）

接種前麻疹平均抗体価および陽性率はNT法で5.71±1.37, 100%であったが，ワクチン接種後は，6.66±0.81, 100%と抗体価の有意上昇を認めた．接種前に比較的低い抗体価を示したのは2.5（1名），3.5（1名），4.0（1名）であり，その他は4.5以上と比較的高い抗体価が維持されていた．接種後抗体価は5.0（1名）と5.5（2名）を除き，6.0以上の抗体価を示した．また，風疹においても接種前平均風疹抗体価および陽性率はHI法で5.93±1.30, 100%であったが，ワクチン接種後は，6.57±0.88, 100%と，抗体価の有意上昇を認めた．接種前最低抗体価は8倍が2名，16倍が2名であり比較的高い抗体価が維持されていたが，接種後抗体価は32倍（3名）を除き，他は64倍以上の抗体価を示した.

3. 副反応

第2期のMRワクチンと水痘ワクチンの同時接種において全身性の有害事象はなかった．17人中接種局所の発赤を2名に，腫脹を2名に認めた程度で特に問題となる副反応はなかった.

考　察

水痘ワクチンのuniversal immunizationの効果は，既に導入後16年が経過した米国からの種々の報告を見れば明らかである[12)13)]．さらに，わが国においての費用対効果研究においても，間接効果を考慮に入れることにより定期接種化が有益なことが示されていることから[9)10)]，一刻も早い水痘ワクチンの定期接種化が望まれている．実際に水痘ワクチンの定期接種化を導入する際，最も現実的なスケジュールは第1期MRワクチンとの同時接種と考えられる．今回の研究の結果，水痘ワクチン単独接種群と水痘ワクチンとMRワクチン同時接種群間でワクチン接種後VZV抗体価に有意差は認められなかったので，水痘ワクチンとMRワクチンの同時接種により，VZVに対する液性免疫誘導に支障のないことが裏付けられた．また，MRワクチン単独接種群と水痘ワクチン，MRワクチン同時接種群間でワクチン接種後の麻疹，風疹抗体価を比較したが，両群間で有意差がなかったことから，同時接種は麻疹ワクチン，風疹ワクチンの抗体誘導能に関しても影響がないことが示された．さらに2期MRワクチンとの同時接種でもVZV抗体価の上昇が確認でき，追加接種の有効性も示唆された．以上の結果から，水痘ワクチンとMRワクチンの同時接種が，両ワクチンの効果に影響を与えないことが示された．これは，対象数は

表4　MRワクチン第2期接種と水痘ワクチンの同時接種における水痘抗体反応（A）と麻疹・風疹の抗体反応（B）

A

採血時期	測定法	陽転率（%）	抗体範囲	平均抗体価	p値
接種前	IAHA法	15/27 (55.6)	<2～>256倍	2.96±3.10*	0.0017
接種後		27/27 (100)	16～>256倍	6.07±1.38*	
接種前	gp-ELISA法	25/28 (89.3)	<50～55,859	3.02±0.92**	0.0003
接種後		28/28 (100)	292～94,256	3.97±0.56**	

*log2を表記：(平均値±標準偏差)
**log10を表記：(平均値±標準偏差)
IAHA抗体価≧2を陽性（+），gp-ELISA抗体価≧50を陽性（+）とした.

B

ウイルス(検査方法)	測定時期	陽転率（%）	抗体価の範囲	平均抗体価	p値
麻疹(NT法)	接種前	28/28 (100)	2.5～8.5*	5.71±1.37*	0.0017
	接種後	28/28 (100)	5.0～8.5*	6.66±0.81*	
風疹(HI法)	接種前	28/28 (100)	8倍～256倍	5.93±1.30*	0.0003
	接種後	28/28 (100)	32倍～256倍	6.57±0.88*	

*log2を表記：(平均値±標準偏差)
麻疹NT抗体価≧2を陽性（+），風疹HI抗体価≧8を陽性（+）とした.

少ないものの，既に欧米でのMMRワクチンと水痘ワクチンの同時接種データで示されている有効性の評価結果を裏付けるものと思われる[14].

本邦の水痘ワクチンは極めて安全性が高く，健康小児に接種した場合ほとんど副反応を生じない[2)15)]．したがって，同時接種時の安全性についての評価に際し，本研究では水痘ワクチン，MRワクチン同時接種群とMRワクチン単独接種群間で接種後1か月間の副反応の発生状況を比較した．発熱，発疹，接種局所反応など全ての項目で両群間に差は認められず，安全性の点でも同時接種は問題のないことが明らかとなった．対象症例が少ないため，極めて頻度の低い副反応については正確な評価は困難と思われ，その点については実際に定期接種が導入された後に注意深く観察してゆく必要がある.

VZVの感染制御においては，液性免疫よりむしろ細胞性免疫が重要なことは良く知られている．我々は既にELISPOTアッセイによるVZV特異的細胞性免疫反応の評価法を報告した[11]．今回その方法を用いて水痘ワクチンとMRワクチン同時接種時のVZV特異的細胞性免疫能の解析を試みた．残念ながら，今回の研究では保存PBMCsをESISPOTアッセイのために融解した際，生存細胞の割合が極めて低く，多くの症例で解析が不可能だった．これは，被検者から末梢血を採取した後，PBMCsを分離，液体窒素保存するまでのタイムラグ(施設間輸送に伴う)がPBMCsの生存状況に影響したと考えられ，今後の検討課題である．しかしながら，解析可能であった一部症例を見ると，7名中5名（71.4%）で細胞性免疫が獲得されていると考えられた．さらに今回VZV抗体陽転を認めなかった5名のうち3名でELISPOTアッセイが実施され，そのうち2名で細胞性免疫の獲得が認められた．抗体測定法の感度の問題もあるが，primary vaccine failureと思われる被接種者の中には弱いながらVZV特異的細胞性免疫を獲得し得るものが存在することがうかがわれる.

