表3 循環器の診断例，非診断例

	診断例	非診断例
中隔欠損	心室中隔欠損症＋大動脈縮窄症 心室中隔欠損症＋肺動脈狭窄	心室中隔欠損症（2例） 心室中隔欠損症＋肺動脈閉鎖 心房中隔欠損症＋心室中隔欠損症
房室・弁異常	三尖弁閉鎖不全症 左心低形成症候群	単心室（双胎）
大血管異常		大血管転移症 大動脈縮窄症
その他	拡張型心筋症 左室腫瘤	

表4 診断例，非診断例とIUGR，羊水過多症との関係

	IUGR	羊水過多
診断例(21例)	7例	3例
非診断例(21例)	2例 (腸回転異常，食道閉鎖)	1例 (咽頭腫瘍)

考 察

国立大蔵病院で行っていた妊娠20週前後と30週前後に産科医または臨床検査技師（日本超音波学会検査士資格所有者）が行う約15分間の胎児超音波スクリーニング検査で，先天異常の出生前診断率は50%であった．胎児超音波スクリーニングの報告は諸外国ではRADIUS study[1]，Eurofetus study[2]などがあるが，残念ながら本邦には大規模な報告はない．諸外国における1987年から1997年までの36のstudyの合計では胎児異常の有病率が2.0%，診断率（sensitivity）の平均が40.4%である．RADIUS studyの前後に分けると1987年から1991年までては診断率の平均は30.0%（14.2～81.8%），1989年から1997年までては平均50.2%（21.4～82.4%）と，年々上昇している[3]．診断率は各国，各施設により大きな開きがあり，その理由としては検者の技術，超音波機器の性能，スクリーニングの時期，回数などが考えられるが，今回の我々の診断率は諸外国での報告の平均とほぼ同等のものであった．

診断率が高かったのは泌尿器，染色体異常，低かったのは消化器（腸回転異常，鎖肛，食道閉鎖），四肢骨格（主に指の異常）であった．指の奇形は今回のスクリーニング項目には含まれていないので診断率は低かった．指の奇形，口唇口蓋裂などは単独であれば出生前に必ずしも診断されなくてもあまり問題はないと思われるが，重篤な疾患，出生後すぐに処置が必要となる疾患は出生前に診断する意義が大きい．特に循環器系は頻度が多く，また動脈管依存性の先天性心疾患は出生後すぐに処置が必要などの理由から重要視すべき臓器である．今回の循環器系疾患の診断率46%は諸外国の報告[2)4)5)]と比較し，症例数が少ないものの平均的な成績と思われる．我国での胎児心臓スクリーニングに関わる研究は少ないが，川滝ら[6]の報告では胎児心臓の出生前診断率は17.3%であり，一般に我が国は諸外国に比較して低いのが現状と思われる．Yooらは胎児心疾患の診断には①上腹部横断面②四腔断面③左室流出路④右室流出路⑤Three vessel view⑥Aortic arch viewの6つの断面の観察が有用だと述べている[7]．今回のスクリーニング項目にも①～④は含まれているが，診断率のさらなる向上のためには⑤⑥をスクリーニング項目に取り入れる必要があるであろう（現在の当センターのスクリーニング項目には入っている）．

IUGR，羊水過多症は胎児奇形のハイリスクとされているが，出生前に診断された先天異常の33%にIUGRを，14%に羊水過多を認めた．Vugtら[8]はIUGRの21.8%に胎児奇形が認められると述べ，Dasheら[9]は胎児超音波スクリーニングで異常を認めなくてもAmnio fluid index（AFI）で35 cm以上の羊水過多症が存在すれば11%にmajor anomalyが存在すると述べている．またSicklerら[10]は超音波検査で異常を認めなくても，IUGRと羊水過多の両方を認めれば67%にmajor anomalyが存在すると述べている．今回我々の報告でもIUGRと羊水量の異常は胎児異常を疑う大きな契機となっている．したがって，スクリーニング項目として胎児発育，羊水量の評価をする意義は大きい．しかし，IUGRを認めても腸回転異常と食道閉鎖が，また羊水過多を認めても咽頭腫瘍の診断ができなかった．

現在，日常産科診療において超音波検査は不可欠なものとなっている．妊婦検診の毎に超音波検査を行っている施設も少なくないが，その時間的制約や技術的問題から出生前の胎児異常の診断率は必ずしも高くはないと予想される．今後，胎児超音波スクリーニングでの診断率を上げるためには，妊婦健診とは別に妊娠中期の超音波スクリーニングの時間を1回もしくは2回設けて行うことを提案したい．そして，異常が疑われたときは時間をかけて再検査を行い，異常と認められたら積極的に専門施設へ紹介することが重要である．我々の成績から先天異常の約50%は出生前に診断可能となるであろう．出生

前診断率の向上は，出生直後に必要となる医療体制の準備や両親の精神的ケアと児の受け入れ促進に役立ち，また胎児治療の実現には不可欠である．

ほとんどの妊婦は超音波検査を「元気な自分の赤ちゃん」を確認する手段だと思っており，胎児に異常が疑われると説明されたときには妊婦は精神的に大きな負担を負う．したがって，あくまでもスクリーニング検査であり，確定診断でないことを十分に説明しなければならないとともに，検査前に場合によっては胎児に異常がみつかる場合があることなどについてあらかじめ説明することが必要である．胎児超音波検査については，今後は診断技術向上のみならず妊婦に超音波検査を正しく理解してもらうために何らかの形でこれらのインフォームドコンセントが必要になってくるであろう．

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Detection of Fetal Abnormalities by Routine Fetal Ultrasound Screenings in Second Trimester

Seiji Wada, Haruhiko Sago, Ryuma Matsumoto*, Rie Kawaguchi*, Kouhei Sugimoto*, Hiroko Omi, Satoshi Hayashi, Nobuaki Ozawa, Eriko Fujii, Yuki Tsukahara, Takahiko Kubo, Michihiro Kitagawa, Tadao Tanaka* and Michiya Natori

Department of Perinatology, National Center for Child Health and Development
10–1, Ohkura 2-chome, Setagaya-ku, Tokyo, 157–8535 JAPAN
Department of Obstetrics and Gynecology, Jikei University School of Medicine*
25–8, Nishishinbashi 3-chome, Minato-ku, Tokyo, 105–0003 JAPAN

The purpose of this study was to evaluate the accuracy of the antenatal detection of malformations by two screening sonograms (20 weeks and 30 weeks) in unselected populations.

There were 42 congenital anomalies (1.2%) in 3,416 infants born at National Okura Hospital from Jan 1996 to Feb 2002. Overall, 50% of the anomalies (21 cases) were detected during pregnancy, and the accuracy of detection depended on the organs. Detection of anomalies was higher for urinary tract (100%) and chromosomal abnormalities (75%) but lower for the digestive system (14%) and the musculoskeletal system (20%). Detection rate of anomalies for the heart and great vessels was 46%. Ultrasonographic screening during pregnacy can now detect only a half of fetal malformations.

双胎間輸血症候群

名取道也*

双胎間輸血症候群（Twin to Twin Transfusion Syndrome；TTTS）は，当然のことながら昔からあった疾患である．旧約聖書創世記25章に述べられているイサクのふたごの子の一方は，赤く全身が毛衣のようであったことからエサウと命名された，との記載がある．このエサウとヤコブが一卵性双胎であったか，TTTSのためにエサウが赤く生まれてきたかは，今は知る由もないが，数千年前から注目されていたことは事実であろう．

その後2000年以上の時を経て，新生児領域の重篤な疾患としてTTTSが認識されるようになってから約30年の時が流れた．1990年にDe LiaらがTTTSに対するレーザー治療を発表して以来，TTTSは胎児治療可能な疾患として急に注目を浴びるようになった．それまで産科医は，TTTSが発症していることを超音波断層像で診断しながら，羊水過多に陥っている児の羊水量を減少させることだけが治療手段であった．それは羊水過多による早産防止を目的とするものであり，双胎両児間で発生している血液の移行というTTTSの本来の病態を治療するものではなく，ある意味では無力感を感じる状況であった．

その意味で，病態の本丸を衝くこの治療法は衝撃的であり，1992年には筆者らは，わが国で最初のTTTSに対するレーザー治療を報告した．しかし当時，TTTSの発症と胎盤におけるシャント血管の研究から，TTTSの原因となるシャント血管には胎盤表面のものと，胎盤表面から内部に入った後にシャントする血管があることが知られており，後者に対し胎盤表面のシャント血管のみを凝固させるとのイメージで登場したこの治療法の有効性に理論的疑問が呈された．現在は，内視鏡で胎盤表面を観察することから，後者の胎盤表面から内部に入るシャント血管は認識可能で，それらの血管を凝固することは可能であり，理論的疑問は解消されている．

