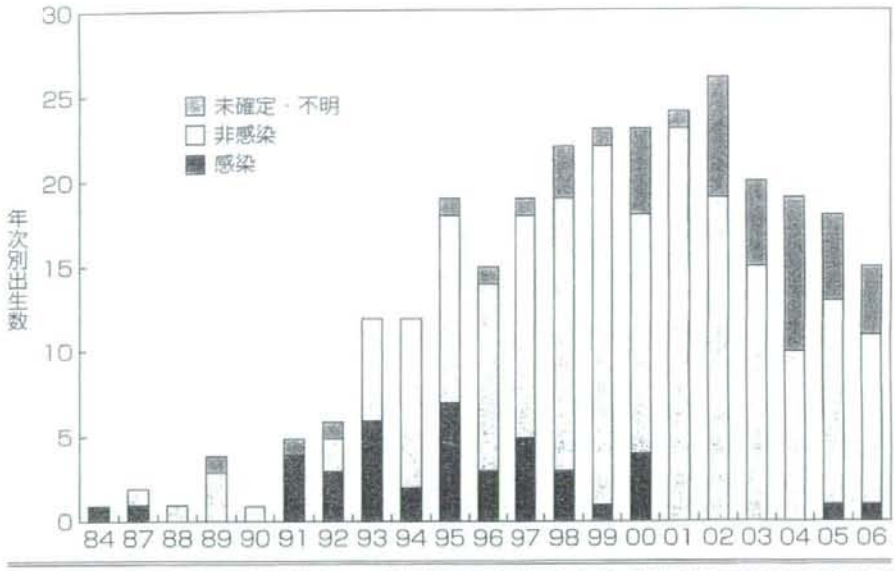


(平成18年度厚生労働科学研究費補助金エイズ対策研究事業「周産期・小児・生殖医療におけるHIV感染対策に関する集学的研究」(主任研究者：稲葉憲之)，分担研究「HIV感染妊婦より出生した児の実態調査とその解析およびHIV感染妊婦とその出生児に関するデータベースの構築」(分担研究者：外川正生)平成18年度総括・分担研究報告書，2007年3月．より引用)

図1 HIV感染妊婦からの年次別出生数と感染の有無

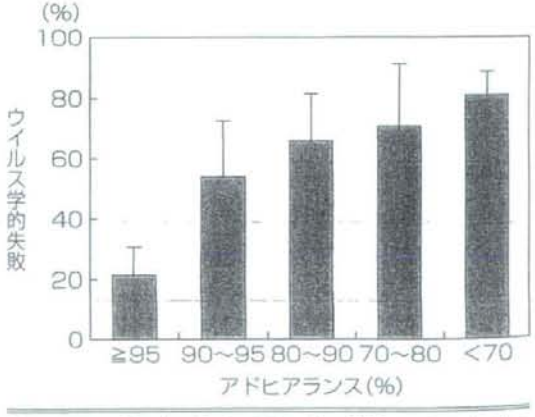
し，分娩時に母体にAZT(ジドブジン)を点滴投与し，出生した児にはAZTを6週間投与，また完全人工栄養とすることである。これらの対策がすべてとられれば，母子感染は日本ではほぼ1％まで防止可能になっている。

2 エイズ孤児，社会的背景の問題

HIV感染児の両親，とくに母親は東南アジア・アフリカ・南アメリカ出身者が多い。家庭環境は，一般的には裕福でない家庭が多く，また育児が十分になされていないこともある。まだまだ社会的な弱者にHIV感染者が多いのである。子どもが母子感染者であれば，その母親も感染者である。両親とも感染者かもしれない。また，エイズ患者の治療コントロールが良好になったとはいえ，さまざまな合併症により両親が死亡することもあり，エイズ孤児という大変な問題もある。このように，HIV感染症には医学的だけでなく，社会的にもさまざまな複雑な問題をかかえていることが多い。

3 小児HIV治療における長期アドヒアランスの問題

小児に対する抗HIV療法が成功するためにもっとも大切な点は，正確かつ継続した服薬の遵守であ



(Paterson, D. L., Swindells, S., Mohr, J., et al.: Adherence to protease inhibitor therapy and outcomes in patients with HIV infection. Ann. Intern. Med., 133: 21-30, 2000. より引用)

図2 アドヒアランスとウイルス学的失敗の関係

る。ウイルス学的失敗の最大の原因は飲み忘れによる血中濃度の低下である。中途半端な服薬は早期に耐性を誘導する。図2[5]に示すように，服薬率を95%以上に保たなければ治療に失敗するとされている。このような高い服薬率を維持するためには，患者は積極的に治療方針の決定に参加し，自ら実行する姿勢が大切であり，これを重視した概念がアドヒアランスという用語である。また，抗 HIV 薬を長期にわたって服薬しなければならないことが，患者にもっとも大きなストレスを与えているとされている。また，抗 HIV 薬は新規に開発がなされているとはいえ，小児にとっては必ずしも飲みやすい形状のものばかりではない。小児期から長期間，正確に飲み続けるということは本当に大変なことである。

母子感染児は，出生直後から HIV 治療および日和見感染予防が開始されるが，自分で服薬が行える年齢までは養育者が服薬管理を行う必要がある。新生児期から乳幼児期は，哺乳やおむつ交換も頻回であり，育児に手間がかかる時期であり，それに加えて毎日規則的な服薬を継続することは精神的にも負担が大きく，母親など養育の中心となる人物一人に育児や治療のすべてを負わせることは治療が中途半端になるばかりか，養育者自身の健康状態にも影響する。家族が家事や育児を分担することで，一人に負担が集中しないようにしたい。

乳児期は，大人に比べて抵抗力が弱いうえに，HIV 感染児は免疫力が低下している場合がある。哺乳や離乳食に生水や生ものの使用を避けることや，日和見感染症につながる動物との接触に注意すること，かぜやその他の感染症を起こしやすいことなど，日常生活での注意点を伝えておくべきである。また，乳幼児期はさまざまな病気に罹りやすいが，使用している抗 HIV 薬との併用を避けたほうがよい薬物や食品についても情報提供しておくべきである。予防接種のなかには集団保育の前に済ませておくほうがよいものもあるが，接種できない生ワクチンもあり，HIV 感染に詳しい小児科での個別接種が望ましい。

4 教育機関への援助

HIV 感染症は治療によりコントロール可能な慢性疾患になってきた。感染者の多くは普通に通園・通学できる。HIV 感染経路は限られており，性行為以外での日常生活での感染は防止可能である。しかし，年少時には引っかき合いの喧嘩や遊び，運動中のけがは多く，日本では HIV 感染小児の数が少ないこともあり，教育現場がその対応に慣れているとは現時点ではいい難い。すべての教育者が「自分の学校に感染者がいても大丈夫」と思える体制を整えるためにも，医療者が教育現場に出向き，HIV 感染症の正しい知識を伝え，受け入れ態勢を整えておくようにしたい。しかし，現実的には，まだ日本の社会は成熟しているとは必ずしもいえず，教育機関に HIV 感染のことを申告するかどうかは慎重に考慮しなければならない。

5 告知の問題

学童期以後は，成長に合わせた服薬管理・体調管理や，二次感染予防のための自己管理能力を高めるためにも，病名の告知が必要となってくる。子どもが自分の病気を受け入れ，自己管理していく過程において不安や疑問を抱き，精神的に不安定になり治療を拒否することもある。とくに，母子感染であれば，子どもに HIV 感染を告知することが母親の感染告知，ひいては母親から子ども本人への感染の告知になるため母親のストレスが大きくなり，告知を拒否することもある。医療者には，身体面のサポートだけではなく，子どもや養育者，あるいは他の家族や周囲の人の気持ちをよく聞き，精神的にもサポートできる存在が必要で，医師だけでなく看護師や心理士なども必要となろう。告知の適切な時期は，性交渉開始年齢から考えても，中学に入る前頃には速やかに考慮したほうがよいと考える。誰が，いつ，どのような状況で告知すべきか事例ごとに検討すべきであるが，非常に困難な問題である。

6 HIV 感染者に対する性教育

告知があっての性教育であり，適切な性教育を実践するにあたり，適切な時期の告知は必要である。もちろん，HIV 感染の有無にかかわらず，性感染症が増加している現在では性教育は非常に大切になってきている。「性教育」とは，性(sex)と人格を結びつけた「命を大切にする」概念として学校教育現場に導入されつつある。

100％確実な避妊法や性感染症予防方法が存在しない以上，性交渉が行われれば妊娠や性感染症が，ある確率をもって誰にでも起こる可能性があると考えるべきである。とくに10代における性行動に関する課題について，妊娠・中絶や性感染症の割合を低下させようとするならば，「性交渉開始年齢」を遅らせることがもっとも効率的であり[6]，このためには，男の子には性の話をしない，女の子には性以外の話をよくすることが効果的といわれている。しかし，このことはHIV 感染者には必ずしも当てはまらず，ある時期に適切かつ正確な告知と並行して性教育をしなければならない。性教育の内容は，性交渉開始年齢を可能な限り遅らせることと，コンドームの装着に尽きる。しかし，やはり「命を大切にする」延長線上に，この概念からくる性行動があることをしっかりと理解させるように話をするべきである。困難を伴うことは承知のうえであるが。