水痘ワクチンは他の弱毒生ワクチンに比べ安全性が高い半面，ワクチン接種後罹患がみられることも知られている[2]．本研究では，同時接種例について1年間の観察期間をもうけワクチン接種後罹患の調査を実施した．その結果，過去の報告[15)16)]同様11%(36名中4名)で接種後罹患が認められた．いずれも，家族内あるいは幼稚園での水痘患児との接種を契機としており，接種後7か月から1年間の間に発生していた．ワクチン接種後罹患患者数の抑制は，今後定期接種化を進める上で重要な課題である．米国では，全員接種方式導入後接種率の上昇に伴い，患者数は減少したがsecondary vaccine failureの増加が問題となった[12)17)]．そのため，現在は水痘ワクチン二回接種が推奨され，追加接種の時期は抗体価の減衰が進む4～6歳が勧められている[18]．現在のわが国での接種後罹患はこの状況とは異なり，primary vaccine failureのみならず1回接種

では十分な免疫誘導ができず接種後罹患を起こすことが問題となっている．したがって，現時点でのわが国での水痘ワクチン接種施策は，米国とは異なり，より早期の追加接種で全体の患者数削減を目指すことが重要と思われる．実際，一部のワクチン接種後水痘未罹患者について1年後のVZV抗体価を調べた結果，IAHA法では9名中6名（67%）の対象者で検出限界以下であった．さらにこれらの患者に水痘ワクチンを追加接種することにより，明確なブースター効果が確認されたことから，今後は適切な追加接種時期を明らかにするために，水痘ワクチン2回接種時期について検討してゆく必要があると思われた．

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Efficacy and Safety of Co-administration of Live Attenuated Varicella Vaccine and Measles-rubella (MR) Vaccine

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The aim of this study was to elucidate the efficacy and safety of the co-administration of a varicella-zoster virus (VZV) vaccine and the measles-rubella (MR) vaccine. One hundred seventy-six children who had no history of varicella were enrolled. Eighty-two children received simultaneous administration of the VZV vaccine and MR vaccine (VZV/MR). As controls, 42 age-matched children received the VZV vaccine only and 51 age-matched children received the MR vaccine only. Serum samples were drawn before vaccination and 4 weeks after vaccination. Antibody titers were measured by immune adherence hemagglutination (VZV), glycoprotein antigen-based enzyme-linked immunosorbent assays (VZV), neutralization tests (measles), and hemagglutination inhibition (rubella). Additionally, cell-mediated immunity (CMI) against VZV was tested in 7 children after vaccination. There were no significant differences in the seroconversion rates and mean antibody titers between the VZV/MR simultaneous vaccination group and the VZV only or MR only vaccination group. Of the 7 (71.4%) cases, 5 had measurable CMI. The incidence of breakthrough varicella in the 1 year following vaccination was 11%. At 1 year after the initial vaccination, a second dose of the VZV vaccine was administered to 9 children without breakthrough varicella, and a booster effect was observed in all subjects. VZV vaccination did not affect the antibody responses induced by the co-administration of the VZV and MR vaccines, and there were no severe side effects. Furthermore, the second dose of VZV vaccine had booster activity.

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Environmental Surveillance of Human Enteroviruses in Shandong Province, China, 2008 to 2012: Serotypes, Temporal Fluctuation, and Molecular Epidemiology

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Environmental surveillance is an effective approach in investigating the circulation of polioviruses (PVs) and other human enteroviruses (EVs) in the population. The present report describes the results of environmental surveillance conducted in Shandong Province, China, from 2008 to 2012. A total of 129 sewage samples were collected, and 168 PVs and 1,007 nonpolio enteroviruses (NPEVs) were isolated. VP1 sequencing and typing were performed on all isolates. All PV strains were Sabin-like, with the numbers of VP1 substitutions ranging from 0 to 7. The NPEVs belonged to 19 serotypes, and echovirus 6 (E6), E11, coxsackievirus B3 (CVB3), E3, E12, and E7 were the six main serotypes, which accounted for 18.3%, 14.8%, 14.5%, 12.9%, 9.0%, and 5.7% of NPEVs isolated, respectively. Typical summer-fall peaks of NPEV were observed in the monthly distribution of isolation, and an epidemic pattern of annual circulation was revealed for the common serotypes. Phylogenetic analysis was performed on environmental CVB3 and E3 strains with global reference strains and local strains from aseptic meningitis patients. Shandong strains formed distinct clusters, and a close relationship was observed between local environmental and clinical strains. As an EV-specific case surveillance system is absent in China and many other countries, continuous environmental surveillance should be encouraged to investigate the temporal circulation and phylogeny of EVs in the population.

Human enteroviruses (EVs) belong to the genus *Enterovirus*, family *Picornaviridae*. Human EVs comprise more than 100 serotypes which are classified into 4 species, EV-A to EV-D (1). EVs usually cause silent infection, but sometimes they are associated with serious diseases, such as acute flaccid paralysis (AFP), aseptic meningitis and encephalitis, acute myocarditis, acute hemorrhagic conjunctivitis (AHC), and hand, foot, and mouth disease (HFMD).