この10年の間にTTTSに対するレーザー凝固法は世界的に多くの症例の集積がなされた．最もインパクトが大きかったのはHeckerらの1999年の報告で，羊水吸引例とレーザー凝固施行例の比較において，生存率には差がないが，脳異常所見の発生率ではレーザー治療が3倍有利であった．またQuinteroらは2003年に，この差において6倍の違いを報告している．

最初の発表以来10年以上の歳月を経て，現在，TTTSに対するレーザー治療は合理的であり，また患者に，より良い予後を提供する方法と認識されるに至ったと考えている．わが国では現在，国立成育医療センターと聖隷浜松病院が多くの症例を手がけている．この技術に限らず，医療のなかにはテクニックの良し悪しが結果を左右するものが多くあるが，テクニックの向上のためには経験が不可欠である．そのためには症例の集積が不可欠であり，今後わが国における成績向上のためには，少数の施設に症例を集積させて技術の向上を図ることが大切であろう．

*なとり・みちや：国立成育医療センター副院長．昭和47年慶應義塾大学医学部卒業．昭和59年慶應義塾大学医学部助手．昭和63年同講師．平成7年国立大蔵病院臨床研究部長．平成14年国立成育医療センター周産期診療部長．平成16年現職．主研究領域／周産期医学，超音波学．

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Determination of HIV-1 Subtypes (A–D, F, G, CRF01_AE) by PCR in the Transmembrane Region (gp41) With Novel Primers

Fumihiro Yagyu,[1] Shoko Okitsu,[1] Kenichi Tanamoto,[2] and Hiroshi Ushijima[1]*

[1]*Department of Developmental Medical Sciences, Institute of International Health, Graduate School of Medicine, The University of Tokyo, Bunkyo-ku, Tokyo, Japan*
[2]*Division of Food Additives, National Institute of Health Sciences, Setagaya-ku, Tokyo, Japan*

HIV-1 has a huge genetic diversity. So far, nine subtypes have been isolated, namely, subtypes A, B, C, D, F, G, H, J, and K. Epidemiological study provides information which may help in the development of HIV-1 prevention programs or health policies. In the future, subtyping may also be critical for vaccine development, and an effective anti-viral drug will need to be effective for different subtypes of HIV virus. The analysis of the nucleotide sequence of the v3 region is considered the most reliable method for determining the HIV-1 subtype. However, the procedures for determining the v3 sequences are complicated and time consuming, requiring expensive reagents, equipment, and well-trained personnel. The polymerase chain reaction (PCR) method using subtype-specific primers for HIV-1 subtyping is easier and faster. The objective of this study was to develop subtype-specific primers for subtyping PCR. The specific primers were designed for subtypes A, B, C, D, F, G, and CRF01_AE, and these primers could be applied to assay for various HIV-1 subtypes in the clinical samples. The specific primers were designed for each subtypes in the gp41 region. The result of PCR was compared with the subtypes which was determined by the v3 sequence. The results of subtyping by PCR using the newly designed primers could detect 29 of 33 patients tested, and all matched those obtained by nucleotide sequencing of the env v3 region except for three subjects, which were differentiated as CRF02_AG. The newly designed primers functioned accurately and conclusively. In comparison with PCR as a method for the determination of subtypes, sequence analysis requires better-trained personnel, more expensive reagents, and more equipment and time. ***J. Med. Virol. 76:16–23, 2005.*** © 2005 Wiley-Liss, Inc.

KEY WORDS: HIV-1; subtyping; PCR

INTRODUCTION

HIV-1 has numerous genetic variations and can be divided by group, subtype, and subsubtype. The majority of HIV-1 strains belong to the M (Major) group, which causes pandemic HIV-1 infection. The O (Outlier) group and the N (Non-M/Non-O) group are seldomly found. Both the O and N groups have high genetic diversity from the M group [Charneau et al., 1994; Gurtler et al., 1994; Vanden Haesevelde et al., 1994; Loussert-Ajaka et al., 1995; Simon et al., 1998].

The M group has thus far been subdivided into nine isolated subtypes: A, B, C, D, F, G, H, J, and K [Carr et al., 1998; Robertson et al., 2000]. Although some isolates from Cyprus and Greece (94CY032, PVMY, and PVCH) were recognized previously as subtype I, they were shown to be a recombinant strain upon reanalysis. Subtype K was recognized initially as subtype F3, and later designated subtype K. Subtypes A and F are divided, respectively, into subsubtypes A1 and A2, and F1 and F2 [Triques et al., 1999, 2000]. Subtypes B and D are similar [Robertson et al., 2000; Triques et al., 2000], and although they should be reclassified as a single subtype, for the sake of historical consistency, they remain classified as different subtypes.

HIV-1 has been divided into not only many subtypes but also many circulating recombinant forms (CRFs), and so far 15 CRFs have been isolated [Carr et al., 1998]. For example, in the CRF01_AE strain, only the env region belongs to subtype E; the rest of the regions

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*Correspondence to: Hiroshi Ushijima, Department of Developmental Medical Sciences, Institute of International Health, Graduate School of Medicine, The University of Tokyo, 7-3-1 Hongo, Bunkyo-ku, Tokyo 113-0033, Japan.
E-mail: ushijima@m.u-tokyo.ac.jp

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belong to subtype A, and the original full-length of subtype E is no longer found [Carr et al., 1996; Gao et al., 1996].

Worldwide, the incidence of HIV-1 subtypes is as follows: subtype C (47.0%), subtype A (27.2%), subtype B (12.3%), subtype D (5.3%), and CRF01_AE (3.2%) [Osmanov et al., 2002]. Certain subtypes prevail in distinct geographical areas, and different subtypes may predominate for each of the different means of infection [Essex, 1999]. In Thailand, subtype B is predominant among those infected through intravenous drug use, and subtype E among those infected through sexual intercourse [Ou et al., 1992, 1993; Weniger and Brown, 1996; Lole et al., 1999].

The gp120 of the HIV-1 envelope gene comprises five variable domains interspersed with conserved regions. The third variable (v3) region plays an important role in biological properties such as cell tropism, cytopathic effect, and pathogenicity [Robert-Guroff et al., 1994; Palker et al., 1988; Takahashi et al., 1988]. Therefore, analysis of the nucleotide sequence of the v3 region is considered as the most reliable method for determining the HIV-1 subtype. However, the procedures for determining the v3 sequences are complicated and time consuming, requiring expensive reagents, equipment, and well-trained personnel. For determining HIV-1 subtypes of a large number of samples, the heteroduplex mobility assay (HMA) and the peptide enzyme-linked immunosorbent assays (PELISAs) are usually used [Cheingsong-Popov et al., 1994; Wasi et al., 1995; Gaywee et al., 1996; Novitsky et al., 1996; Delwart et al., 1998]. Both of these methods often show cross-reaction or are not reliable. Recently, new methods were attempted to detect subtypes and recombinants [Hoelscher et al., 2002; Plantier et al., 2002]. However, these new methods are more difficult and complicated than PCR. Furthermore, the cost of the single PCR method in particular is approximately 10 times less than that of the sequence method, and thus consideration should be given to its preferential use in developing countries in the future [Yagyu et al., 2002].

The objective of this study was to develop subtype-specific primers for subtyping PCR, since the PCR method is easy, fast, cheap, and accurate. Therefore, specific primers were designed for subtypes A, B, C, D, F, G, and CRF01_AE, and these primers could be applied to examine various HIV-1 subtypes in clinical samples. This method could be applied with several advantages in developing countries, which face a large number of people living with HIV/AIDS.

MATERIALS AND METHODS

Clinical Specimens

Blood samples were collected from HIV-1 carriers with informed consent (Table I). All the subjects were positive originally for anti-HIV-1 antibody. Six samples were from Brazil and the patients had a history of treatment. Six samples from Japan, 2 samples from Kenya, 2 samples from Thailand, 1 sample from Tanzania, and 15 samples from Africa, for all of which a history of treatment was not available. The collection site of one sample was not specified nor the history of medication was known. Blood sample was obtained without any anti-coagulant and incubated at 56°C for 1 hr and stored at −20°C.

DNA Extraction

Stored samples were thawed at room temperature, centrifuged at 12,000*g*, and the supernatants were discarded. The cell pellets were suspended in 500 μl of DNA extraction buffer (150 mM NaCl, 10 mM Tris-HCl [pH 8.0], 10 mM EDTA), 5 μl of 10% SDS and 5 μl of Proteinase K (25 mg/ml) were added, and incubated at 56°C for 1 hr. Following phenol and phenol/chloroform extractions, DNA was ethanol-precipitated at −85°C for 1 hr. After centrifugation at 12,000*g* for 15 min, DNA pellets were rinsed with ice-cold 70% ethanol and dried. The dried pellet in each tube was dissolved in 15 μl of distilled H_2O and stored at −20°C until use.

PCR of Cellular Beta-Actin

To examine the integrity of the DNA samples, cellular *beta-actin* gene was amplified as described previously with b-F and b-R primers [Yagyu et al., 2002] (Table II). The PCR products were subjected to 1% agarose gel electrophoresis at 100 V for 30 min and stained with ethidium bromide.