7 結婚・妊娠・出産

A 女性 HIV 感染者の場合

HAART の進歩により，HIV 感染女性も比較的安全に妊娠・出産ができるようになった。パートナーが HIV 陰性者の場合，通常の性交渉ではパートナーに HIV を感染させる可能性があるため，コンドームを使用しなければならない。パートナーに感染を引き起こさない安全な妊娠のためには，体外受精という方法がある。国立国際医療センターでもすでに３例が体外受精で出産されており，出生した児は感染もなく他にとくに異常を認めていない。そのためにも，HIV に熟知したカウンセラー，内科医，産婦人科医，小児科医がいる総合病院で，妊娠前から計画性をもって妊娠・出産に臨むべきであり，また，そうすればほぼ安全な妊娠・出産が可能な時代になってきた。前述したように，母子感染予防策が完全に実施されたスタンダードな治療[4]では，母子感染率は約１％に抑えられている。すなわち，妊娠前からフォローされていれば母子感染はほぼ防止可能といえる。

B 男性 HIV 感染者の場合

精子中に HIV ウイルスが存在する可能性は低いが，精液中にはリンパ球などの感染細胞が存在し，パートナー(女性)が HIV 陰性の場合，通常の性交渉ではパートナーが感染の危険に曝される。しかし，精子で HIV が増殖することはないと考えられており，特殊で専門的な方法にて体外受精を実施しての妊娠・出産は施設により可能と考えられる[7]。詳細は参考文献を参照していただきたい。

8 まとめ

繰り返して述べるが，医療の進歩によりエイズは死の病気ではなくなりつつある。しかし，最近の小児科領域の HIV 感染は，血液製剤を介しての感染の頻度は低下し，そのほとんどが母子感染になっている[2]。また，母子感染した小児は，HIV ウイルスを根治する治療方法がない現在では，一生 HIV ウイルスと闘っていかなければならない。しかも乳児期発症のエイズは重症になりやすい，また，アドヒアランスを維持したうえでの内服の困難さ，思春期を迎えての病名の告知・自覚の難しさ，結婚・妊娠・出産にわたっての社会的・医学的な問題など，問題は山積している。

母子感染は妊娠初期の妊婦スクリーニングで発見して医学的なフォローが十分なされれば，その感染率はほぼ１％まで抑えることができるため，まず妊婦の HIV スクリーニング検査実施率を100％にすることが，根本から絶つ意味でももっとも大切であると考えている。検査実施率は今も地域間格差が存在し，低い県では約70％の実施率に過ぎない。また，

万一 HIV 陽性の小児にとっても，現代の医学をもってすれば，コントロールが可能な慢性疾患となってきたといえる。ただし，まだまだ小児の HIV 患者の治療に熟練した小児科医ばかりではない。小児科医のみならず，さまざまな職種の人が専門的な知識をもって HIV 感染小児をフォローしていくことによって，その子の QOL は著明に改善されると思われる。また，この領域の新薬の開発も含めた医学の進歩は目覚しく，今後ワクチンを含めた画期的な治療法の出現も期待したいところである。人間の繁殖のための営みである性交渉により感染する HIV ウイルスの脅威は，性行動が乱れてきている現代に対する警鐘と思われ，「命・愛を大切にする」生活をわれわれは再認識しなければならない。

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〈國方徹也〉

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Use of sequence analysis of the VP4 gene to classify recent Vietnamese rotavirus isolates

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ABSTRACT

Twenty-eight strains of P(8), four of P(4) and one of P(19) rotavirus, isolated in Ho Chi Minh City, Vietnam, during 2002–2003, were investigated by sequence analysis of the VP4 gene. Seven of the 28 P(8) rotavirus VP4 sequences clustered in the P(8)-3 lineage, or the rare, so-called OP354-like lineage. Amino-acid sequence comparison revealed that Vietnamese P(8)-3 rotaviruses were generally very similar to Malawian strains, including the prototype OP354 strain. The numerical severity scores of diarrhoeal disease caused by the Vietnamese P(8)-3 rotaviruses were statistically higher than those of diarrhoeal disease caused by rotaviruses in the more common P(8)-2 lineage. Sequence and phylogenetic analysis of the VP4 gene of a Vietnamese G9P(19) rotavirus isolate showed a high degree of homology with the cognate genes of other human and porcine rotaviruses, including the prototype 4F strain.

Keywords Diarrhoeal disease, epidemiology, phylogenetic analysis, rotavirus, sequence analysis, Vietnam

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INTRODUCTION

Group A rotaviruses are members of the family Reoviridae and are a major cause of acute gastroenteritis in infants and young children. Rotaviruses cause *c.* 22% of childhood hospitalisations associated with diarrhoea, and are responsible worldwide for *c.* 611 000 childhood deaths annually [1]. The rotavirus genome consists of 11 segments of double-stranded RNA enclosed in a triple-layered capsid. The outer capsid layer is composed of two proteins: VP7, which defines G types (derived from glycoprotein); and VP4, which defines P types (derived from protease-sensitive protein) [2]. At least 15 different G genotypes and 27 P genotypes have been established, based on sequence analysis of the VP7 and VP4 genes, respectively [2–4]. Epidemiological studies worldwide have revealed that rotaviruses of types G1–G4, P(4) and P(8) are responsible for most infections, and four G–P combinations, G1P(8), G2P(4), G3P(8), and G4P(8), have been linked to 88.5% of the cases of rotavirus diarrhoea among children worldwide. However, these four G–P combinations accounted for <70% of rotavirus infections in South America, Asia and Africa [5].

Since VP7 is one of the two neutralising proteins, extensive studies on the molecular characteristics of the gene encoding this protein have been conducted. However, epidemiological studies of the distribution of P genotypes are also necessary for the development of a rotavirus vaccine, as sequence analyses of the VP4 gene could help to identify novel genotypes or subgenotypes in the human population, including recombinant viruses containing genes of animal origin. Data from epidemiological studies have indicated that P(8) is the predominant type, and that types P(8) and P(4) account for >90% of all cases of rotavirus diarrhoea in most parts of the world [5]. Sequence analysis of available P(8)

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rotavirus strains has revealed that they can be divided into three lineages: the P(8)-1 or Wa-like lineage; the P(8)-2 or F45-like lineage; and the P(8)-3 or OP354-like lineage, with the last being a rare subtype comprising only a few rotavirus strains isolated from Malawi [6]. It has also been reported that, besides the dominance of the P(8) type, unusual P genotypes, e.g., P(19), which are normally predominant in animals, have been found increasingly in humans [7]. Further investigation of these strains could provide important insights into the evolution of rotaviruses, including the emergence of new P serotypes or evidence of human–animal recombinant viruses [8,9].

Vietnam is a developing country with a population of 80 million individuals. There are 1.52 million births annually, and a high mortality rate among children aged <5 years (42.2 deaths/1000/year), with 15.4% of deaths being related to diarrhoeal disease [10]. Several epidemiological studies of viruses causing acute gastroenteritis have been conducted, and these have revealed the significant impact of rotavirus on diarrhoeal disease in Vietnam [11–14]. The burden of rotavirus diarrhoea in Vietnam has also been estimated in order to consider the feasibility of a vaccine trial in the near future [15,16]. However, studies concerning the molecular characteristics of rotaviruses, especially the gene encoding the VP4 protein, have been very limited. The first study of the VP4 gene of Vietnamese rotavirus strains reported the presence of P(6) rotaviruses in Khanh Hoa Province, with these viruses probably originating from porcine rotavirus [17]. The present study determined the characteristics of the VP4 gene of rotaviruses belonging to the P(8), P(4) and P(19) genotypes that were detected during surveillance in Ho Chi Minh City in 2002–2003.

MATERIALS AND METHODS

Viruses and patients

A 1-year surveillance study of common viruses causing acute gastroenteritis among children in Ho Chi Minh City was performed and has been described in detail previously [13]. In brief, 1010 faecal specimens were collected from paediatric patients who were admitted to Children's Hospital 1 with a clinical diagnosis of acute gastroenteritis between October 2002 and September 2003. Disease severity was recorded using the 20-point Vesikari score [18]. Group A rotavirus was the most common cause, accounting for 681 (67.4%) of the 1010 cases. Among the 640 samples in which the P genotype could be determined, P(8) was the most frequent (362/640), followed by P(4), P(6) and P(19) (202, five and one cases, respectively). Infections involving more than one P genotype were found in 70 cases. Representative specimens from among the rotavirus-positive samples belonging to each P type were chosen randomly for investigation in the present study.

RT-PCR and nucleotide sequencing of the VP4 gene

A 687-bp fragment of the gene encoding the VP4 protein, including the majority of the hypervariable region VP8* cleavage and a small part of the more conserved region VP5*, was amplified by primers HumCom5 (sense, nucleotides 200–221, 5′-CTCTCGATGGTCCATATCAACC) [19] and Con2 (antisense, nucleotides 887–868, 5′-ATTTCGGACCATTTATA ACC) [20]. PCR products were purified using a QIAquick PCR Purification Kit (Qiagen, Hilden, Germany) and were sequenced using a Big Dye Terminator Cycle Sequencing Kit v.3.1 and an ABI Prism 310 Genetic Analyser (Applied Biosystems, Foster City, CA, USA) according to the manufacturer's instruction. The HumCom5 and Con2 primers were also used for sequencing.

RT-PCR and nucleotide sequencing of the VP6 gene

The full length of the gene encoding the VP6 protein of the G9P(19) strain, VN375/2003, was amplified as described previously [21]. Primers VP6R (antisense, nucleotides 1356–1339, 5′-GGTCACATCCTCTCACTA), derived from primer 1 [21], and VP6_694 (sense, nucleotides 694–711, 5′-CCTTAT TACCAGATGCTG), designed for this study, were used for sequencing. Sequencing was performed as described above.