Polioviruses (PVs) belong to EV-C and have three serotypes. Infection with PVs is known to be associated with acute paralytic poliomyelitis. The global incidence of poliomyelitis has dropped a lot since the Global Polio Eradication Initiative (GPEI), and no cases due to indigenous wild poliovirus (WPV) have been identified in Shandong Province since 1991. However, WPV importation from countries where it is endemic maintains a threat to the polio-free status of China, and several incidents of WPV importation have been reported, including the importation of WPV1 in Xinjiang in 2011. The standard approach recommended by the WHO for polio surveillance is the detection and investigation of AFP cases, and environmental surveillance offers a supplementary method which has been demonstrated to play an important role in early warning of WPV importation and vaccine-derived poliovirus (VDPV) circulation (2). In several countries, WPVs and VDPVs have been detected in sewage in the absence of reported AFP cases (3–6).

Although environmental surveillance has served primarily as part of PV surveillance in many parts of the world, gradually more focus has been put on the circulation and molecular characterization of environmental nonpolio enteroviruses (NPEVs) (7–12). In China, information on the circulating NPEVs is limited due to the absence of a specific enterovirus surveillance system. Surveillance based on human specimens is limited and mainly includes testing of specimens collected through AFP and hand, foot, and HFMD surveillance and occasional testing of patients with meningitis or encephalitis. So, despite the increasingly detailed information on temporal/geographical circulation and molecular epidemiology of EVs from various parts of the world, substantial geographical gaps remain in mainland China.

Environmental surveillance has been conducted in Shandong Province, China, since 2008. Previously we reported the isolation of a rare recombinant poliovirus with chimeric capsid VP1 protein from sewage in 2009 (13) and molecular epidemiology and intercity spread of echovirus 6 (E6) in 2008 to 2011 (10, 11), demonstrating the high sensitivity of the surveillance. In this report, we present an overview of serotype distribution and temporal dynamics of PVs and NPEVs from environmental surveillance from 2008 to 2012 and phylogenetic comparison of the relationship of

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predominant serotypes between environmental and clinical strains, to reflect the circulation and molecular characterization of EVs in Shandong, China.

MATERIALS AND METHODS

Sampling sites, frequency, and method. Sewage samples were collected in the cities of Jinan and Linyi, with metropolitan populations of 2.6 million and 1.9 million, respectively. The sampling sites were the inlet collector canals of the sewage treatment plants of each city—Jinan Everbright Water (JNEW) and Linyi Shouchuang (LYSC).

Generally, sewage samples were collected monthly in Jinan between February 2008 and July 2010 and semimonthly in Jinan between August 2010 to December 2012 and in Linyi from April 2010 to April 2012. Approximately 1 liter of sewage was collected by the grab sampling method in the afternoon between 2:00 and 3:00 p.m. A cold temperature (approximately 4°C) was maintained during sample transport to the laboratory, storage (<24 h), and processing.

Concentration and virus isolation. Sewage samples were concentrated approximately 40-fold by using membrane absorption/elution as described previously (8, 11). Briefly, 800 ml of the sewage was centrifuged at 3,000 × *g* for 30 min at 4°C. $MgCl_2$ (2.5 M) was added to the supernatant to a final concentration of 0.05 M. The pH was adjusted to 3.5 with 0.5 M hydrochloric acid. Then the solution was filtered through a 0.45-μm mixed-cellulose ester membrane filter (ADVANTEC, Tokyo, Japan). Absorbents on the filter were then eluted with three additions of 10 ml of 3% beef extract solution, followed by ultrasonication for 1 min each time (for sewage specimens collected from 2008 to June 2011) or twice for 1.5 min each time (samples collected from July 2011 to December 2012). After centrifugation at 3,000 × *g* for 30 min, the supernatant was filtered through a 0.22-μm filter and was ready for cell inoculation. L20B, RD, and HEp-2 cell lines were used for virus isolation. Cells were seeded in each tube at 1×10^5. For each cell line and each sewage sample, 18 parallel cell vials with standard monolayer cell cultures were inoculated with 200 μl of concentrated solution for each vial.

Comparison of elutions at different pHs. During the 5-year surveillance, the pH of elution fluid (3% beef extract solution) had been changed from 7.0 to 9.0 since July 2011. Its influence on virus elution was examined by a laboratory-based recovery experiment. Briefly, a sewage specimen was collected from the Jinan treatment plant. After inactivation at 56°C for 2 h and centrifugation at 3,000 × *g* for 30 min, the PV type 1 (PV1) Sabin strain was seeded into the supernatant (400 ml) to a final titer of 256 to 512 50% tissue culture infective doses ($TCID_{50}$)/100 μl. After absorption and sonication, elution was performed with beef extract at pH 7.0 and 9.0. The microtiter assay on prefiltration fluid, filtrated fluid, and elution fluid (20 ml) was performed. Serial 4-fold dilutions were prepared with minimal essential medium (MEM), and 100 μl of each was transferred to the RD cell monolayer in a microplate. The titer ($TCID_{50}$/100 μl) was determined by reading cytopathic effect (CPE) microscopically after 5 days. The assays were performed in triplicate under both pH conditions.

Clinical isolates. The EVs isolated from cerebrospinal fluid (CSF) specimens from local acute meningitis and encephalitis patients were used for VP1 sequencing and phylogenetic analysis. A total of 8 coxsackievirus B3 (CVB3) strains and 1 E3 strain were used in this study, and they were isolated according to the WHO's *Polio Laboratory Manual* (14).