Differentiation of Subtype B and E by PCR With Subtype-Specific Primers

Nested PCR with two sets of primers were carried out for differentiating subtypes B and E as described previously [Yagyu et al., 2002]. The primers were BECO5 and BECO3 for the first round and BE-ANCH, B-SPEC, and E-SPEC for the second round in PCR reaction (Table II). The PCR products were subjected to 1% agarose gel electrophoresis at 100 V for 30 min and stained with ethidium bromide.

Determination of Subtypes by PCR With Novel Designed Primers

The primers were BECO5 and BECO3 for the first round and BE-ANCH, B-SPEC, C-SPEC, E-SPEC, and F-SPEC for the second round in PCR reaction (Table II, Fig. 1). The primers were 5′D and 3′D, or 5′A, 3′A, 5′E, 3′E, 5′G, and 3′G for the alternative second round PCR (Table II).

A reaction mixture was made by adding 5.0 μl of 10 × PCR buffer with 22.5 mM $MgCl_2$ (Roche Diagnostics, Indianapolis, IN), 0.3 μl of enzyme mix (Roche Diagnostics), 1.5 μl each of dATP, dCTP, dGTP, and dTTP (25 mM each), 1.5 μl each primer (33 pM each), 1.0 μl of template DNA solution and adding distilled H_2O, up to 50 μl. The cycle condition was 93°C for 1 min, 50°C for 1.5 min, and 72°C for 2 min, for 30 cycles.

The second round PCR products were subjected to 2% agarose gel electrophoresis at 100 V for 1 hr and stained with ethidium bromide.

TABLE I. Samples and Subtyping Results

Sample	Sex	Age	Sample taken in	HV+ from when	Symptom	The way of infection	Drug therapy	Sampling date	v3 sequence	PCR differentiate subtype B and E[b]	PCR with newly designed primer[c]	PCR with newly designed primer[d]	PCR with newly designed primer[e]
BRON01	M	34	Brazil	Sep/95	AC	Sexual	+	Nov/99	B	B	B	—[g]	B
BRON02	M	24	Brazil	Aug/95	AC	Sexual	+	Nov/99	B	B	B	—[g]	B
BRON03	M	54	Brazil	Nov/96	AC	Sexual	+	Nov/99	B	B	B	—[g]	B
BRON04	F	31	Brazil	Aug/98	AC	Sexual	+	Nov/99	F	—[f]	F	—[g]	—[g]
BRON05	M	33	Brazil	Mar/97	AIDS	Sexual	+	Nov/99	B	B	B	—[g]	B
BRON06	M	36	Brazil	Mar/93	AIDS	Sexual	+	Nov/99	B	B	B	—[g]	B
YoD	—[a]	—[a]	Japan	—[a]	—[a]	—[a]	—[a]	—[a]	D	B	B	—[g]	D
OS01	F	—[a]	Tanzania	—[a]	—[a]	Sexual	—[a]	—[a]	D	B	B	—[g]	D
OS02	F	—[a]	Japan	—[a]	—[a]	Sexual	—[a]	—[a]	D	B	B	—[g]	D
OS03	F	—[a]	Thailand	—[a]	—[a]	Sexual	—[a]	—[a]	CRF01 AE	CRF01 AE	CRF01 AE	CRF01 AE	—[g]
OS04	F	—[a]	Thailand	—[a]	—[a]	Blood transfusion	—[a]	—[a]	CRF01 AE	CRF01 AE	CRF01 AE	CRF01 AE	—[g]
OS05	M	—[a]	Japan	—[a]	—[a]	Sexual	—[a]	—[a]	CRF01 AE	CRF01 AE	CRF01 AE	CRF01 AE	—[g]
OS06	—[a]	—[a]	—[a]	—[a]	—[a]	Sexual	—[a]	—[a]	CRF01 AE	CRF01 AE	CRF01 AE	CRF01 AE	—[g]
OS07	F	—[a]	Japan	—[a]	—[a]	Sexual	—[a]	—[a]	CRF01 AE	CRF01 AE	CRF01 AE	CRF01 AE	—[g]
OS08	F	—[a]	Kenya	—[a]	—[a]	Sexual	—[a]	—[a]	CRF02 AG	CRF01 AE	CRF01 AE	A	—[g]
OS09	M	—[a]	Japan	—[a]	—[a]	Sexual	—[a]	—[a]	CRF01 AE	CRF01 AE	CRF01 AE	CRF01 AE	—[g]
OS10	F	—[a]	Kenya	—[a]	—[a]	Blood transfusion	—[a]	—[a]	G	CRF01 AE	CRF01 AE	G	—[g]
NG01	F	41	Africa	—[a]	—[a]	Sexual	—[a]	Sep/2002	A	CRF01 AE	CRF01 AE	A	—[g]
NG02	F	38	Africa	—[a]	—[a]	Sexual	—[a]	Sep/2002	A	—[f]	—[f]	—[f]	—[g]
NG03	F	21	Africa	—[a]	—[a]	Sexual	—[a]	Sep/2002	D	B	B	—[f]	D
NG04	M	14	Africa	—[a]	—[a]	Sexual	—[a]	Sep/2002	C	CRF01 AE	C	—[g]	—[g]
NG05	F	36	Africa	—[a]	—[a]	Sexual	—[a]	Sep/2002	A	CRF01 AE	CRF01 AE	A	—[g]
NG06	F	26	Africa	—[a]	—[a]	Sexual	—[a]	Sep/2002	CRF02 AG	CRF01 AE	CRF01 AE	A	—[g]
NG07	F	20	Africa	—[a]	—[a]	Sexual	—[a]	Sep/2002	C	CRF01 AE	C	—[g]	—[g]
NG08	F	61	Africa	—[a]	—[a]	Sexual	—[a]	Sep/2002	CRF02 AG	—[f]	—[f]	—[g]	—[g]
NG09	F	34	Africa	—[a]	—[a]	Sexual	—[a]	Sep/2002	A	CRF01 AE	CRF01 AE	A	—[g]
NG10	F	35	Africa	—[a]	—[a]	Sexual	—[a]	Sep/2002	C	CRF01 AE	C	—[g]	—[g]
NG11	M	33	Africa	—[a]	—[a]	Sexual	—[a]	Sep/2002	D	B	B	—[g]	D
NG12	M	40	Africa	—[a]	—[a]	Sexual	—[a]	Sep/2002	D	—[f]	—[f]	—[g]	—[f]
JP02	M	31	Japan	1994	AC	Sexual	+	Jul/1999	B	—[f]	—[f]	—[g]	—[f]
KA14	—[a]	—[a]	Africa	—[a]	—[a]	—[a]	—[a]	—[a]	G	CRF01 AE	CRF01 AE	G	—[g]
KA18	—[a]	—[a]	Africa	—[a]	—[a]	—[a]	—[a]	—[a]	F	—[f]	—[f]	—[g]	—[g]
KA43	—[a]	—[a]	Africa	—[a]	—[a]	—[a]	—[a]	—[a]	G	CRF01 AE	CRF01 AE	G	—[g]

[a] No data.
[b] Using primer BECO5 and BECO3 for the first round PCR and BE-ANCH, B-SPEC, and E-SPEC for the second PCR.
[c] Using primer BECO5 and BECO3 for the first round PCR and BE-ANCH, B-SPEC, E-SPEC, and newly designed subtype-specific primers C-SPEC and F-SPEC for the second round PCR.
[d] Using primer BECO5 and BECO3 for the first round PCR and BE-ANCH, B-SPEC, E-SPEC, and newly designed subtype-specific primers 5′A, 3′A, 5′E, 3′E, 5′G, and 3′G for the second round PCR.
[e] Using primer BECO5 and BECO3 for the first round PCR and BE-ANCH, B-SPEC, E-SPEC, and newly designed subtype-specific primers 5′D and 3′D for the second round PCR.
[f] No product generated.
[g] Not done.