Sequence and phylogenetic analysis

Similarities of the sequenced strains with other strains were assessed by BLAST searches of partial nucleotide sequences using the default options (DNA DataBank of Japan). Multiple sequence alignments were calculated using the CLUSTALW program, and the phylogenetic trees were constructed using the neighbour-joining method with the MEGA 3.1 software package [22], with the different rotavirus sequences available in GenBank being used for comparisons and as outgroups.

RESULTS

The nucleotide sequences of a 687-bp fragment of the P gene were determined for 28 strains of P(8), four strains of P(4), and one strain of P(19) rotavirus. Results from the BLAST searches confirmed the P genotyping results obtained previously by nested type-specific PCR (data not shown). Table 1 summarises the clinical data of the patients studied. The age distribution of the patients ranged from 2 to 31 months. None showed dehydration, except for one patient, VN964, who was moderately dehydrated. Evaluation of diarrhoea severity, using a 20-point numerical score, showed a range between 7 and 15 points, with a mean score of 10.5 (Table 1).

Table 1. Clinical data and P genotypes of selected Vietnamese P(8) and P(4) rotavirus strains isolated from hospitalised children in Ho Chi Minh City, Vietnam, 2002–2003

Isolate	Date of collection	P type	Age (months)	Dehydration	Vesikari's score	Mean Vesikari score ± SE (number)[a]
VN545	April 2003	P[8]-3	15	None	9	12.6 ± 1.0 (n = 6)
VN546	April 2003	P[8]-3	7	None	12	
VN564	April 2003	P[8]-3	NA	NA	NA	
VN645	May 2003	P[8]-3	11	None	15	
VN827	July 2003	P[8]-3	10	None	15	
VN929	September 2003	P[8]-3	21	None	11	
VN952	September 2003	P[8]-3	9	None	14	
VN6	October 2002	P[8]-2	18	None	7	9.8 ± 0.7 (n = 16)
VN21	October 2002	P[8]-2	14	None	8	
VN23	October 2002	P[8]-2	8	None	8	
VN45	October 2002	P[8]-2	12	NA	NA	
VN318	February 2003	P[8]-2	NA	NA	NA	
VN368	March 2003	P[8]-2	NA	NA	NA	
VN517	April 2003	P[8]-2	15	None	13	
VN532	April 2003	P[8]-2	8	None	12	
VN537	April 2003	P[8]-2	6	None	8	
VN538	April 2003	P[8]-2	4	None	7	
VN543	April 2003	P[8]-2	NA	NA	NA	
VN544	April 2003	P[8]-2	31	None	15	
VN548	April 2003	P[8]-2	NA	NA	NA	
VN550	April 2003	P[8]-2	22	None	12	
VN553	April 2003	P[8]-2	10	None	12	
VN574	April 2003	P[8]-2	24	None	7	
VN621	April 2003	P[8]-2	9	None	8	
VN626	May 2003	P[8]-2	14	None	9	
VN828	July 2003	P[8]-2	30	None	9	
VN851	August 2003	P[8]-2	18	None	8	
VN964	September 2003	P[8]-2	9	Moderate	14	
VN271	February 2003	P[4]	12	None	12	9.5 ± 2.5 (n = 2)
VN322	February 2003	P[4]	NA	NA	NA	
VN580	April 2003	P[4]	4	None	7	
VN594	April 2003	P[4]	NA	NA	NA	

NA, not available or not known.
[a]Mean severity scores ± standard error of diarrhoeal disease caused by rotaviruses in lineages P[8]-3, P[8]-2, and P[4], respectively. Numbers of patients evaluated for the severity scores are also shown.

Sequence analysis of the VP4 gene of P[8] rotaviruses

The phylogenetic tree of the nucleotide sequences demonstrated that the 28 Vietnamese P[8] rotaviruses clustered into two different lineages, lineage P[8]-2 or F45-like (21/28), and lineage P[8]-3 or OP354-like (7/28) (Fig. 1). The results of amino-acid (aa) comparisons, spanning aa 86–263, for seven Vietnamese P[8]-3 rotavirus strains, revealed that they had a high degree of homology with other strains within lineage P[8]-3 (98.3–98.8%), and a lower homology with other P[8] rotaviruses in lineages 1 and 2 (85.9–88.2%) (data not shown). Amino-acid alignments within the same region are shown in Fig. S1 (see Supplementary material), and revealed that the highly conserved proline molecules at residues 224 and 225 were seen in all strains studied. The seven Vietnamese P[8]-3 strains were, in general, identical with other rotaviruses in the OP354-like lineage, within the studied region, and had several unique residues that were characteristic of OP354-like viruses (aa 140-F, aa 149-S, aa 178-K, aa 182-G, aa 184-V, aa 187-G, SD at position 191–192, aa 211-I, aa 221-Y). However, the Vietnamese P[8] rotavirus strains did not have the same conserved residues as some Malawian strains at two positions (aa 194, I instead of T; and aa 258, A instead of T).

In view of the hypothesis that the rare OP354-like viruses might cause more severe diarrhoeal disease than other viruses in common lineages because they could escape protective immunity in communities with common P[8] rotaviruses, the severity of diarrhoea in the patients was investigated using Vesikari's numerical score. The mean score for disease caused by rotaviruses in lineages P[8]-3, P[8]-2 and P[4] was 12.6 ± 1.0 (n = 6), 9.8 ± 0.7 (n = 16) and 9.5 ± 2.5 (n = 2), respectively. The difference between the mean score for the rare P[8]-3 lineage and that for the other lineages was statistically significant ($p < 0.05$).

Sequence analysis of the VP4 gene of P[4] rotaviruses

All four Vietnamese P[4] rotavirus strains in this study were identical with each other, and clustered into a single lineage with other recent P[4] rotaviruses isolated worldwide (Fig. 2).

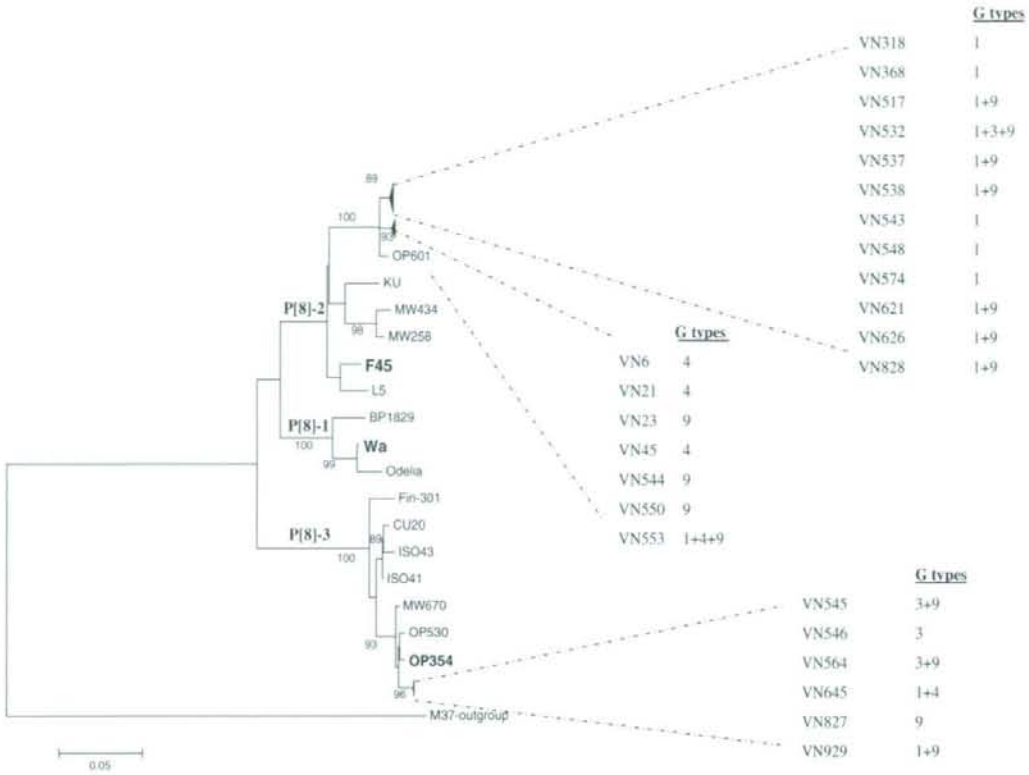

Fig. 1. Phylogenetic tree of the VP8* fragment of 28 Vietnamese P(8) rotavirus strains. Bootstrap values >70% are indicated at the branch nodes. The prototype strains of three lineages are in shown bold type. The associated G types are also shown next to each isolate. Three sub-lineages are indicated. The P(6) rotavirus strain M37 was used as an outgroup.

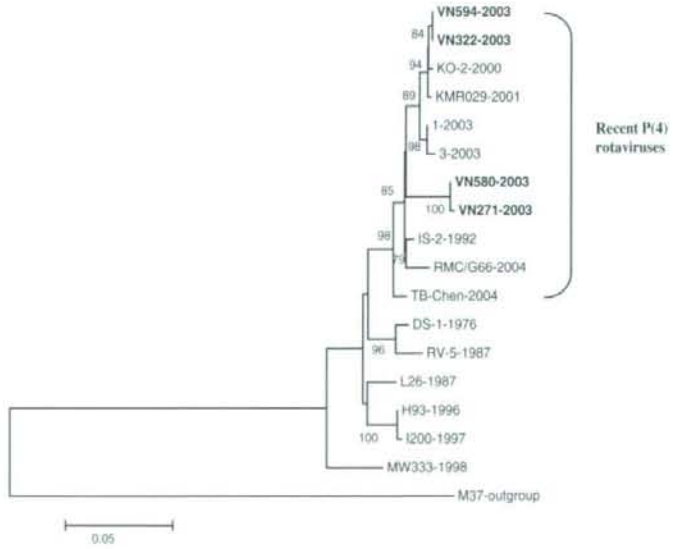

Fig. 2. Phylogenetic tree of the VP8* fragment of four Vietnamese P(4) rotavirus strains. Bootstrap values >70% are indicated at the branch nodes. Year of isolation is also shown. Vietnamese P(4) rotaviruses are shown in bold type. The P(6) rotavirus strain M37 was used as an outgroup.