Serotyping. According to standard protocols recommended by the WHO (14), PV serotyping was carried out via microneutralization assays in 96-well tissue culture plates using polyclonal antisera against PV types 1, 2, and 3 (National Institute for Public Health and the Environment, RIVM, the Netherlands). NPEV serotyping was performed by using RIVM antiserum pools A to G (15).

VP1 amplification, sequencing, and molecular typing. VP1 sequencing was performed on all the NPEV isolates. Total RNA was extracted from 140 μl of the viral isolates using a QIAamp viral RNA minikit (Qiagen, Valencia, CA) according to the manufacturer's recommended procedure. Reverse transcription-PCR (RT-PCR) was performed using the Access RT-PCR system (Promega, USA). Primer pair UG1/UC11 (16) was used to amplify the entire VP1 coding region of PV isolates. Primer pair 187/011 (17), which corresponds to the 3′ end of VP1 and 5′ end of 2A, was used for amplification of an ~750-nucleotide (nt) sequence of NPEVs. In order to detect cross contamination, an RT-PCR using the RNA extracted from normal RD cells served as a blank control, and a negative control containing all the components of the reaction except for the template was also included. PCR-positive products were purified and sequenced bidirectionally with the BigDye Terminator v3.0 cycle sequencing kit (Applied Biosystems, Foster City, CA), and sequences were analyzed with an ABI 3130 genetic analyzer (Applied Biosystems, Hitachi, Japan). Molecular typing based on VP1 sequences was performed using the online Enterovirus Genotyping Tool, version 0.1 (18).

TABLE 1 Rate of recovery of PV1 Sabin strain by elution solution under different pHs[a]

Expt no.	pH	Prefiltration $TCID_{50}$/100 μl	Elution $TCID_{50}$/100 μl	Recovery rate (%)	Avg (%)
1	7.0	362	256	7.1	
2	7.0	304	256	8.4	8.5
3	7.0	512	512	10.0	
1	9.0	362	2,048	56.6	
2	9.0	512	3,444	67.2	63.7
3	9.0	431	2,896	67.2	

[a] The volume for prefiltration was 400 ml in all experiments, and the volume for elution was 40 ml in all experiments.

Homologous comparison and phylogenetic analysis. Nucleotide sequence alignments were carried out by BioEdit 7.0.5.3 software (19). Phylogenetic trees were constructed by Mega 4.0 (20) using the neighbor-joining method after estimation of genetic distance using the Kimura two-parameter method (21). A bootstrapping test was performed with 1,000 duplicates, and the transition/transversion rate was set at 10 (22). In order to reduce excessive computational load, the 100% identical sequences collected at the same location and time were manually removed.

Nucleotide sequence accession numbers. The VP1 nucleotide sequences of environmental and clinical isolates described in this report were deposited in the GenBank database under the accession numbers GU272011 to GU272015, JQ364885, KF747380 to KF747467, KF747491 to KF747578, KF246751, KF246758, GQ329744, GQ329767, GQ246518, FJ919564, FJ919566, JQ364849, and JQ364850.

RESULTS

Comparison of elution at different pH conditions. Virus recovery in laboratory-based experiments showed that the recovery rates for the PV1 Sabin strain were 7.1% to 10.0% at pH 7.0 and 56.6% to 67.2% at pH 9.0 (Table 1). The titer of the filtrated fluid is 0 $TCID_{50}$/100 μl, reflecting efficient absorption of the virus to the membrane.

Virus isolation. A total of 129 sewage samples (80 in Jinan and 49 in Linyi) were collected from February 2008 to December 2012. EVs were detected from 99 samples (63 in Jinan and 36 in Linyi), for a positivity rate of 76.7%.

A total of 168 PVs and 1,007 NPEVs were isolated during the 5-year surveillance (Table 2). Multiple serotypes could be detected simultaneously from a single sewage sample. For all the 99 EV-positive samples, the numbers of serotypes in a single sewage sample ranged from 1 to 12, although most samples ($n = 90$) contained no more than 8 serotypes (Fig. 1). Of the 1,007 NPEV strains isolated, 677 (67.2%) were isolated from the RD cell line and the other 330 isolates were from the HEp-2 cell line (Table 3).

In concentration of the 53 sewage samples collected from 2008 to 2010, the absorbents on the filter were eluted with 10 ml of 3%

TABLE 2 Number of isolates of different serotypes from environmental surveillance conducted in the two cities of Shandong from 2008 to 2012

Serotype	No. of isolates in city: Jinan 2008	2009	2010	2011	2012	Sum	Linyi 2010	2011	2012	Sum	Total no. of isolates
NPEV											
E1	0	0	0	1	1	2	0	2	0	2	4
E3	6	3	0	19	78	106	0	9	15	24	130
E6	1	0	29	66	10	106	3	70	5	78	184
E7	1	0	11	17	12	41	1	14	1	16	57
E11	0	0	22	32	59	113	32	4	0	36	149
E12	0	0	0	24	8	32	0	59	0	59	91
E13	0	0	9	2	0	11	6	1	1	8	19
E14	1	0	1	0	0	2	0	0	0	0	2
E19	0	4	0	3	3	10	5	0	0	5	15
E24	0	0	0	0	2	2	0	0	0	0	2
E25	0	0	1	3	5	9	0	9	8	17	26
E29	0	0	1	0	0	1	0	3	0	3	4
E30	0	0	0	3	14	17	4	2	2	8	25
CVA21	0	0	0	0	2	2	0	0	0	0	2
CVB1	0	0	0	2	0	2	2	8	0	10	12
CVB2	1	0	4	1	0	6	2	0	0	2	8
CVB3	7	1	1	40	49	98	0	8	40	48	146
CVB4	0	0	1	0	0	1	4	0	0	4	5
CVB5	0	4	3	8	14	29	0	0	0	0	29
Mix[a]	0	7	4	12	44	67	4	23	3	30	97
Total	17	19	87	233	301	657	63	212	75	350	1,007
PV type											
1	0	0	3	7	26	36	1	2	2	5	41
2	0	0	4	13	44	61	4	3	5	12	73
3	0	1	3	8	31	43	3	6	2	11	54
Total	0	1	10	28	101	140	8	11	9	28	168
Total EV	17	20	97	261	402	797	71	223	84	378	1,175

[a] "Mix" indicates NPEV mixtures. They could not be serotyped by RIVM antibody pools, and VP1 sequencing revealed mixed peaks.