TABLE II. Sequence of Primers and Positions

Name	Sequence (5′–3′)	HXB2 no.[a]
J5′-2KSI	ATAAGCTTGCAGTGTAGCAGAAGAAGA	7003–7029
5′C2V3	TGTACACATGGAATTAGGCCAG	6963–6984
3′V3	ATGAATTCATTACAGTAGAAAAATTCCC	7363–7391
3′C2V3	ATTTCTGGGTCCCCTCCTGAGG	7313–7334
BECO5	GGCATCAAACAGCTCCAGGCAAG	7938–7960
BECO3	AGCAAAGCCCTTTCTAAGCCCTGTCT	8766–8791
BE-ANCH	TCCTGGCTGTGGAAAGATACCTA	7963–7985
B-SPEC	GTCCCCTCGGGGCTGGGAGG	8384–8403
E-SPEC	GTCTCAGTCCCTTGAGACTGCTG	8585[b]
F-SPEC	AACAGCTCTACCAGCTCTTTGCAAA	8720–8744
C-SPEC	AGACCCCAATACTGCACAAGACTT	8615–8638
5′E	CAGGAAAGGAATGAAAAGGATTTGTTA	8181–8207
3′E	ATAACCCTATCTGTCCACCCC	8693–8713
5′A	GANAACATGACCTGGCTGC	8094–8112
3′A	TCTATAACCCTATCTGTCCAGCCA	8693–8716
5′G	ACAATTACACATACCACATATACAGCC	8131–8757
3′G	TCTATAACCCTATCTGTCCAGTT	8694–8716
5′D	ACCACTAATGTGCCCTGGAACT	8037–8058
3′D	AGGAGGGTCTGAAATGACAGA	8356–8386
b-F	AGAGATGGCCACGGCTGCTT	
b-R	ATTTGCGGTGGACGATGGAG	

[a] HXB2 no. indicates primer position corresponding to nucleotide number of HXB2.
[b] HXB2 does not have sequence corresponding to primer, because of gap.

PCR of the v3 Region of the *gp120* Gene

The v3 region of HIV-1 provirus was amplified by nested PCR using primers 5′C2V3 and 3′V3 for the first round PCR reaction, and J5′-2KSI and 3′C2V3 for the second round reaction as described previously [Yagyu et al., 2002] (Table II). The PCR products were then subjected to 1% agarose gel electrophoresis at 100 V for *30 min*, recovered from the gel, and used as a template for nucleotide sequencing.

Nucleotide Sequencing and Analysis

The sequencing reaction was carried out with a dideoxynucleotide cycle sequencing kit (Perkin Elmer, Wellesley, MA) using J5′-2KSI and 3′C2V3 as sense

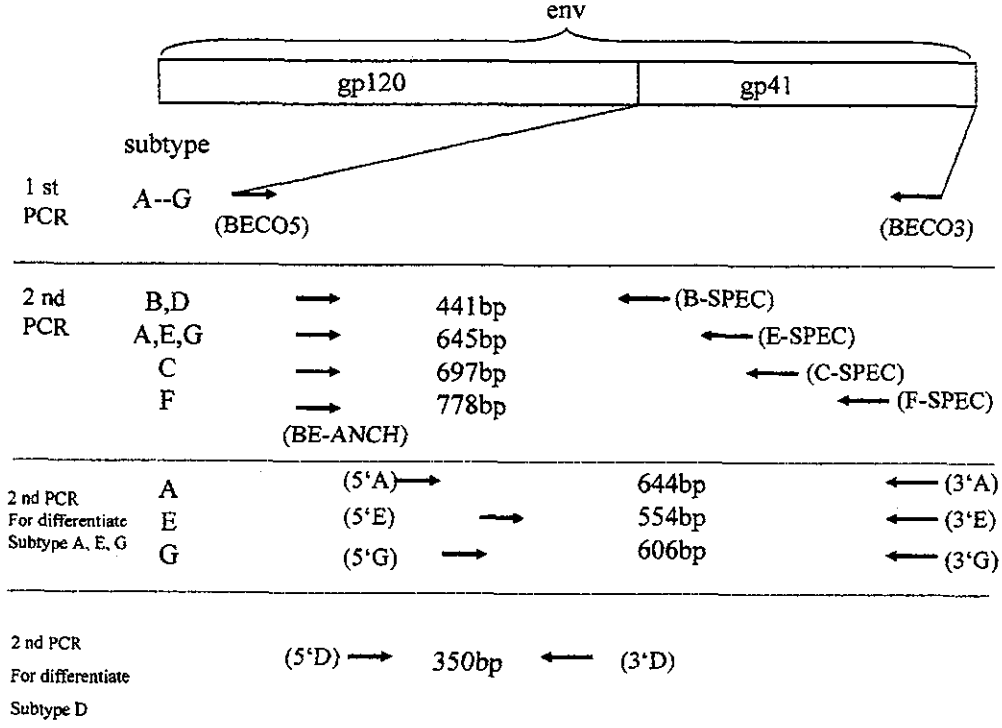

Fig. 1. Primer location and length of polymerase chain reaction (PCR) products.

and anti-sense primers for v3 region, respectively (Table II). The reaction products were analyzed by an automated DNA sequencer (ABI). The sequences of samples were aligned with references strains of subtypes A, B, C, D, F, G, CRF01_AE, and CRF02_AG, and SIV for analysis of v3 region by Clustal X [Thompson et al., 1997]. The subtypes were determined by phylogenetic trees, which were constructed using the neighbor-joining (NJ) method [Saitou and Nei, 1987].

RESULTS

Differentiation of Subtypes B and E by PCR With Subtype-Specific Primers

PCR to differentiate subtypes B and CRF01_AE was performed using samples from 33 subjects. A 441-bp PCR product, which was amplified between BE-ANCH and B-SPEC, was generated from the samples of 10 subjects, and a 645-bp PCR product, which was amplified between BE-ANCH and E-SPEC, was generated from the samples of 17 subjects. However, six subjects were negative, no PCR product was generated (Table I). All samples were positive for cellular beta-actin. This experiment was carried out to determine whether or not the primers B-SPEC and E-SPEC would react with the different subtypes. This information was considered useful for designing of new primers.

Determination of HIV-1 Subtype by Nucleotide Sequence Analysis of the v3 Region

In order to differentiate correct subtypes of the subjects, sequence analysis of the v3 region was performed, since this is considered the most reliable method. PCR of the v3 region of all samples generated successfully products of approximately 350 bp, which were then directly sequenced. The phylogenetic tree showed that the number of subjects of subtypes A, B, C, D, F, G, CRF01_AE, and CRF02_AG were 4, 6, 3, 6, 2, 3, 6, and 3, respectively (Tables I and III).

Designing of Env-Specific Primers for Differentiating Subtypes A, B, C, D, F, G, and CRF01_AE

For the first round PCR, the subtype-common primers, BECO5 and BECO3, were designed to differentiate subtypes B and CRF01_AE. Between BECO5 and BECO3, the subtype-unique conserved sequences of subtypes C and F were found, and used to design subtype C- and F-specific primers, respectively. However, the unique sequences specific for subtypes A, D, G, and CRF01_AE that could be used as anti-sense primers were not found. The results showed that the samples which were positive by PCR using BE-ANCH and subtype E-specific primers could be either subtype A, E, or G according to the sequence alignment, and this observation was in good agreement with a previous study [Yagyu et al., 2002]. Therefore, when the sample was positive for subtype A, E, or G by using the subtype E-specific primer, it was necessary to perform the second round PCR by using the first PCR product as the template with the alternative sets of subtype-specific primers, 5′A, 3′A, 5′E, 3′E, 5′G, and 3′G (Table II, Fig. 2). Similarly, the sample which was positive for subtype B or D by using the subtype B-specific primer should be differentiated between subtypes B and D in the second round PCR using the first round PCR product as the template with the alternative set of primers 5′D and 3′D (Table II, Fig. 2).

Determination of Subtypes With Subtype-Specific Primers

The novel subtype-specific primers designed in this study were verified for specificity by testing with clinical specimens of the known subtypes. The primers used for the first round PCR were BECO5 and BECO3, and then the second round PCR was performed using the first round PCR product as the template, BE-ANCH as the 5′ primer and B-SPEC, C-SPEC, E-SPEC, and F-SPEC as the 3′ primers (Table II, Figs. 1, 2, and 3A). The primers C-SPEC and F-SPEC generated products of 675 and 778 bp, and the samples were identified as subtype C and F, respectively. A PCR product of 440 bp was also generated, possibly due to the annealing of the B-SPEC primer to the proviral DNA of subtypes B and D in the sample. Then, using the first PCR product as template, the second PCR was performed with 5′D and 3′D primers for detecting subtype D, and a 350-bp product was generated (Figs. 1, 2, and 3B). Similarly, E-SPEC annealed to subtypes A, E, and G, and generated a 650-bp product. Then, using the first PCR product as template, the second PCR was performed with a mixture of the primers 5′A, 3′A, 5′E, 3′E, 5′G, and 3′G, and

TABLE III. Subtypes Determined by v3 Sequence and PCR Using Novel Primers

Subtype by v3 sequence	No. of samples with subtype by PCR using novel primers								
	A	B	C	D	F	G	CRF01_AE	ND	Total (n = 33)
A	3							1	4
B		5						1	6
C			3						3
D				5				1	6
F					2				2
G						3			3
CRF01_AE							6		6
CRF02_AG	2							1	3