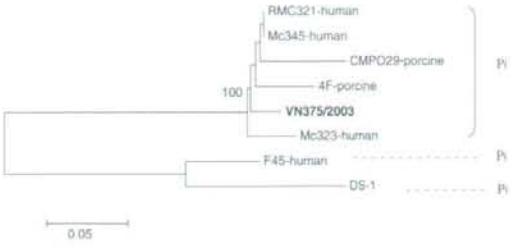

Fig. 3. Phylogenetic tree of the VP8* fragment of Vietnamese and other human and porcine P[19] rotavirus strains. The Vietnamese P[19] isolate is shown in bold type.

Sequence analysis of the VP4 and VP6 genes of a P[19] rotavirus

Nucleotide and amino-acid sequence analysis of the VP4 gene of the VN375/2003 isolate detected during 2002–2003 revealed that this strain had greater identity with P[19] rotaviruses (94.5–96.7% for nucleotides; 94.4–97.2% for amino-acids) than other P genotypes (24.2–68.1% for nucleotides; 44.6–72.2% for amino acids). The phylogenetic tree indicated that the Vietnamese P[19] strain clustered in the same group as other human and several porcine P[19] rotaviruses (Fig. 3). A deduced amino-acid comparison, from aa 88 to aa 273, showed that these P[19] strains were identical. However, the VN375 strain had several unique residues at positions aa 89-I, aa 147-N and aa 195-K. Interestingly, only the Vietnamese strain and the prototype porcine strain 4F had a valine at position 254 (Fig. S2, see Supplementary material).

A 462-bp nucleotide sequence of the VP6 gene of the VN375 strain was also analysed. The results revealed that this gene had closer identity with cognate genes of porcine rotavirus strains (91.1–93.7% for nucleotides; 98.7–99.3% for amino-acids) than with other human strains (82.4–83.7% for nucleotides; 91.5–92.2% for amino-acids).

DISCUSSION

Genotype P[8] has been found to be the most frequent P type among rotaviruses worldwide, with a prevalence of 51% in Africa and 87.8% in Europe [5]. Reports from several surveillance studies in Vietnam have also indicated a high prevalence of P[8] rotavirus (70.7–71.8%) [11,14]. The present study analysed the VP4 gene sequences of these isolates and found that, surprisingly, seven of 28 randomly chosen P[8] strains clustered in the rare P[8]-3, or so-called OP354-like lineage. Since the first report of OP354-like viruses in Malawi during 1998–1999 [6], these rare rotavirus strains have not been isolated elsewhere. A BLAST search of P[8] nucleotide sequences available from GenBank showed that only a few P[8] rotavirus strains had a high identity with the OP354 virus, including some strains from Malawi [6] and India [23], and one each from Thailand and Finland. The detection of these viruses from various countries in different continents suggests that these viruses are widespread around the world. The rather low rate of detection of these rare strains could be explained by large-scale mutation in the nucleotide sequence of the VP4 gene at the primer-binding site of the primer, 1T-1, specifically for P[8], which may make recognition of these rotavirus strains more difficult [6].

Cunliffe *et al.* [6] reported that Malawian P[8] rotaviruses predominantly comprised strains belonging to a distinct G type, in which the G4 type for OP354, the G3 type for MW258, and the G1 type for OP601 were clustered. In the present study, the phylogenetic tree indicated clearly that Vietnamese P[8] rotaviruses belonged to two separate clusters, the OP354 cluster and the OP601 cluster. The strains in the OP601 cluster could be divided further into two variants, in which one variant had a tendency to belong to G types 1 and/or 9, and the second variant had the characteristics of types G9 or G4. However, the concordance between P and G type in the OP354-like viruses was unclear (Fig. 1).

A rotavirus vaccine, which includes a P[8]-1 rotavirus strain as a component, has begun to be used widely in some countries. The finding in the present study that viruses of the P[8]-3 lineage caused, on average, more severe diarrhoeal disease may be important for the development and application of rotavirus vaccines, especially in countries in which OP354-like rotaviruses are prevalent.

The P[19] rotaviruses are known to commonly infect pigs, with the prototype virus being the porcine 4F strain [24]. To our knowledge, there have been only two reports of the isolation of the P[19] rotavirus in humans worldwide, one from Thailand and one from India [7,9]. The detection of this virus in Vietnam emphasises that the P[19]

rotavirus is circulating in Asia. Sequence analysis of the VP6 gene of the Vietnamese G9P(19) strain also showed closest identity with the cognate genes of other porcine rotaviruses, suggesting gene reassortment or a human infection by a virus similar to one that infects animals. Further investigation of this virus might provide interesting data concerning the relationship between human and porcine rotaviruses, especially in Asian countries in which farming is the main activity and close contact between humans and animals could promote inter-species transmission.

ACCESSION NUMBERS

The VP4 nucleotide sequences of the Vietnamese isolates described in this study have been deposited in GenBank under the following accession numbers: EF673752–EF673779 for the P(8) strains; and EF673780–EF673783 for the P(4) strains. The accession numbers of the nucleotide sequences of the VP4 and VP6 genes of the VN375/2003 strain are EF063154 and EU042135, respectively.

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SUPPLEMENTARY MATERIAL

The following supplementary material is available for this article online at http://www.blackwell-synergy.com:

Fig. S1. Partial amino-acid sequence alignment (aa 86-263) for selected P(8) rotaviruses. Lineages and sub-lineages are indicated.

Fig. S2. Partial amino-acid sequence (aa 88-273) alignment within selected P(19) rotaviruses. Human and porcine rotaviruses are indicated.

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Multiple Combinations of P[13]-Like Genotype with G3, G4, and G5 in Porcine Rotaviruses▽

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Epidemiological surveillance of porcine rotavirus (PoRV) strains was carried out in Chiang Mai Province, Thailand, from 2002 to 2003, and eight rotavirus isolates could not be completely typed by PCR. Of these, six were G3 and one was G4 and displayed a P-nontypeable genotype, while another isolate was both G and P nontypeable. Analysis of a partial VP4 gene of all eight P-nontypeable strains revealed a high degree of amino acid sequence identities (94.7% to 100%), suggesting that they belonged to the same P genotype. Comparison of the amino acid sequences of two representative strains (namely, strains CMP178 and CMP213) with those of 27 other known P genotypes revealed a high degree of amino acid sequence identity with those of P[13] porcine rotavirus reference strains HP113 and HP140, which were recently isolated in India. However, amino acid sequence comparison with non-P[13] rotavirus strains revealed relatively low identities, ranging from 58.2% to 84.8% for full-length VP4 sequences and 35.1% to 80.6% for VP8* sequences. Phylogenetic analysis revealed that CMP178 and CMP213 clustered together in a monophyletic branch with P[13]-like genotypes HP113 and HP140 which was clearly separated from the other lineages of P[13] or P[22] strains. Altogether, these findings indicate that PoRV strains CMP178 and CMP213 should be considered the P[13]-like VP4 genotype, a rare genotype that has been identified only in pigs. This study provides additional evidence of increasing genetic diversity among group A rotaviruses in nature.

Group A rotaviruses are the most important etiologic agents of severe diarrhea in young children and young animals worldwide. In developing countries, these severe diarrhea cases lead to an estimated 454,000 to 705,000 deaths annually among children under 5 years of age (32). Group A rotaviruses are members of the *Reoviridae* family with nonenveloped icosahedral particles. The mature virion is formed by three concentric layers of proteins that enclosed a genome of 11 segments of double-stranded RNA. Rotaviruses are classified according to the genetic and antigenic diversity of the two outer capsid proteins, VP4 and VP7. These proteins independently induce type-specific neutralizing antibodies and form the basis of the dual classification of group A rotaviruses into the P (protease sensitive) and the G (glycoprotein) serotypes, respectively (5, 17).

Rotaviruses express extensive antigenic and genomic diversities. To date, at least 15 G genotypes and 26 P genotypes have been identified from humans and a variety of animal species (5, 22, 26, 28, 30, 35, 36). Most recently, several groups of investigators have proposed a novel genotype, P[27], which was isolated from diarrheic piglets (18, 25, 38). Generally, rotaviruses of the same G genotypes share at least 90 to 91% VP7 amino acid sequence identity (11, 12, 13, 16). Rotavirus strains sharing ≥89% VP4 amino acid sequence identities are considered to belong to the same P genotype, while those sharing VP4 amino acid sequence identities of <89% belong to different genotypes (2, 5, 7). Moreover, the VP8* trypsin-cleavage product of VP4 coding for amino acids (aa) 13 to 250, including the greatest sequence divergent region (aa 71 to 204), correlates well with VP4 genotype specificity (20, 21).

Several epidemiological studies have demonstrated that among porcine rotaviruses (PoRVs), G3, G4, and G5, are the most common G genotypes and usually associate with the P[6] or P[7] genotype (5, 23, 39, 40). In addition, other G and P types, such as G1, G2, G6, G8, G9, and G10 and P[13], P[19], P[23], P[26], and P[27], have also been identified in various geographical settings (1, 3, 5, 9, 10, 16, 18, 27, 29, 33, 34, 37, 39, 40, 41). Accordingly, a comprehensive genotypic characterization of the rotavirus strains circulating in domestic animal populations, especially pigs, is important to define the extent of rotavirus diversity.