TABLE 3 Number of NPEV isolates from RD and HEp-2 cell lines

Serotype	No. of isolates detected in cell line: RD	HEp-2	Total no. of isolates
E1	1	3	4
E3	111	19	130
E6	93	91	184
E7	52	5	57
E11	132	17	149
E12	84	7	91
E13	16	3	19
E14	2	0	2
E19	11	4	15
E24	2	0	2
E25	21	5	26
E29	4	0	4
E30	23	2	25
CVA21	2	0	2
CVB1	0	12	12
CVB2	5	3	8
CVB3	35	111	146
CVB4	1	4	5
CVB5	8	21	29
Mix	74	23	97
Total	677	330	1,007

beef extract solution by ultrasonication three times (1 min each time). Among the 205 EV isolates from this period, 101 (49.3%), 64 (31.2%), and 40 (19.5%) were recovered from the first, second, and third elutions by ultrasonication, revealing that the greater the degree of sonication, the lower the titer in the eluted fluid. Hence, the sonication protocol for the sewage collected from July 2011 was changed to two sonications of 1.5 min each.

Polioviruses. Of the 129 sewage samples during the 5-year surveillance, 50 were positive for PV (positivity rate, 38.8%), and altogether, 168 PVs were isolated. Numbers of PV1, PV2, and PV3 isolates were 41 (24.4%), 73 (43.5%), and 54 (32.1%), respectively. All these PVs were Sabin strains, with no WPV. The numbers of VP1 substitutions ranged from 0 to 7 (Fig. 2). Except for one VDPV2 isolate with 7 substitutions in VP1 coding region, no VDPV strains were identified. The monthly PV isolation in the two cities is illustrated in Fig. 3F and 4F. PV detection peaked in winter and spring, while in NPEV active seasons of summer and early autumn, PV detection decreased dramatically.

NPEVs. Of the 129 sewage samples during the 5-year surveillance, 96 were positive for NPEV (positivity rate, 74.4%), and altogether, 1,007 NPEVs were isolated. Serotyping and molecular typing were performed on all isolates, and 19 serotypes were identified (Table 2). Except for two coxsackievirus A21 (CVA21) isolates of species EV-C, all isolates belonged to EV-B. E6, E11, CVB3, E3, E12, and E7 were the six main serotypes and accounted for 18.3%, 14.8%, 14.5%, 12.9%, 9.0%, and 5.7% of the NPEVs isolated, respectively. There were still 97 isolates that could not be typed by serological methods, and VP1 sequencing revealed mixed peaks, demonstrating the existence of multiple NPEV serotypes in the isolates. Generally, the serotype constitutions of the two cities are similar, except for four serotypes (E14, E24, CVA21, and CVB5) that were detected only in Jinan.

Typical summer-fall peak of detection of NPEV was observed in the monthly distribution of isolation (Fig. 3 and 4). In different years, the constitutions of most common serotypes were different. In Jinan, the annual most common serotypes were E3 plus CVB3, E3 plus E19, E6 plus E11, E6 plus E11 plus CVB3, and E3 plus E11

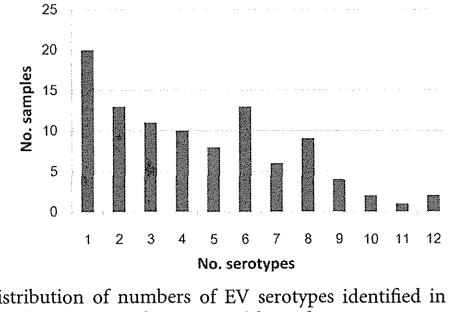

FIG 1 Distribution of numbers of EV serotypes identified in EV-positive sewage samples. Most samples contained fewer than 8 serotypes, while up to 12 EV serotypes could be detected in a single sample.

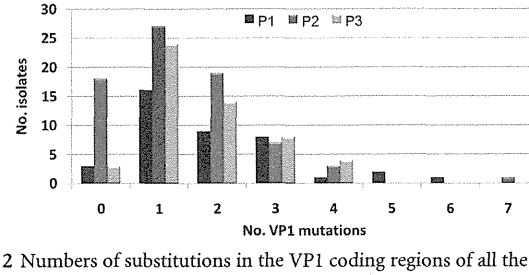

FIG 2 Numbers of substitutions in the VP1 coding regions of all the 168 PV isolates from environmental surveillance in Shandong, 2008 to 2012.