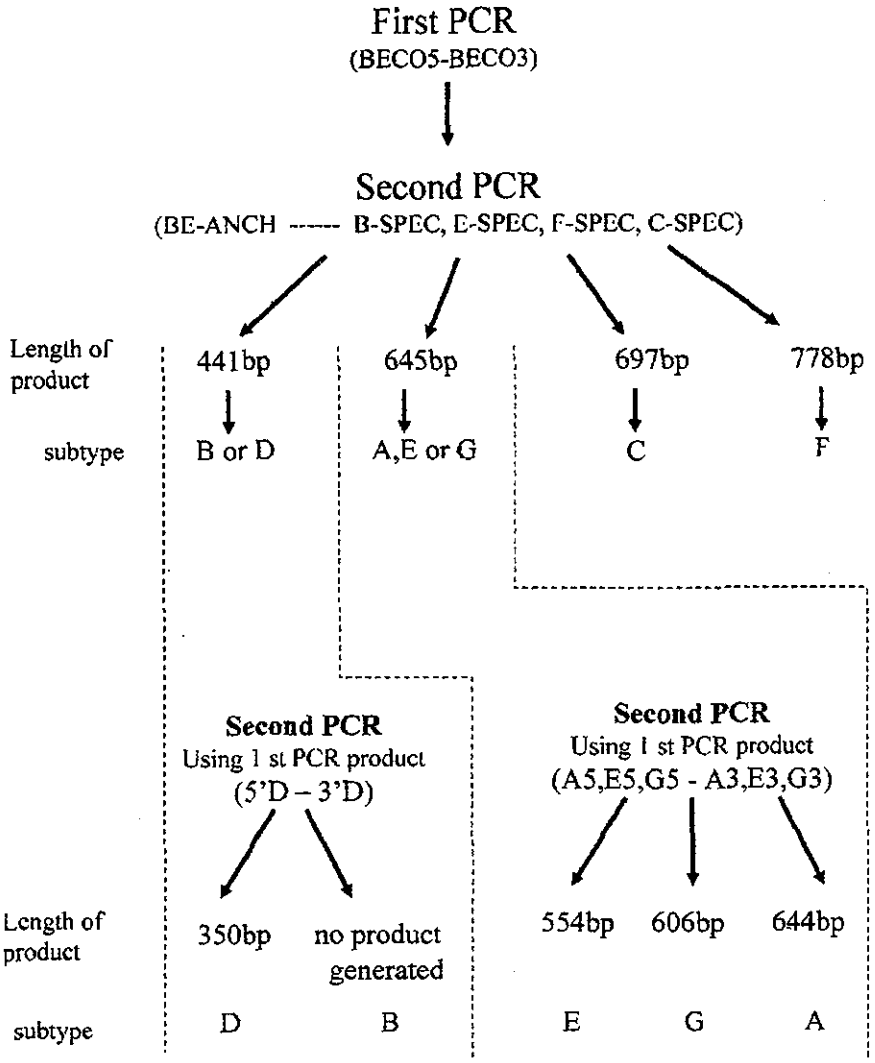

Fig. 2. Procedure of PCR for differentiation of subtypes with newly designed primers.

products of 644, 554, and 606 bp were generated for subtypes A, E, and G, respectively (Figs. 1, 2, and 3C). A total of 33 subjects were tested by this procedure using the newly designed primers. When BE-ANCH was used as a 5′ end primer in combination with B-SPEC, E-SPEC, C-SPEC, and F-SPEC as 3′ end primers, and the first round product was used as the template, PCR products of 441, 645, 697, and 778 bp were generated from 10, 14, 3, and 2 out of the 33 subjects, respectively (Tables I and III). In addition, when the alternative second round PCR for differentiation of subtypes A, G, and CRF01_AE was performed using the first round PCR product as a template and a mixture of the 5′A and 3′A, 5′E and 3′E, 5′B and 3′G primer pairs, 644 bp (subtype A), 554 bp (CRF01_AE), and 606 bp (subtype G) products were generated from 5, 6, and 2, out of the 13 subjects, respectively. Furthermore, when 5′D and 3′D were used in the alternative second round PCR to differentiate subtype D from subtype B, a 350 bp (subtype D) product was generated from 6 out of 12 subjects. The other 6 of the 12 subjects were therefore identified as subtype B.

DISCUSSION

The PCR for differentiation of subtype B and CRF01_AE was carried out as described previously [Yagyu et al., 2002]. The primers B-SPEC and E-SPEC have cross-reaction with other subtypes, except for subtype B and CRF01_AE. The B-SPEC primer anneals subtypes B and D, and the E-SPEC primer anneals subtypes A, C, G, and CRF01_AE. The result of PCR of cellular beta-actin was positive for all samples, suggesting that the DNA in samples was intact by the time of testing and the extraction procedure was successful. The samples that did not generate any products were considered not to have been amplified by the B-SPEC and E-SPEC primers, possibly because there was an insufficient copy number of the HIV-1 provirus or because it was subtype F [Yagyu et al., 2002].

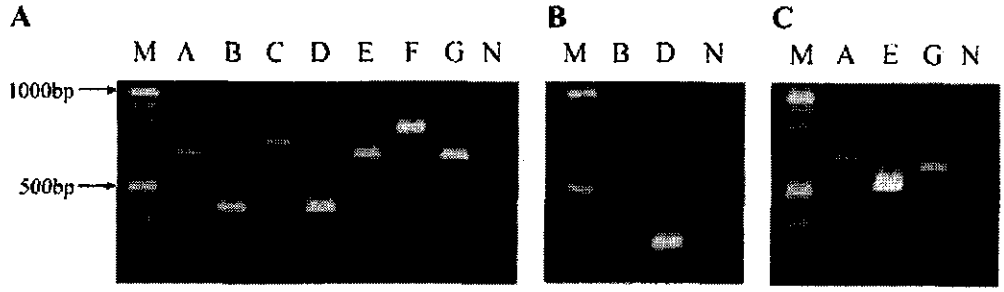

Fig. 3. Determination of subtypes with subtype-specific primers. A: PCR with primers (BECO5 and BECO3 for the first round PCR and, BE-ANCH, B-SPEC, E-SPEC, and newly designed subtype-specific primers C-SPEC and F-SPEC for the second round PCR) of subtype A (**lane A**), B (**lane B**), C (**lane C**), D (**lane D**), CRF01_AE (**lane E**), F (**lane F**), and G (lane F). PCR products were subjected to agarose gel electphoresis with 100 bp ladder markers (**lane M**) and negative control (**lane N**). **B**: PCR with primers (BECO5 and BECO3 for the first round PCR and, 5'D and 3'D for the second round PCR) of subtype B (**lane B**) and D (**lane D**). PCR products were subjected to agarose gel electrophoresis with 100 bp ladder markers (**lane M**) and negative control (**lane N**). **C**: PCR with primers (BECO5 and BECO3 for the first round PCR and, 5'A, 3'A, 5'E, 3'E, 5'G, and 3'G for the second round PCR) of subtype A (**lane A**), CRF01_AE (**lane E**), and G (**lane G**). PCR products were subjected to agarose gel electphoresis with 100 bp ladder markers (**lane M**) and negative control (**lane N**).

Among the patients (BRON04, NG02, NG08, NG12, JP02, and KA18) who were negative for PCR for differentiation of subtypes B and E, BRON04 and KA18 were subtype F as expected. And, the subjects, NG02, NG08, NG12, and JP02, were subtypes A, CRF02_AG, D, and B, respectively.

The newly designed subtype-specific primers could be used as anti-sense primers against BE-ANCH, and could generate PCR products of different lengths. Specific primers for subtypes C and F which act as the anti-sense primers against BE-ANCH were also designed. However, it was rather difficult to design a subtype-specific for A, B, D, G, or CRF01_AE, because subtypes A, E, and G and subtypes B and D, respectively, were similar to each other. After the second PCR with primers BE-ANCH, B-SPEC, C-SPEC, E-SPEC, and F-SPEC, the sample which was positive for the subtype B-specific primer was further differentiated between subtypes B and D by using the subtype D-specific primers 5'D and 3'D. By this strategy, the primers were able to recognize only subtype D, since it was impossible to design a subtype B-specific primer for the second PCR. Since the subtypes B and D are very closely related, an attempt was made to differentiate the subsubtypes by PCR. The results showed that PCR was able to separate them incompletely in this experiment. However, because subtypes B and D are predominant in different countries, the differentiated subtypes B and D could provide important information on the course of an HIV strain newly imported to a particular country.

Three samples (OS08, NG06, NG08) of CRF02_AG determined by nucleotide sequence were differentiated as subtype A by PCR with the novel primers. The envelope gene of CRF02_AG consists of subtype A and G. Therefore, it may be impossible to design specific primers for CRF02_AG, when only the genome of gp41 is used for subtype determination.

The subtypes of all subjects determined by PCR using newly designed primers were in complete agreement with those determined by nucleotide sequence analysis of the v3 region except for CRF02_AG. Nevertheless, there were four patients, who did not generate any positive signals. This indicates that the relatively lower sensitivity of PCR with novel primers might not be due to the low copy number of proviral DNA; rather, it might be due to mismatched of the primer, since point mutations readily occur in the HIV-1 genome.