Rotavirus strains bearing P[13] genotype specificity are host restricted. The P[13] rotavirus genotype is commonly detected among pigs and has not been identified from other animal sources or humans (5, 7, 39, 16). Nevertheless, only a few studies have reported on the distribution of the P[13] genotypes circulating worldwide. Most recently, genome characterization of two PoRV strains, HP113 and HP140, isolated from eastern India revealed that their VP4 sequences were similar to those of P[13] genotypes, while their VP7 sequences were closely related to those of the G6 genotype reference strains (7). In our study, eight strains of P[13] in combination with either G3, G4, or G5 were isolated from diarrheic piglets raised in several farms in Chiang Mai, Thailand. The VP4 genes of these strains were most closely related to those of

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P[13]-like PoRV strains HP113 and HP140. These findings affirm the evidence that rotavirus strains bearing the P[13] genotype are frequently detected in pigs.

MATERIALS AND METHODS

Fecal specimens and rotavirus detection. A total of 250 fecal specimens were collected from diarrheic piglets on six different farms in Chiang Mai, Thailand, in 2002 and 2003. The specimens were evaluated for the presence of group A rotavirus by enzyme-linked immunosorbent assay (ELISA) with a polyclonal antibody against group A rotavirus as the capture antibody (15). The samples that were positive for group A rotavirus by ELISA were investigated further for their G and P genotypes by multiplex reverse transcription-PCR (RT-PCR), as described elsewhere (4, 6, 9, 10, 11, 24, 31, 40).

RNA extraction and RT-PCR genotyping. Viral double-stranded RNA was extracted from a 20% fecal sample suspension with a QIAamp viral RNA mini kit (Qiagen, Hilden, Germany), according to the manufacturer's protocol. For G typing, the VP7 gene was reverse transcribed and amplified by using the consensus primer pair Beg9-End9 (11). Then, the G type was determined by using different pools of primers currently reported to be specific for human and animal G types (G1 to G6 and G8 to G11) (4, 10, 11, 40). For P typing, the consensus primer pair Con2-Con3 was used to generate a 876-bp fragment (VP8*) of VP4, and the P type was determined by using different pools of primers currently reported to be specific for human and animal P types (P[1], P[3] to P[11], P[14], and P[19]) (6, 9, 24, 31, 40).

Nucleotide sequencing. Rotavirus strains that failed to be genotyped by multiple sets of primers were further investigated by nucleotide sequencing. The full-length VP7 (1,062-bp) and partial VP4 (876-bp) genes of these strains were reverse transcribed and were amplified by using the primer pairs Beg9-End9 (10) for VP7 and Con2-Con3 (8) for partial VP4 genes. In addition, the full-length VP4 genes of two representative strains, CMP178 and CMP213, were also amplified by using reverse primer 170 (nucleotides [nt] 2344 to 2368; 5′-GGTCAC AWCCTCTAGMMRYTRCTTA-3′) (26) in combination with forward primer VP4-5F (nt 1 to 23; 5′-GGCTATAAAATGGCTTCDCTCAT-3′). The PCR amplicons were purified with a QIAquick PCR purification kit (Qiagen) and sequenced in both directions by using a BigDye Terminator cycle sequencing kit (Applied Biosystems, Foster City, CA) on an automated sequencer (ABI 3100; Applied Biosystems). For sequencing of the full-length VP4 genes, four additional primers were used as internal sequencing primers. The nucleotide positions and sequences of these primers (5′ to 3′) were as follows: forward primer P28F650, nt 685 to 704, CTTCCACCAATGCAGAATAC; forward primer P28In1, nt 1262 to 1283, CGGATTATGTATCTCTTAACTC; forward primer P28In2, nt 2007 to 2027, GGAGAAATTCATACCAAATAG; and reverse primer P28In3, nt 375 to 396, CACTAGGTTAACTTGTTGA CCG.

Sequences and phylogenetic analysis. The nucleotide sequences were assembled and analyzed by using Bioedit software packages (14). The sequences were compared with those available in the GenBank database by use of the BLAST program in order to determine their genotypes. Phylogenetic and molecular evolutionary analyses were conducted by using the MEGA program (version 3.1) (19). A phylogenetic tree was elaborated by both the parsimony and the distance methods and by supplying statistical support with bootstrapping over 100 replicates.

Nucleotide sequence accession numbers. The full lengths of the VP7 and VP4 gene sequences of strains CMP178 and CMP213 described in this study have been deposited in the GenBank sequence database under accession numbers DQ515961 (VP7) and DQ536362 (VP4) for CMP178 and DQ786576 (VP7) and DQ786578 (VP4) for CMP213.

RESULTS

G and P genotypes. Of a total of 250 fecal specimens tested, 43 (17.2%) were positive for group A rotavirus by ELISA. Characterization of their G and P genotypes by multiplex RT-PCR revealed that 29 isolates were G3, 1 was G4, 4 were G8, 2 were G9, and 7 were G nontypeable. These seven G-nontypeable strains were further characterized by nucleotide sequence analysis, and six were identified to be G3 and 1 was G5. The P genotype was also characterized; and 20 were P[6], 10 were P[7], 5 were P[19], and 8 were P nontypeable. Among these P-nontypeable strains, six were found in combination with G3 (strains CMP213, CMP214, CMP215, CMP225, CMP234, and CMP239), one was found to carry G4 genotype (strain CMP077), while another one was found to be both G and P nontypeable (strain CMP178). Therefore, representative strains CMP178 and CMP213 were selected and their full-length VP4 and VP7 gene sequences were characterized further.

TABLE 1. Comparison of the VP7 deduced amino acid sequence identities of strains CMP178 and CMP213 with the amino acid sequences of 15 known G genotypes

Strain (origin)[a]	G genotype	% VP7 amino acid identity[b]	
		CMP178	CMP213
Wa (human)	1	78.2	80.7
Hu/5 (human)	2	72.3	74.1
YO (human)	3	84.6	**94.4**
AU-1 (human)	3	84.6	**95.4**
P (human)	3	85.5	**95.1**
CP-1 (bovine)	3	85.5	**96.8**
PP-1 (bovine)	3	85.5	**96.8**
A131 (porcine)	3	85.8	**94.7**
A138 (porcine)	3	84.9	**96.0**
4F (porcine)	3	86.1	**95.0**
Gottfried (porcine)	4	74.5	77.2
OSU (porcine)	5	**92.3**	86.0
JL49 (porcine)	5	**92.6**	86.3
H-1 (equine)	5	**92.9**	86.3
A46 (porcine)	5	**92.6**	86.7
134/04-15 (porcine)	5	**93.2**	86.3
NCDV (bovine)	6	81.5	83.5
PO-13 (avian)	7	58.9	56.7
B37 (bovine)	8	78.2	82.5
116E (human)	9	80.9	83.5
B223 (bovine)	10	78.8	81.1
YM (porcine)	11	88.3	86.7
L26 (human)	12	79.4	80.7
L338 (equine)	13	77.6	80.7
CH3 (equine)	14	78.5	84.2
Hg18 (bovine)	15	77.6	77.2

[a] The GenBank accession numbers of the VP7 genes are as indicated for the following strains: Wa, K02033; Hu/5, A01028; YO, D86284; AU-1, D86271; P, AB118024; CP-1, AF448852; PP-1, AF427124; Gottfried, X06386; OSU, X04613; JL49, AY538665; H-1, AF242393; A46, L35054; 134/04-15, DQ062572; NCDV, M12394; PO-13, D82979; B37, J04334; 116E, L14072; B223, X57852; YM, M23194; L26, M58290; L338, D13549; CH3, D25229; and Hg18, AF237666.

[b] Boldface data indicate percentages of the highest sequence identity that CMP178 and CMP213 shared with the corresponding reference genotypes.

VP7 sequence analysis. The deduced amino acid sequences of strains CMP178 and CMP213 were compared with those of representative strains of 15 other known G genotypes. The VP7 amino acid sequence of CMP178 was the most closely related to the amino acid sequences of G5 rotavirus strains, with the amino acid sequence identities ranging from 92.3% to 93.2%, while the VP7 amino acid sequence of CMP213 was the most closely related to the amino acid sequences of the G3 genotypes (94.4% to 96.8%) (Table 1). The data indicated that CMP178 belongs to the G5 genotype and also confirmed that CMP213 has the G3 genotype. Phylogenetic analysis of the deduced amino acid sequences corroborated the results of the analysis of the VP7 sequences by demonstrating that CMP178 and CMP213 clustered with the reference strains of the G5 and G3 rotaviruses, respectively (Fig. 1).

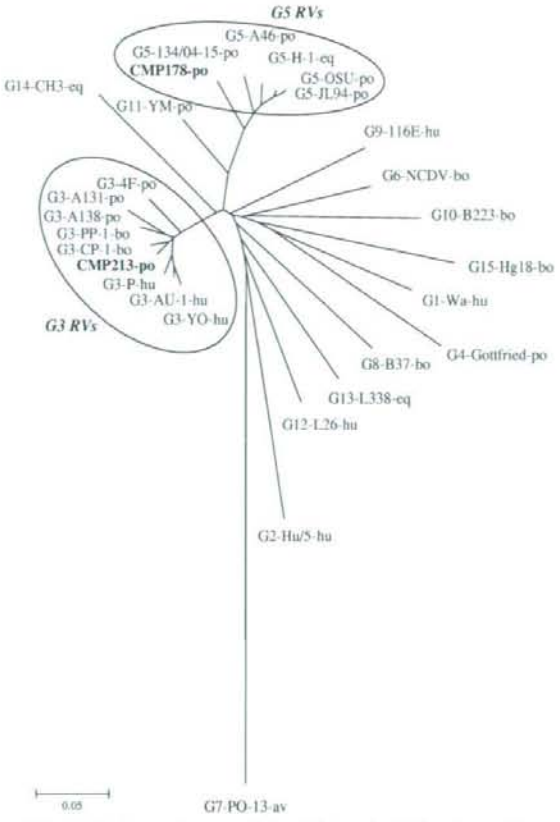

FIG. 1. Phylogenetic tree of the full-length VP7 amino acid sequences displaying the relationships between PoRV strains CMP178, CMP213, and representative strains of all the VP7 genotypes recognized to date. The following abbreviations are used to identify the species of strain origin: hu, human; eq, equine; po, porcine; bo, bovine; av, avian.