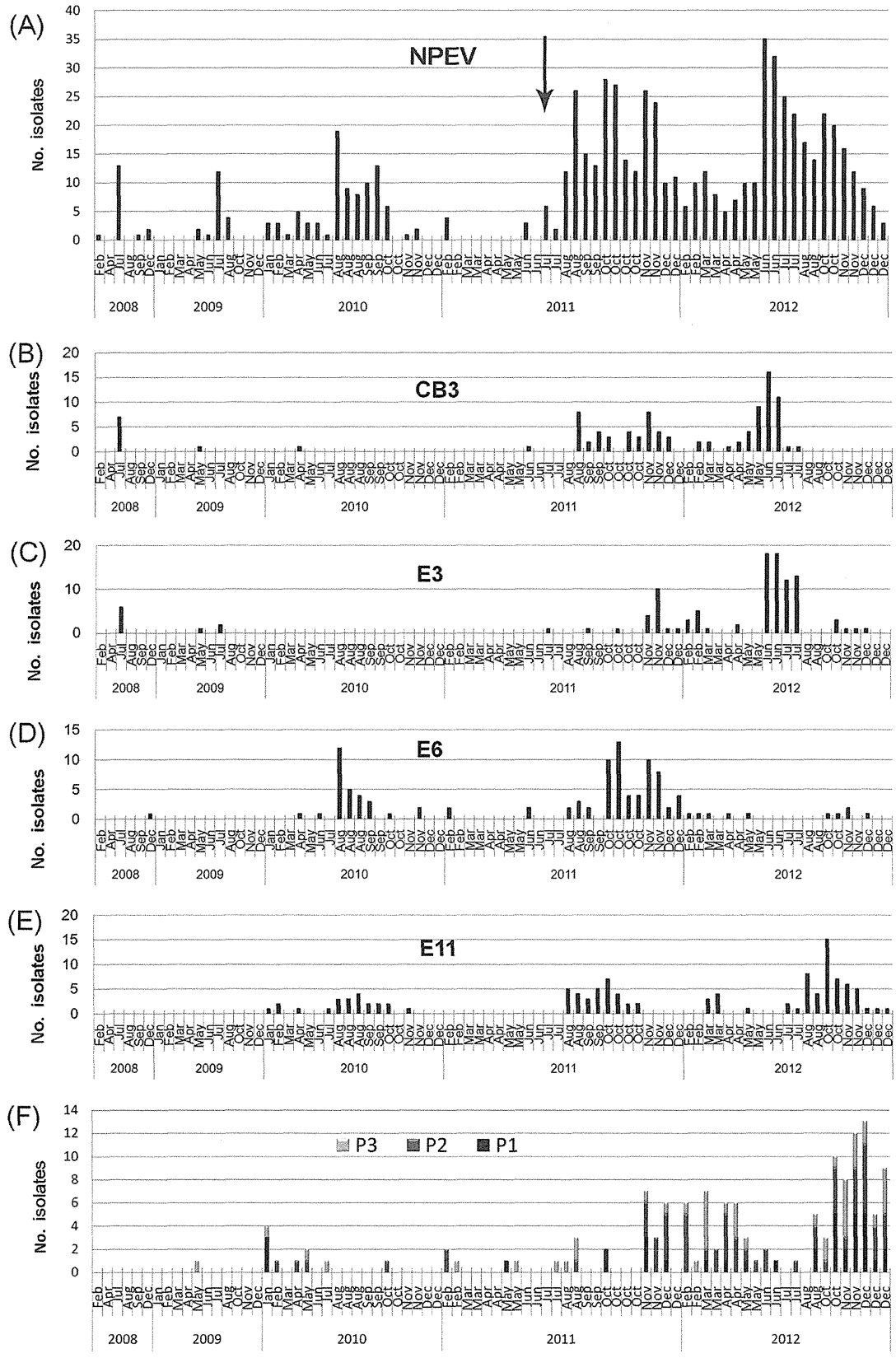

FIG 3 Temporal distribution of EVs in different samples collected in Jinan, 2008 to 2012. Monthly distributions of total NPEV, CVB3, E3, E6, E11, and PVs are illustrated in panels A to F, respectively. The black arrow in panel A indicates the time when the modified method was used. (Panels A and D are adapted from reference 11.)