The newly designed primers functioned accurately and conclusively. In comparison with PCR as a method for the determination of subtypes, sequence analysis requires better-trained personnel, more expensive reagents, and more equipment and time. The PCR method is useful for developing countries in which the burden of HIV/AIDS has increased dramatically. However, although the PCR method has the above advantages, it also has a shortcoming in that it differentiates subtypes only in the gp41 region. In order to evaluate this method accurately, a larger number of samples should be tested.

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A novel RT-multiplex PCR for enteroviruses, hepatitis A and E viruses and influenza A virus among infants and children with diarrhea in Vietnam

T. G. Phan, T. A. Nguyen, H. Yan, S. Okitsu, and **H. Ushijima**

Department of Developmental Medical Sciences, Institute of International Health, Graduate School of Medicine, The University of Tokyo, Tokyo, Japan

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Summary. A novel reverse transcription-multiplex polymerase chain reaction (RT-multiplex PCR) assay that can detect enteroviruses, hepatitis A and E viruses and influenza A virus from various hosts (avian species, human, swine and horse) was developed. The identification of that group of viruses was performed with the mixture of four pairs of published specific primers (F1 and R1, P3 and P4, 2s and 2as, MMU42 and MMU43) for amplifying viral genomes and specifically generated four different amplicon sizes of 440, 267, 146 and 219 bp for enteroviruses, hepatitis A and E viruses and influenza A virus, respectively. A total of 276 fecal specimens (previously screened for rotavirus, adenovirus, norovirus, sapovirus and astrovirus-negative) from infants and children admitted into hospital with acute gastroenteritis in Ho Chi Minh city, Vietnam during October 2002 and September 2003 were collected and further tested for the presence of those viruses by RT-multiplex PCR. Enteroviruses were identified in 27 specimens and this represented 9.8%. No hepatitis A and E viruses and influenza A virus was found among these subjects. The sensitivity and specificity of RT-multiplex PCR were also assessed and demonstrated the strong validation against RT-monoplex PCR. Taken together, the findings clearly indicated that this novel RT-multiplex PCR is a simple and potential assay for rapid, sensitive, specific and cost-effective laboratory diagnosis to investigate molecular epidemiology of acute gastroenteritis caused by enteroviruses, hepatitis A and E viruses and influenza A virus. This report is the first, to our knowledge, detecting these kinds of viruses in diarrheal feces from infants and children in Vietnam.

Introduction

Viral gastroenteritis is one of the most common illnesses in humans worldwide and it has a great impact on people, particularly in infants and the elderly.

The mortality among children due to acute gastroenteritis is greater in developing than in developed countries [16]. Annual mortality associated with acute gastroenteritis was estimated to be 2.1 million in 2000. More than 20 viruses have been recognized as important causes of this illness. Apart from rotavirus as the major cause of gastroenteritis in infants and children, enteroviruses, hepatitis A and E viruses and influenza A virus are also considered as agents of this illness.

Enteroviruses are non-enveloped enteric RNA viruses belonging to the family *Picornaviridae*. Enteroviruses (EVs) infect billions of people worldwide and cause clinical manifestations such as poliomyelitis, aseptic meningitis, encephalitis, myocarditis, hand-foot and mouth diseases and other acute and chronic illness. EVs are subgrouped into poliovirus, coxsackievirus A and B, echovirus and enterovirus 68 to 71 on basis of their pathogeneses in humans and in experimental animals [13, 14].

Hepatitis A and E viruses are recognized as the common causes of infectious jaundice in the world today. The principal mode of transmission for these viruses is by the fecal-oral route, often resulting in community-wide outbreak [9, 22]. The course of viral hepatitis may be extremely variable. Patients with inapparent or subclinical hepatitis have neither symptoms nor jaundice. Symptoms of viral hepatitis are characterized by anorexia, myalgia, fever, malaise which range from mild and transient to severe and prolonged. Diarrhea, nausea and vomiting, however, are more frequent in children than in adults [9, 22]. To date, adequate information on molecular epidemiology of hepatitis A and E viruses in Vietnam has been not available so far, even through several reports were published [6, 7].

Influenza viruses, belonging to the *Orthomyxoviridae* family, are of three types A, B and C based on antigenic differences on the nuclear and matrix proteins. However, influenza A virus is the most predominant and is associated with pandemics of influenza. Influenza A virus can infect swine, horse, and a large variety of birth as well as humans. Recently, the largest outbreak of highly pathogenic avian influenza occurred among poultry in 8 Asian countries and caused the deaths in human [25]. Infection with influenza A virus results in a spectrum of clinical responses from asymptomatic infection to a primary viral pneumonia that rapidly progresses to a fatal outcome. A part from respiratory symptoms, a high incidence of gastrointestinal manifestations in children especially under 3 years old is reported [18].

At present, RT-PCR is a sensitive and specific method that has been used with the success to accurately define the true burden of disease due to viruses [1, 4, 10]. Recently, RT-PCR with specific primers individually or combined for multiple human pathogens have proved to be comparable to or better that cell culture or other immuno-diagnostic methods for viral detection [3, 8]. In addition, conventional monoplex PCR with a single pair of primers thus can detect only one target virus and is potentially expensive. In contrast to monoplex assay, RT-multiplex PCR with different pairs of specific primers for amplifying different viral genomes in one reaction tube enables to detect for two or more targets in a single test. Moreover, RT-multiplex PCR assay is a cost-effective laboratory

diagnosis because of the reduction in labor and reagent costs and faster detection [19, 23, 27, 28, 30]. Thus, in the present study we developed the RT-multiplex PCR method for simultaneous detection of enteroviruses, hepatitis A and E viruses and influenza A virus in fecal specimens collected from infants and children with acute gastroenteritis.

Materials and methods

Fecal specimens

Two hundreds and seventy six fecal specimens were collected from hospitalized infants and children with acute gastroenteritis in Ho Chi Minh city, Vietnam, during the period of October 2002 to September 2003. No patient was found to manifest the other symptoms of the hepatitis after the onset of diarrhea. Among those patients, 54.7% (151 cases) had the fever. The fecal specimens were diluted with distilled water to 10% suspensions, and clarified by centrifugation at 10,000 *g* for 10 min. The supernatants were collected and stored at −30 °C until use for the detection of viruses.

Extraction of viral genomes

The viral genomes were extracted from 140 μl of 10% fecal suspensions using a spin column technique according to the manufacturer's instructions (QIAGEN®, Germany).

Reverse transcription (RT)

For RT, 7.5 μl of extracted RNA was added with a reaction mixture consisting of 2.05 μl of 5× First Strand Buffer (Invitrogen, USA), 0.75 μl of 10 mM dNTPs (Roche, Mannheim, Germany), 0.75 μl of 10 mM DTT (Invitrogen), 0.75 μl (200 U/μl) of SuperScript Reverse Transcriptase II (Invitrogen), 0.375 μl (1 μg/μl) of random primer (hexa-deoxyribonucleotide mixture) (Takara, Shiga, Japan), 0.5 μl (33 U/μl) of RNase Inhibitor (Toyobo, Osaka, Japan), and 2.325 μl MilliQ water. The total of reaction mixture is 15 μl. The RT step was carried out at 42 °C for 1 h, followed by 99 °C for 5 min and then held at 4 °C.

PCR primers

A total of four pairs of specific primers for target viral genomes as previously published were used in the present study. A pair of primers F1 and R1 for the 5′ noncoding region (NCR) amplifies enteroviruses, P3 and P4 for the capsid region of hepatitis A virus, 2s and 2as for ORF2 of hepatitis E virus, and MMU42 and MMU43 for nucleoprotein (NP) of a wide range of influenza A virus from various hosts (avian species, human, swine and horse) [7, 12, 15, 28]. These primers specifically generated four different sizes of amplicons of 440, 267, 146 and 219 bp for enteroviruses, hepatitis A and E viruses and influenza A virus, respectively.

Monoplex PCR

In monoplex PCR, only a pair of primers was used to detect the target virus. Exactly, 2.5 μl of cDNA was mixed with a reaction mixture containing 2.5 μl of 10× TaqDNA polymerase buffer (Promega, Madison, WI), 2.0 μl of dNTPs (2.5 mM/μl), 0.4 μl of each specific primer (33 μM), 0.125 μl (2.5 mM/μl) of Tag DNA polymerase (Promega). MilliQ water was added to make up a total volume of 25 μl. The PCR was performed at 94 °C for 3 min followed by 35 cycles of 94 °C 30 s, 55 °C 30 s, 72 °C 60 s, and a final extension at 72 °C for 7 min, and then held at 4 °C.

Multiplex PCR

On contrast to monoplex PCR, up four pairs of specific primers for enteroviruses, hepatitis A and E viruses and influenza A virus were mixed in multiplex PCR. The same volume and concentration of primers, reagents and thermal cycler program described above were used.