VP4 sequence analysis. In order to determine the P genotypes of all eight P-nontypeable PoRV strains, their partial VP4 amplicons (876 bp), which had been generated by the consensus primer pair Con2-Con3, were first subjected to nucleotide sequencing. Comparison of the partial VP4 amino acid sequences of these strains revealed high degrees of amino acid sequence identity (94.7% to 100%) among themselves (data not shown), suggesting that they all belong to the same P genotype.

Analysis of the full-length VP4 amino acid sequences revealed that both representative strains, CMP178 and CMP213, were almost identical (98.4% amino acid identity; data not shown). In addition, when the amino acid sequences of CMP178 and CMP213 were compared with those of 27 known P genotypes (Table 2), the highest level of amino acid sequence identity was found to be with P[13]-like PoRV strains HP113 and HP140 (88.1% and 87.2% for the VP8* portion and 92.2 and 92.4% for the complete VP4 sequences for CMP178 and CMP213, respectively). In contrast, they showed somewhat lower levels of VP8* amino acid sequence identity with other P[13] reference strains, strains MDR-13, JP35-7, JP13-3, ICB2212, ICB2219, and A46, ranging from 79.2% to 83.7% amino acid identity for CMP178 and 79.2% to 83.2% amino acid identity for CMP213. Additionally, they also showed lower levels of amino acid sequence identity with P[22] lapine rotavirus reference strains 160/01, 229/01, 308/01, and 3489/3, ranging from 77.9% to 81.0% for CMP178 and 78.4% to 81.0% for CMP213. Therefore, all eight isolates were considered to be P[13]-like viruses since they had the highest level of sequence identity with P[13] PoRV strains and revealed remarkably lower levels of amino acid sequence identity with

TABLE 2. Comparison of the VP4 and VP8* deduced amino acid sequence identities of strains CMP178 and CMP213 with the amino acid sequences of 27 known P genotypes[a]

Strain (origin)	P genotype	% Amino acid identity[b]			
		VP4		VP8*	
		CMP178	CMP213	CMP178	CMP213
RF (bovine)	P[1]	76.9	77.1	59.8	59.3
SA11 (simian)	P[2]	78.1	78.2	60.5	60.9
RRV (simian)	P[3]	77.3	77.3	59.7	59.7
RV-5 (human)	P[4]	68.8	68.8	51.4	51.4
UK (bovine)	P[5]	70.5	71.0	53.9	55.1
Gottfried (porcine)	P[6]	71.5	71.8	54.3	54.7
OSU (porcine)	P[7]	77.7	78.0	62.6	63.0
Wa (human)	P[8]	69.1	69.1	51.8	51.8
K8 (human)	P[9]	65.9	66.0	49.8	49.3
69M (human)	P[10]	76.3	76.7	57.2	57.6
B223 (bovine)	P[11]	58.2	58.2	35.1	35.1
H-2 (equine)	P[12]	74.8	74.8	58.0	57.2
MDR-13 (porcine)	P[13]	87.5	87.9	79.2	79.2
JP35-7 (porcine)	P[13] or P[22]?	—[c]	—	81.9	83.2
JP13-3 (porcine)	P[13] or P[22]?	—	—	81.9	83.2
ICB2212 (porcine)	P[13]?	—	—	83.2	82.3
ICB2219 (porcine)	P[13]?	—	—	82.3	81.4
A46 (porcine)	P[13]	89.3	89.7	83.7	83.2
HP113 (porcine)	P[13]	**92.2**	**92.2**	**88.1**	**87.2**
HP140 (porcine)	P[13]	**92.4**	**92.4**	**88.1**	**87.2**
PA169 (human)	P[14]	68.1	68.3	51.8	52.2
Lp14 (ovine)	P[15]	76.7	76.9	59.3	59.7
EB (murine)	P[16]	72.4	72.7	52.6	52.2
993-83 (bovine)	P[17]	59.9	59.9	60.0	60.0
L338 (equine)	P[18]	74.6	75.0	56.4	56.8
4F (porcine)	P[19]	71.8	71.8	52.6	52.6
EHP (murine)	P[20]	74.1	74.5	57.2	57.6
Hg18 (bovine)	P[21]	75.1	75.8	55.4	58.0
160/01 (lapine)	P[22]	—	—	80.6	80.6
229/01 (lapine)	P[22]	—	—	79.7	79.7
308/01 (lapine)	P[22]	—	—	77.9	78.4
3489/3 (lapine)	P[22]	—	—	81.0	81.0
A34 (porcine)	P[23]	—	—	61.8	62.2
TUCH (rhesus macaque)	P[24]	77.6	77.7	61.8	61.8
Dhaka6 (human)	P[25]	66.1	66.0	51.4	51.0
134/04-15 (porcine)	P[26]	84.7	84.8	69.2	69.2
CMP034 (porcine)	P[27]	71.0	71.0	57.1	56.0

[a] The GenBank accession numbers of VP4 genes are as indicated for the following strains: RF, U65924; SA11, M23188; RRV, M18736; RV-5, M32559; UK, M22306; Gottfried, M33516; OSU, X13190; Wa, M96825; K8, D90260; 69M, M60600; B223, D13394; H-2, L04638; MDR-13, L07886; PA169, D14724; Lp14, L11599; EB, U08419; 993-83, D16352; L338, D13399; 4F, L10359; EHP, U08424; Hg18, AF237665; 160/01, AF526374; A34, AY174094; TUCH, AY596189; Dhaka6, AY773004; 134/04-15, DQ061053; and CMP034, DQ534016.

[b] Boldface data indicate percentages of the highest sequence identity that CMP178 and CMP213 shared with the corresponding reference genotypes.

[c] —, no full-length VP4 sequence is available in the GenBank database.

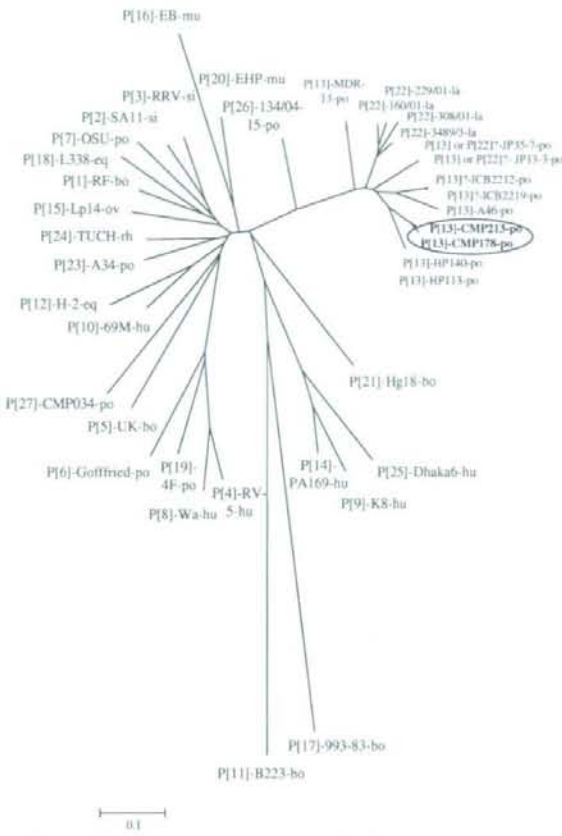

FIG. 2. Phylogenetic tree of the partial VP4 amino acid sequences displaying the relationships between PoRV strains CMP178, CMP213, and selected strains of the 27 P genotypes recognized to date. The following abbreviations are used to identify the species of strain origin: hu, human; eq, equine; po, porcine; bo, bovine; av, avian; pi, pigeon; mu, murine; si, simian; ov, ovine; la, lapine.

other reference strains of non-P[13] rotavirus (35.1% to 81.0% for both CMP178 and CMP213). Phylogenetic analysis supported the amino acid sequence alignments by showing that strains CMP178 and CMP213 clustered in the same branch with strains HP113 and HP140. Of note, although strains CMP178 and CMP213 were closely related to PoRV strains HP113 and HP140, they were rather distantly related to other P[13] PoRV strains and formed a distinct lineage (Fig. 2).

Taken together, the eight strains of P[13] PoRV described in this study comprised six strains of G3P[13] genotype and one strain each of the G4P[13] genotype and the G5P[13] genotype.