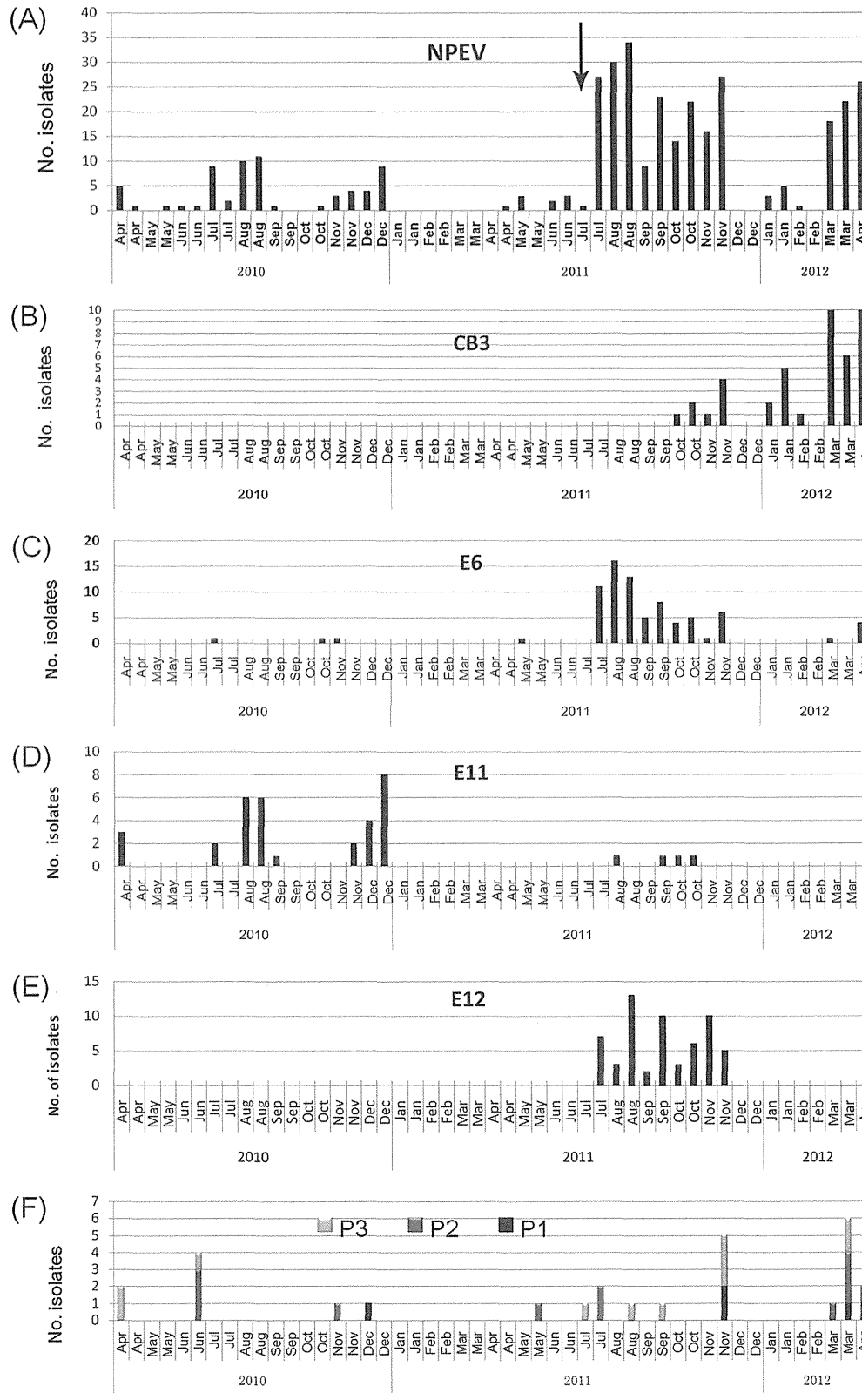

FIG 4 Temporal distribution of EVs in different samples collected in Linyi, 2010 to 2012. Monthly distributions of total NPEV, CVB3, E6, E11, E12, and PV are illustrated in panels A to F, respectively. The black arrow in panel A indicates the time when the modified method was used. (Panels A and C are adapted from reference 11.)

plus CVB3 from 2008 to 2012, respectively. In Linyi, the annual most common serotypes were E11, E6 plus E12, and CVB3 plus E3 from 2010 to 2012, respectively.

An epidemic pattern of annual circulation was revealed for these common serotypes by environmental surveillance (Fig. 3 and 4). CVB3 had low detection rates in 2009 and 2010 in Jinan. Then, an abrupt occurrence in sewage in August 2011 initiated a period of high CVB3 activity for about 12 months. However, in August 2012, CVB3 could not been detected from sewage, and since then, it has come into quiescence. E6 had low detection rates in 2008 and 2009, and after the high activity in 2010 and 2011, it came into quiescence in 2012 again. Similar temporal fluctuation was also observed for other common serotypes such as E3 and E11.

E30 is an important serotype associated with aseptic meningitis throughout the world (23). In the 5-year surveillance, E30 was present at a relatively higher frequency but lower number of isolates for each sewage sample, implying a low titer. From October 2011 to December 2012, a total of 13 sewage samples in Jinan were positive for E30, but the number of isolates for each sewage specimen was less than 3. Similar results were observed in Linyi. CVB5 is also associated with aseptic meningitis in Shandong Province (24). Its isolation had a characterization similar to that of E30. A total of 18 sewage samples in Jinan were positive for CVB5, and the number of CVB5 isolates for each sewage sample was less than 4 in all cases.

Homologous comparison and phylogenetic analysis. Phylogenetic analysis was performed on partial VP1 sequences of CVB3 and E3 strains from the environment, local aseptic meningitis patients, and global reference strains. Both serotypes had all been previously demonstrated to be associated with aseptic meningitis in Shandong Province and were present at a high frequency of isolation in sewage.

Environmental CVB3 segregated into two lineages (Fig. 5A). Both lineages contained isolates from Jinan and Linyi, and a close relationship was observed for the CVB3 isolates from the two cities, suggesting the occurrence of frequent intercity spread. Strain AM002/JN/CHN/12, isolated from an aseptic meningitis patient in Jinan in 2012, had a high similarity (99.8%) to environmental strain JN12180, isolated in Jinan in 2012. Phylogenetic analysis also revealed a close relationship between them.

Most Shandong environmental E3 isolates formed an exclusive cluster with no foreign strains (Fig. 5B). However, an E3 isolate (JNEW111125.R1-5) had a remote relationship with other Shandong strains and segregated into a cluster together with other foreign strains. Strain AM177/JN/CHN/12 was isolated from an aseptic meningitis patient in Jinan in 2012. It had a close relationship with strain JN12213 (similarity, 99.7%), isolated from sewage in Jinan in 2012.

DISCUSSION

Environmental surveillance has been demonstrated to be a sensitive method for monitoring PV and NPEV and estimating the extent and duration of EV circulation in a population (25, 26). In 2008, it was initiated in two provincial poliovirus laboratories (Shandong and Guangdong) in China as a supplementary method of poliovirus surveillance. This report presents an overview of serotypes distribution, temporal dynamics, and molecular epidemiology of EVs from environmental surveillance conducted in Shandong Province from 2008 to 2012.