Positive viral controls

The positive controls of poliovirus 2 (Sabin) with 10^9 PFU/ml, genotype IA hepatitis A virus (09030/Shizuoka/Japan/1990) with 2.9×10^9 PFU/ml, genotype I hepatitis E virus (01/Myanmar) and human influenza A virus (1129/H3/Kawasaki/Japan/2003) with 10^8 PFU/ml used in this study were provided by Dr Osamu Nishio, Dr Kenji Abe from National Institute of Infectious Diseases (NIID), Tokyo and Dr Hideaki Shimizu from Kawasaki City Institute of Public Health, Kawasaki City, Japan.

Specificity testing of the four primer pairs

Specificity testing was carried out for the positive controls of poliovirus 2 (Sabin), genotype IA hepatitis A virus (09030/Shizuoka/Japan/1990), genotype I hepatitis E virus (01/Myanmar) and human influenza A virus (1129/H3/Kawasaki/Japan/2003) in order to determine the specificity of the mixture of four primer pairs. Two combinations were tested: i) the PCR mixture containing four primer pairs and each single template (cDNA), and ii) four primer pairs and four templates. Specificity testing was also performed for negative controls of group A rotavirus, adenovirus, norovirus (GI, GII), sapovirus and astrovirus.

Sensitivity testing of mutiplex PCR and monoplex PCR

To compare the sensitivity level of mutiplex PCR and monoplex PCR, 10-fold serial dilution (10^{-1} to 10^{-4}) in MilliQ water of the four different viral cDNA, four positive controls of poliovirus 2 (Sabin), genotype IA hepatitis A virus (09030/Shizuoka/Japan/1990), genotype I hepatitis E virus (01/Myanmar) and human influenza A virus (1129/H3/Kawasaki/Japan/2003) were tested by mutiplex PCR and monoplex PCR. The mutiplex PCR and monoplex PCR were carried out simultaneously for the same dilution series with the same PCR machine.

Electrophoresis

The PCR products were electrophoresed in a 1.5% agarose gel, followed by staining with ethidium bromide (original stock EtBr, 5 mg/ml) for 20 min then visualized under ultraviolet (UV) light, and the results were recorded by photography.

Results

Specificity testing of the four primer pairs

The specificity of the mixture of four pairs of specific primers used in the present study was tested and shown in Fig. 1. Each pair of primers amplified the viral genomes of positive controls and specifically and independently generated four different sizes of amplicons of 440, 267, 146 and 219 bp by F1 and R1, P3 and P4, 2s and 2as, MMU42 and MMU43 for poliovirus 2 (Sabin), genotype IA hepatitis A virus (09030/Shizuoka/Japan/1990), genotype I hepatitis E virus (01/Myanmar) and human influenza A virus (1129/H3/Kawasaki/Japan/2003), respectively. No cross-reaction with non-targets was identified. For the negative controls group A rotavirus, adenovirus, norovirus (GI, GII), sapovirus and astrovirus, no amplicon was demonstrated (data not shown).

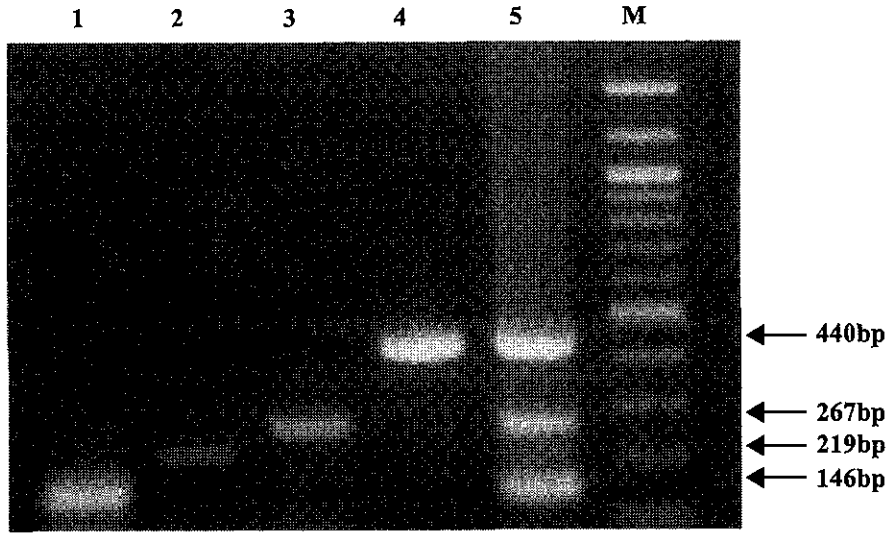

Fig. 1. Specificity testing of the multiplex PCR assay with a mixture of four primer pairs for four positive controls. Lane: *1–4*, genotype I hepatitis E virus (01/Myanmar), human influenza A virus (1129/H3/Kawasaki/Japan/2003), genotype IA hepatitis A virus (09030/Shizuoka/Japan/1990) and poliovirus 2 (Sabin) positive controls, respectively; *5*, the mixture of hepatitis E virus, human influenza A virus, hepatitis A virus and enterovirus positive controls; *M*, molecular marker

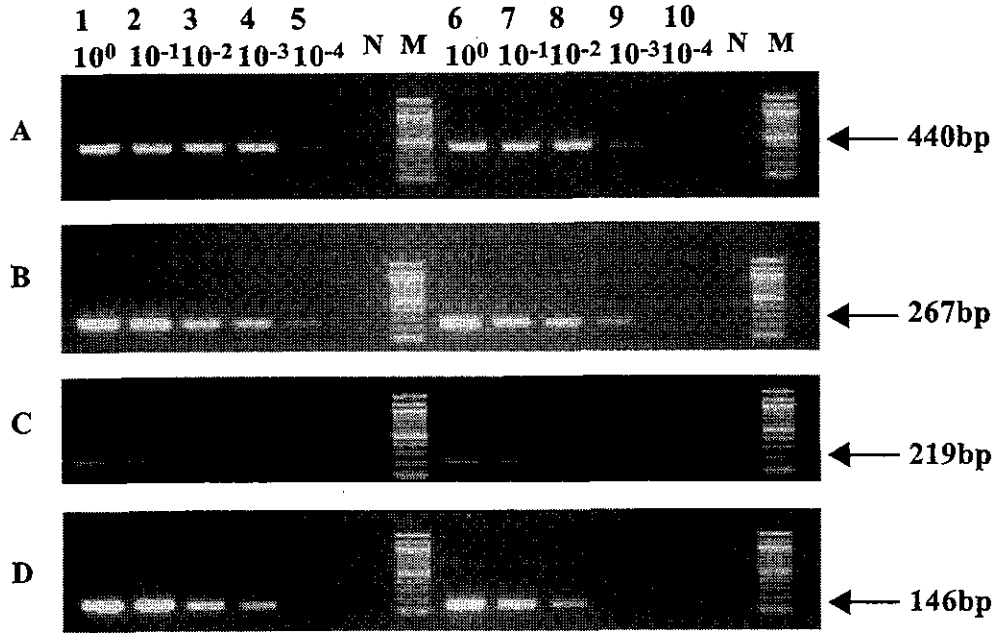

Fig. 2. Comparison of the sensitivity between multiplex PCR and monoplex PCR for the 10-fold serial dilutions of positive controls. **A**, poliovirus 2 (Sabin); **B**, genotype IA hepatitis A virus (09030/Shizuoka/Japan/1990); **C**, human influenza A virus (1129/H3/Kawasaki/Japan/2003); **D**, genotype I hepatitis E virus (01/Myanmar), *N*, negative control; *M*, molecular marker. *1–5*, sensitivity testing of monoplex PCR; *6–10*, sensitivity testing of multiplex PCR

Table 1. Specific primers for amplifying the genomes of enteroviruses, hepatitis A and E viruses and influenza A virus were used in mutiplex PCR

Viruses and primers	Target region	Polarity[a]	Sequence (5′ to 3′)[b]	Position	Amplicon size (bp)
Enterovirus					
F1	5′ noncoding region (NCR)	+	CAAGCACTTCTGTTTCCCCGG	160–180	440
R1	5′ noncoding region (NCR)	–	ATTGTCACCATAAGCAGCCA	599–580	
Hepatitis A virus					
P3	capsid region	+	TATTTATCTGTCACAGAACAATCAG	2949–2973	267
P4	capsid region	–	AGGAGGCGGAAGCACTTCATTTGA	3215–3190	
Hepatitis E virus					
2s	ORF2	+	CCGTCGTCTCAGCCAATGGCGAGC	6345–6368	146
2as	ORF2	–	CTCATGTTGGTTGTCATAATCCTG	6490–6467	
Influenza A virus					
MMU43	nucleoprotein (NP)	+	CATCCCAGTGCTGGGAARGAYCCTAAGAA	288–317	219
MMU42	nucleoprotein (NP)	–	AGAGCTCTTGTTCTCTGATAGGTG	506–483	

[a] +, Forward primer; –, Reverse primer

[b] Within nucleotide sequence of primers, R = A or G; Y = C or T

Sensitivity testing of mutiplex PCR and monoplex PCR

For the 10-fold dilution series, the highest dilution at which mutiplex PCR showed positive results was 10^{-4}, 10^{-3}, 10^{-2} and 10^{-2} for poliovirus 2 (Sabin), genotype IA hepatitis A virus (09030/Shizuoka/Japan/1990), genotype I hepatitis E virus (01/Myanmar) and human influenza A virus (1129/H3/Kawasaki/Japan/2003), respectively. On the other hand, the positive results of highest dilution in monoplex PCR were 10^{-4} for poliovirus 2 (Sabin), 10^{-4} for genotype IA hepatitis A virus (09030/Shizuoka/Japan/1990), 10^{-3} for genotype I hepatitis E virus (01/Myanmar) and 10^{-3} for human influenza A virus (1129/H3/Kawasaki/Japan/2003) (Fig. 2). Taken together, the results indicated that the sensitivity of mutiplex PCR decreased slightly in comparison with that of monoplex PCR.