DISCUSSION

The eight strains of PoRV described in the present study were isolated from diarrheic piglets on several farms in Chiang Mai, Thailand, during epidemiological surveillance in 2002 and 2003. The initial characterization of the G and P genotypes of these strains by PCR by the use of multiple specific primer sets reported previously (6, 9, 24, 31, 40) revealed that six were G3, one was G4, and the other one was G nontypeable, while all were P nontypeable, as determined by PCR-based genotyping. We therefore further characterized those nontypeable strains by sequencing their VP7 and partial VP4 (VP8*) genes. The amino acid sequence of the VP8* fragment of these strains showed high degrees of identity, ranging from 94.7% to 100%, suggesting that they all belong to the same P genotype. In contrast, when the VP8* amino acid sequences of these strains were compared with those of the existing 27 P genotypes, only a low level of identity (35.1% to 88.1%) was observed (Table 2). In order to verify whether these strains belong to a novel P genotype or a variant strain of an existing known P genotype, the entire VP4 amino acid sequences were analyzed. Comparison of the full-length VP4 amino acid sequences of two representative strains, CMP178 and CMP213, with those of previously reported 27 P genotypes revealed sequence identities that ranged from 58.2% to 92.4% (Table 2). The high levels of amino acid identity were shared with two strains of PoRV recently isolated in India (7), strains HP113 and HP140, at 92.2% and 92.4%, respectively. On the basis of full-length VP4 amino acid sequence analysis, our strains most likely belong to the P[13] genotype. Phylogenetic analysis of the full-length amino acid sequences of the VP4 genes of strains CMP178 and CMP213 supported the sequence alignment data. CMP178 and CMP213 clustered tightly together with strains HP113 and HP140 in a single branch separated from other P[13] PoRV strains (Fig. 2).

Although strain CMP178 was initially identified as a G- and P-nontypeable strain by PCR-based genotyping, it was identified as a P[13] genotype strain (Table 2) that associated with the G5 genotype (Table 1) as a G5P[13] strain by amino acid sequence analyses of the VP4 and VP7 genes. Alignment of the G5-specific primer (SG 5) sequence (40) with the VP7 nucleotide sequence of CMP178 revealed four point mutations in the primer binding region (nt 180 to 200) at positions 183 (A to T), 186 (G to A), 187 (C to A), and 196 (G to A) (data not shown). These point mutations may lead to the failure of the binding of primer SG 5 at the target sequence in the VP4 gene of strain CMP178. This may explain why CMP178 was initially identified as G nontypeable by PCR-based genotyping. Additionally, CMP178 was also initially identified by PCR as P nontypeable, even though it was later identified as being of the P[13] genotype by VP4 sequence analysis, because no primer specific for P[13] has previously been reported in the literature.

Strain CMP213 was initially identified by PCR-based genotyping, and it was confirmed by VP7 sequence analysis (Table 1) to be genotype G3 with a P-nontypeable genotype and was later identified by VP4 sequence analysis to have the P[13] genotype (Table 2). On the basis of these data, CMP213 has a G3 genotype that exists in association with the P[13] genotype. In addition, among the P[13] strains of PoRV isolated in this study, one was found to have P[13] in combination with a G4 genotype (strain CMP077).

Altogether, the strains of PoRV isolated in the present study were P[13] in combination with several G types, including G3, G4, and G5. This finding implies multiple reassortment events and shows the diversity of the P[13] PoRV strains circulating in the pig population in Chiang Mai, Thailand.

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ORIGINAL ARTICLE

Genetic variations in the gB, UL144 and UL149 genes of human cytomegalovirus strains collected from congenitally and postnatally infected Japanese children

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Abstract Human cytomegalovirus (CMV) is the leading cause of intrauterine viral infection. The association of genetic polymorphisms in some particular genes with the incidence and severity of congenital infection has been controversial. To address this issue, we analyzed the genotypes of the glycoprotein B (gB), UL144 and UL149 genes of CMV clinical strains obtained from 33 congenitally and 31 postnatally infected Japanese children. Our results demonstrated that (1) CMV strains with any combination of genotypes could be vertically transmitted from mother to fetus, potentially causing neurological abnormalities, (2) the gB3 genotype was more prevalent in the congenital cases than in postnatally infected children ($P < 0.05$), particularly in congenital cases with sensorineural hearing loss ($P = 0.009$), (3) there was no relationship between gB genotype and viral load in the urine and dried umbilical cord specimens in the congenital cases, and (4) the UL144 and UL149 genotype distributions had no bias for congenial infection. In future studies, it would be interesting to see whether the gB genotypes serve as a prognostic indicator of CMV-associated diseases.

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Abbreviations

CMV	Cytomegalovirus
PCR	Polymerase chain reaction
SNHL	Sensorineural hearing loss

Introduction

Human herpesvirus 5, also known as human cytomegalovirus (CMV), belongs to the subfamily *Betaherpesvirus* of the family *Herpesviridae*. CMV infects most people during their childhood, usually without any clinical symptoms, and establishes latent infection for the rest of their life. Congenital CMV infection occurs in 0.2–2% of all births worldwide [16, 32] and causes developmental abnormalities in >10% of infected fetuses and/or newborns. In addition to those who are symptomatic at birth, a proportion of asymptomatic newborns face a significant risk of late-onset sequelae, such as sensorineural hearing loss (SNHL) and developmental delay [16, 32]. Through retrospective diagnosis using dried umbilical cord specimens, which are generally preserved in households in Japan [23], we demonstrated that 15% of cases of severe SNHL could be ascribed to congenital CMV infection and that at least half of the CMV-related cases developed SNHL after the age of 6 months [31].

Although the genomic sequences of CMV are generally well conserved among strains, a certain number of genes exhibit a high degree of inter-strain variation [14, 37]. In addition, CMV strains passaged in vitro usually contain deletions and/or mutations in several genes that may influence cell tropism [17, 38]. The nucleotide polymorphisms of

the genes encoding viral envelope glycoproteins, such as glycoprotein B (gB), and the genes encoding cellular homologs of chemokines and chemokine receptors, such as UL144, have been studied extensively due to their potential relevance to cell tropism and virulence in the host [37, 39, 41]. However, the relationship between the genotypes of the gB and UL144 genes and clinical outcome has been controversial [37]. Some of the inconsistent results are due to the demographic heterogeneity of subjects, geographical bias, the lack of a proper control group, and the small number of subjects. The genotypes of the gN and gO genes are useful for the classification of isolates, and gN genotype may be correlated to chronic clinical outcome, but not acute clinical manifestations of congenitally infected cases [27, 36]. Recently, a degree of association between the genotypes of UL149 gene and clinical outcome has been proposed [21]. As we are interested in the association of CMV genotypes with the incidence and severity of congenital infection, we compared genotypes of congenitally infected cases, whose infections were confirmed by clinical and laboratory tests with those of postnatally infected children. Here, we report our analysis of the genetic variations in the gB, UL144 and UL149 genes.

Materials and methods

Study subjects

This study was approved by the Ethical Committee on Human Subjects of National Institute of Infectious Diseases, Asahikawa Medical College, and Fukushima Medical University. Informed consent was obtained from the parents of all children. A total of 72 clinical specimens were collected from 64 Japanese children, including 33 congenitally infected children and 31 postnatally infected children. Clinical specimens included 46 urine, 24 dried umbilical cord, 1 amniotic fluid, and 1 saliva specimen.

Eleven of the congenitally infected cases were identified in a retrospective study on severe SNHL cases [31]. Six of the congenital cases were identified by detection of CMV-specific IgM in maternal or cord blood specimens, and another six by our newborn CMV screening program [19]. The rest of the cases were identified by clinical manifestations at birth ($n = 6$) or by late-onset developmental delay ($n = 4$). All congenital CMV infections were confirmed by the detection of CMV DNA in urine specimens collected within 2 weeks after birth or in dried umbilical cord specimens.

Congenitally infected cases were classified as symptomatic ($n = 11$) or asymptomatic ($n = 22$) at birth. Symptomatic infection was defined as having at least one of the following manifestations: petechial/purpuric rash, jaundice with conjugated hyperbilirubinemia (>2 mg/dl direct bilirubin), hepatosplenomegaly, seizures, thrombocytopenia ($<1 \times 10^5/mm^3$), neuroimaging abnormalities (cerebral calcifications and/or ventriclemegaly), and microcephaly (occipital frontal head circumference at birth, <10th percentile). Premature infants or those with low birth weight (<10th percentile) alone were classified as asymptomatic.

Twenty-two of the 33 congenitally infected cases exhibited one or more of the following neurological abnormalities at birth or during subsequent evaluation in childhood: microcephaly ($n = 1$), cerebral calcifications and/or ventriclemegaly ($n = 4$), seizures ($n = 2$), developmental delay ($n = 16$), and SNHL ($n = 19$). Evaluation of the severity of developmental delay and SNHL was based on the criteria described previously [30, 31].

Postnatally infected children included 23 healthy infants between 12 and 24 months of age, and 8 infant patients between 6 and 29 months of age with one of the following manifestations: hepatic damage ($n = 3$), pneumonitis ($n = 3$), bone marrow transplantation ($n = 1$), and systemic infection due to premature birth ($n = 1$). Postnatally infected healthy children were chosen for the presence of CMV in urine specimens from more than 100 volunteers who attended routine medical check-ups at pediatric hospitals or who inquired regarding their natural infection status. Congenital CMV infection in most of these children was excluded by the absence of CMV DNA in their dried umbilical cord specimens. Dried umbilical cord specimens or dried blood spots were not available in a few cases. However, their congenital infection was unlikely, as the incidence rate of congenital CMV infection in our screening program has been less than 0.5% [19].

DNA preparation and real-time PCR

DNA samples were purified from body fluid specimens and/or dried umbilical cord specimens using commercial kits (QIAampViral RNA mini kit and QIAamp DNA mini kit, QIAGEN), and the CMV copy numbers in the purified DNA samples were determined by real-time PCR assays. The details of the real-time PCR assays were described previously [31].