A dramatic increase of PV and NPEV isolation was observed for sewage samples collected since July 2011 (Fig. 3 and 4). This is supposedly due to the optimization of the concentration method at that time. In the membrane absorption/elution method used for sewage concentration, beef extract solution was used to elute EVs from the membrane. Since July 2011, the pH of beef extract solution has been adjusted from neutral to alkaline (~9). The subsequent increased isolation from July 2011 to December 2012 demonstrated the importance of the alkaline condition for eluting EVs from the membrane. Moreover, the laboratory-based recovery test on PV1 further demonstrated the importance of the alkaline condition for EV elution (Table 1).

In this study, the sensitivity of the environmental surveillance was reinforced in two ways. First, besides the L20B and RD cell lines routinely used in poliovirus laboratory network, the HEp-2 cell line was used in this study for the isolation of group B coxsackieviruses. The frequent detection of CVB3 and CVB5 demonstrated the necessity of including the HEp-2 cell line in the environmental surveillance. Second, the number of parallel vials inoculated (18 vials of each cell line for each sewage specimen) was increased compared with that in other studies (7–9). This may increase the laboratory workload. However, our study suggested that by increasing the number of parallel vials inoculated, more serotypes can be detected from a single specimen (especially for the minor circulating serotypes), more sequences can be obtained for further molecular epidemiological study, and temporal dynamics can be manifested more clearly.

In Shandong Province, the last WPV-associated paralytic poliomyelitis patient was identified in 1991. Since then, no WPV has been detected, and VPDVs were detected in Shandong AFP cases in 2007, 2009, and 2011. In the 5-year environmental surveillance, all the 168 PVs isolated were Sabin strains, and only one VDPV2 strain with 7 substitutions in VP1 coding region was isolated. No further circulation of VPDV was detected. Previously, we reported the isolation of a recombinant poliovirus with chimeric capsid VP1 protein from sewage in 2009 (13). No such virus was identified from the local AFP surveillance system at that time. In this study, the environmental VDPV also had no related cases via AFP surveillance. So, our study demonstrates that AFP surveillance combined with continuous environmental surveillance is of great importance in improving the sensitivity of poliovirus detection.

An epidemic pattern is the characterization of temporal circulation of many EV serotypes (27). It is characterized by substantial fluctuations in circulation levels over time, including large peaks when the serotype was among the most prevalent serotypes in a given year. In this study, the monthly isolation of the common serotypes such as E3, E6, E11, E12, and CVB3 also reflected a distinct epidemic pattern. An interesting observation was made for the monthly detection of E30 and CVB5. They both are important pathogens of aseptic meningitis. During the surveillance period, their detection in sewage was frequent. However, the numbers of isolates were low. Case-based enterovirus surveillance in the United States in 1970 to 2005 had demonstrated an epidemic pattern for both serotypes (23, 27). So, the low isolate number of E30 and CVB5 might due to the low activity in the surveillance period or is just the result of low copy numbers in the excreta of infected individuals. Further investigation and surveillance are needed for clarification.

In this study, a total of 19 NPEV serotypes were identified from the sewage. Except for two isolates of CVA21 of EV-C, the serotypes belonged to EV-B. The predominance of EV-B in sewage is similar to findings described in previous reports (8, 9, 12, 26). As EV-A71- and CVA16-associated HFMD has become an emerging

FIG 5 Phylogenetic relationships of CVB3 (A) and E3 (B) isolates from sewage and clinical specimens in Shandong, 2008 to 2012, and strains from other regions. The phylogenetic trees were constructed using Mega, version 4.0, using the neighbor-joining method based on 704-nt (positions 2601 to 3304 of strain Nancy) and 716-nt (positions 2608 to 3323 of strain Morrisey) partial VP1 sequences of CVB3 and E3, respectively. ▲ and ▼, isolates from sewage in Jinan and Linyi, respectively. ●, isolates from patients of aseptic meningitis. The isolates before 2012 are identified by a code that consists of JNEW or LYSC, followed by the sample date (presented as YYMMDD [i.e., year, month, and day]) and the tube number. The isolates from 2012 are identified by JN or LY, followed by a 3-code serial number.

concern in mainland China since 2007 (28, 29), the absence of detection of these two serotypes from sewage is not correlated to the clinical situation. One reason may be the cell culture method used in this study. Because most echoviruses propagate more quickly in RD cells, if echoviruses and EV-A71 are inoculated into the same RD cell tube, the growth of EV-A71 can easily be suppressed by echovirus 6. Another reason may be that the viral copy numbers in the excreta of EV-A71-infected individuals are possibly lower than those from HEV-B-infected individuals, which will result in low titers in sewage. These explanations may partly account for the absence of detection.

The phylogenetic analysis revealed that Shandong environmental strains had a high degree of genetic diversity from foreign strains. CVB3 had been demonstrated to be the causative agents of aseptic meningitis outbreaks in Shandong in recent years (30), and CVB3 and E3 were also isolated from sporadic aseptic meningitis patients in 2012. A close relationship was found between environmental stains and clinical strains, demonstrating the sensitivity and significance of environmental surveillance, especially in regions with limited case surveillance. Moreover, the increase in CVB3 and E3 isolation from sewage initiated in the latter half of 2011 (Fig. 3) and the sporadic clinical cases present since the first half of 2012 reflect the importance of environmental surveillance in early warning of associated diseases.

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