Detection of target viruses in fecal specimens by RT-monoplex PCR and RT-multiplex PCR

A total of 276 fecal specimens (previously screened for rotavirus, adenovirus, norovirus, sapovirus and astrovirus-negative) were collected from infants and children with acute gastroenteritis in Ho Chi Minh city, Vietnam, during the period of October 2002 to September 2003. All fecal specimens were tested for the presence of enteroviruses, hepatitis A and E viruses and influenza A virus from various hosts (avian species, human, swine and horse) by monoplex PCR and multiplex PCR with specific primers as previously published (Table 1). The results shown in Table 2 revealed that there was no difference of viral detection

Table 2. Results of detection of target viruses in clinical fecal specimens by monoplex PCR and multiplex PCR

Target viruses (%)					
Number of clinical specimen tested	Laboratory method	Enteroviruses	Hepatitis A virus	Hepatitis E virus	Influenza A virus
276	Monoplex PCR	27 (9.8%)	0 (0)	0 (0)	0 (0)
276	Multiplex PCR	27 (9.8%)	0 (0)	0 (0)	0 (0)

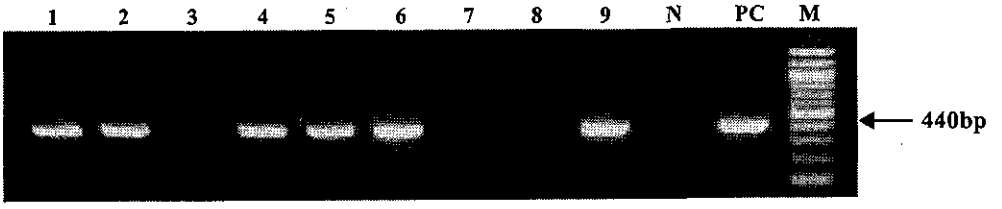

Fig. 3. Detection of enteroviruses in clinical fecal specimens collected from infants and children with acute gastroenteritis in Ho Chi Minh city, Vietnam. *1*, *2*, *4*, *5*, *6* and *9*, enteroviruses found in clinical fecal specimens; *3*, *7* and *8*; no viral amplification in clinical fecal specimens; *N*, negative control; *PC*, poliovirus 2 (Sabin) positive control; *M*, molecular marker

rate between monoplex PCR and multiplex PCR in clinical fecal specimens. Enterovirus was identified in 27 specimens and this represented 9.8%. No hepatitis A and E viruses and influenza A virus was found among these subjects. Fig. 3 showed an example of enteroviruses detected from Vietnamese clinical feces.

Discussion

Viral gastroenteritis is a common disease with a high morbidity reported worldwide especially in infants and the elderly. Acute gastroenteritis consistently ranks as one of the top six causes of all deaths. Among different kinds of enteropathogens, rotavirus is recognized to be the leading global agent of severe acute gastroenteritis [17]. Enteroviruses, hepatitis A and E viruses and influenza A virus, however, are also recognized as causes of this disease. Over the past decades, neutralization test, ELISA or virus isolation had been usually done to identify the causative viruses by different groups of investigators. However, these methods are complicated, labor intensive, time consuming, low sensitive and sometimes require the cell culture techniques, special cell lines or animal models from which certain viruses have been isolated [8, 14]. Furthermore, attempts to isolate viruses are frequently unsuccessfully because of the low viral titer in clinical specimens. In addition, some viruses grow poorly or are refractory to isolation in cell culture [5, 21, 26]. So many disadvantages lead to severely limit these tests' usefulness [3]. At present, amplification of the genome by RT-PCR in clinical specimens has been introduced as a more convenient and powerful alternative as for molecular diagnosis [2, 24]. Highly sensitive and specific RT-PCR assay is currently available for the detection of hepatitis A and E viruses as well as influenza A virus [19, 23]. Additionally, genome amplification allows further characterize viruses according to type by sequencing analysis. RT-PCR is successfully used to detect most of enteroviruses, including those, which cannot readily be propagated in cell culture [20]. Even with many advantages, monoplex PCR with a single pair of primers thus can detect only one target virus and is potentially expensive. On the other hand, RT-multiplex PCR with different pairs of specific primers for amplifying different viral genomes enables to detect for two or more targets in one reaction tube. As the results, RT-multiplex PCR assay is a cost-effective laboratory diagnosis because of the reduction in labor and reagent costs and faster detection [29, 30].

In the present study, we developed a rapid assay to detect simultaneously enteroviruses, hepatitis A and E viruses and influenza A virus in fecal specimens. Up to date, there are several similar studies on mutiplex PCR as previously reported such as norovirus, sapovirus and astrovirus as well as all subgenera A to F adenovirus, rotavirus [29, 30]; hepatitis A virus, norovirus and enteroviruses [23]; influenza virus A, B and C [19]; and other pathogens [27]. Our multiplex assay was designed in which a total of four primers pairs were used to detect four target viruses in one PCR reaction tube. A pair of primers F1 and R1 can detect a wide range of subgroups of enteroviruses (poliovirus, coxsackievirus A and B, echovirus and enterovirus 68 to 71) with numerous serotypes.

Recently, viral infections in human due to interspecies were reported. The largest outbreak of severe avian influenza occurred in Asian countries and caused the loss of deaths in human beings infected [25]. Apart from respiratory symptoms, a higher incidence of gastrointestinal manifestations such as vomiting, abdominal pain, diarrhea has been seen in children especially those younger than 3 years [18]. Therefore, the primers MMU42 and MMU43 were used to identify influenza A virus not only from human but also from various animal hosts such as avian species, swine and horse in clinical fecal specimens [15].

In our multiplex PCR, primer selection for the four viruses was based on sizes of the amplicons generated. These primers produced four different PCR products of 440, 267, 146 and 219 bp for enteroviruses, hepatitis A and E viruses and influenza A virus, respectively. Such that these amplicons could be visualized and easily differentiated with agarose gel electrophoresis. For thermal cycler program, the annealing temperature was selected appropriately in order to decrease nonspecific priming or other artifacts. Times for annealing and extension were minimized to reduce the possibility of nonspecific and unexpected amplification.

In this experiment, the specificity of the mixture of four primer pairs was tested for the positive controls and also for negative controls (group A rotavirus-, adenovirus-, norovirus (GI, GII)-, sapovirus- and astrovirus-positive specimens) by using multiplex PCR. The lack to obtain an amplification signal from these negative controls demonstrated the high specificity of the multiplex reaction. It was found that the sensitivity of multiplex PCR remained at the same level or dropped from 10 to 100 fold in comparison with monoplex PCR [11, 29]. In the present study, the multiplex reaction with primers R1 and F1 for detection of enteroviruses was shown to be equally sensitive to the monoplex PCR. However, the mutilplex PCR for hepatitis A and E viruses, and influenza A virus was demonstrated slightly less sensitive than monoplex assay. Furthermore, out of 276 clinical fecal specimens collected from infants and children with acute gastroenteristis in Vietnam, enteroviruses were interestingly identified in rather high percentage (9.8%). No hepatitis A and E viruses and influenza A virus was found among these subjects by either multiplex PCR or monoplex PCR. The result was also identical to that of nested PCR even as the most sensitive method (data not shown). Taken together, the findings clearly indicated that our novel multiplex PCR was highly sensitive and specific for detection of those four viruses in the laboratory research and in the clinical specimens.

This is the first study to detect simultaneously enteroviruses, hepatitis A and E viruses and influenza A virus by RT-multiplex PCR in fecal specimens from infants and children with acute gastroenteritis in Vietnam. This assay is recognized as a simple, potential and applicable assay for rapid, sensitive, specific and cost-effective laboratory diagnosis to investigate molecular epidemiology of acute gastroenteritis caused by those viruses. Our report is also the first to underscore the presence and the importance of enteroviruses in association with acute gastroenteritis in Ho Chi Minh city, Vietnam and warn of the threat that they pose.