Genotyping

DNA fragments encoding the variable regions of the gB, UL144, and UL149 genes were amplified by nested PCR using PfuI polymerase (Promega) in a 50-μl reaction volume. For the first-round PCR, 10–100 copies of CMV DNA were used as templates in the reaction, and 2 μl of the first-round PCR products was used for the second-round PCR. Optimized PCR conditions and primer sets are shown in Table 1. The PCR products were separated on

Table 1 Primers and PCR conditions

Genes and primers	Round	Sequence (5′–3′)[b]	Amplicon (bp)[a]	PCR conditions	References
gB					
gBout2	First	GCAGCACCTGGCTCTATCG	974	96C/5 m, [94C/45 s, 65C/45 s, 72C/2 m] × 40, 72C/10 m	[8]
gBout		GCACCTTGACGCTGGTTTGG			
gB1319	Second	GGAAYTSGAACGTTTGGC	304	94C/2 m, [94C/45 s, 60C/45 s, 72C/1 m] × 40, 72C/10 m	[12, modified]
gB1604		GAAACGCGCGGCAATCGG			
UL144					
UL144-F	First	TCTCGTATTACAAACCGCGGAGAGGATG	738	96C/5 m, [94C/45 s, 55C/45 s, 72C/2 m] × 40, 72C/10 m	[31]
UL144-R		ACTCAGACACGGTTCCGTAAAGTGCTTC			
UL144-F2	Second	TTCCGGTAGGAGGCATGAAG	587	94C/2 m, [94C/45 s, 55C/45 s, 72C/1 m20 s] × 40, 72C/10 m	
UL144-R2		GTGACTTCATCGTACCGTGA			
UL149					
UL149-F	First	ACTCCTCTTCCTTCTGCTC	776	96C/5 m, [94C/45 s, 55C/45 s, 72C/90 s] × 40, 72C/10 m	[21]
UL149-R		CCGACGTCTCGTACACTAC			
UL149-F2	Second	GTCTGGTGGCCCTGATGTAC	289	94C/2 m, [94C/45 s, 60C/45 s, 72C/1 m] × 40, 72C/10 m	This study
UL149-R2		CGATTGGGGAGCGACAAGAC			

[a] Based on the Merlin sequence (Accession no: AY446894)

[b] R = A/G, S = G/C, Y = C/T

agarose gels and purified using a DNA extraction kit (QIAEX II, QIAGEN). The purified DNA fragments were sequenced with a BigDye Terminator Cycle Sequencing kit (ver. 3.3, PE Applied Biosystems). The primers for the second-round PCR were used as primers for sequencing.

Obtained sequences were assembled with ATGC software version 4.0 (Genetyx, Tokyo, Japan) and aligned with Genetyx 7.0. Phylogenic analysis was conducted using MEGA software version 3.1 (Biodesign Ins., Tempe, USA [24]). The schema for the genotypes of gB, UL144, and UL149 were based on prior publications [11, 12, 21, 25].

Statistical analysis

Genotype distributions and the relationship between genotype and the outcome of CMV infections were analyzed using the chi-square test. P values of <0.05 were considered significant. Comparison of viral loads among genotypes was performed by the Mann–Whitney U test using SPSS software (ver. 11).

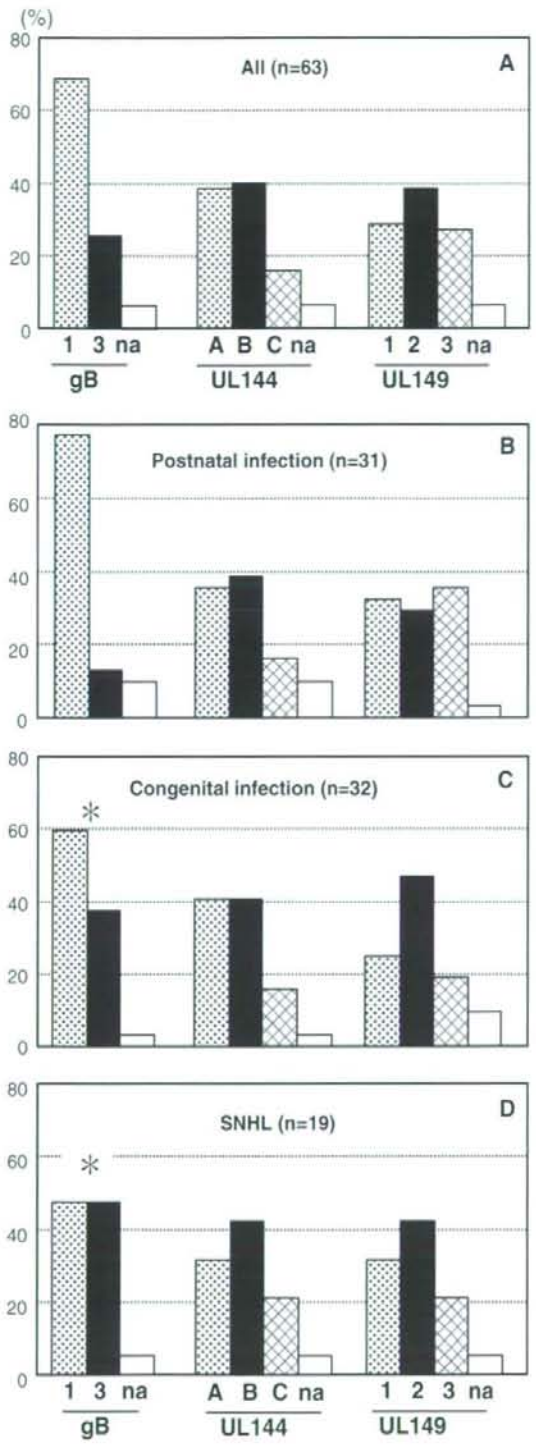
Results

Comparison of strains obtained from different sources of newborns with congenital CMV infection

In this study, sequences of the variable regions of the gB, UL144 and UL149 genes from 72 specimens obtained from 64 children were determined. Infection with multiple strains was not identified at a level detectable in raw sequencing data (>25% in the population). The 64 individuals included one pair of twin newborns with congenital CMV infection. Since the twins had identical sequences for the three genes tested, they were counted as a single entity. Both urine and dried umbilical cord specimens were collected from five congenital CMV cases. The sequences of the gB, UL144 and UL149 variable regions from these five cases were identical regardless of the specimen source. In one of the five cases, the urine specimen was collected 31 months after birth, suggesting the stability of CMV sequences. Urine, amniotic fluid, and dried umbilical cord specimens were collected from one additional case of congenital CMV, and the sequences of the UL144 gene in the three specimens were found to be identical.

Overall distribution of gB, UL144 and UL149 genotypes

The gB, UL144 and UL149 genotypes were analyzed for the 63 strains (the strains from the twins were counted as one). The overall distribution of the 63 gB genotypes was as follows: 43 gB1, no gB2, 16 gB3, no gB4, and 4 not available (na). That of the UL144 genotypes was 24 UL144-A, 25 UL144-B, 10 UL144-C, no UL144-A/B, no UL144-A/C and 4 na; and that of the UL149 genotype was 18 UL149-1, 24 UL149-2, 17 UL149-3, and 4 na (Fig. 1a). Since only one study to date has analyzed the UL149 genotype, the phylogenic tree of the UL149 sequences



◀ **Fig. 1** Distribution of CMV genotypes. *Each panel* shows the genotyping results of the following subjects: **a** all analyzed cases ($n = 63$); **b** postnatally infected children ($n = 31$); **c** congenitally infected cases ($n = 32$); **d** congenital cases with SNHL ($n = 19$). *na* sequence data not available. *Asterisks* indicate a significant difference in the distribution of the genotypes from that of the postnatally infected children

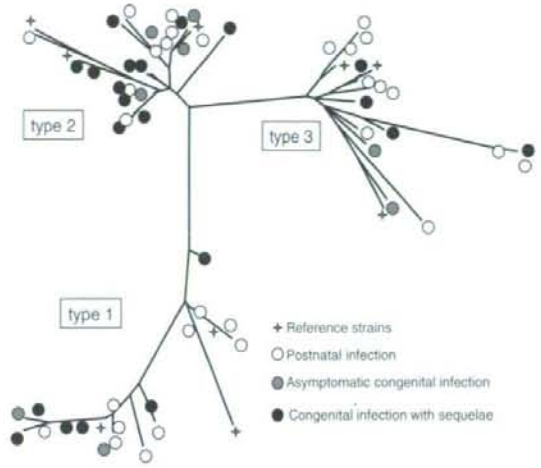


Fig. 2 Unrooted phylogenic dendrogram of CMV UL149 sequences. The tree was generated by the neighbor-joining method based on an approximately 300-bp nucleotide sequence region. *Open circles* postnatal CMV infection ($n = 30$); *shaded circles* congenital CMV infection without clinical symptoms at birth ($n = 8$); *closed circles* congenital CMV infection with neurological abnormalities ($n = 21$); *stars* reference strains ($n = 9$)

obtained from our study is shown in Fig. 2, confirming the previous grouping of CMV strains into three UL149 genotypes.

Comparison of genotype distributions between congenital and postnatal infection

The genotype distributions of each gene were compared between congenitally and postnatally infected children (Fig. 1b, c). The proportion of the gB3 genotype in the congenital group was significantly ($P = 0.035$) higher than that in the postnatally infected group. In contrast, the genotype distributions of the UL144 and UL149 were similar between the two populations.

Relationship between genotype and clinical outcome

A comparison of genotype distribution between strains from the congenital cases with clinical symptoms at birth and those of the cases without symptoms at birth did not reveal any significant differences.

Next, the relationship between genotype and neurological abnormalities was examined (Table 2). Among the 32 congenitally infected cases, 11 cases were identified from the severe SNHL cohort. Among the