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CYP2C19 Genetic Variants Affect Nelfinavir Pharmacokinetics and Virologic Response in HIV-1–Infected Children Receiving Highly Active Antiretroviral Therapy

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Abstract

Background—The objective of this research was to identify the impact of genetic variants of P-glycoprotein (ABCB1) and cytochrome P450 (CYP) on nelfinavir pharmacokinetics and response to highly active antiretroviral therapy (HAART) in HIV-1–infected children.

Methods—HIV-1–infected children (n = 152) from Pediatric AIDS Clinical Trial Group 366 or 377 receiving nelfinavir as a component of HAART were evaluated. Genomic DNA was assayed for ABCB1 and CYP genetic variants using real-time polymerase chain reaction Nelfinavir oral clearance (CL/F), M8 to nelfinavir ratios, $CD4^+$ T cells, and HIV-1-RNA were measured during HAART.

Results—Nelfinavir CL/F and M8 to nelfinavir ratios were significantly associated with the CYP2C19-G681A genotypes ($P < 0.001$). Furthermore, the CYP2C19-G681A genotype was related to virologic responses at week 24 ($P = 0.01$). A multivariate analysis demonstrated that age ($P = 0.03$), concomitant protease inhibitor use ($P < 0.001$), and the CYP2C19-G681A genotype ($P < 0.001$) remained significant covariates associated with nelfinavir CL/F.

Conclusions—CYP2C19 genotypes altered nelfinavir pharmacokinetics and the virologic response to HAART in HIV-1–infected children. These findings suggest that CYP2C19 genotypes are important determinants of nelfinavir pharmacokinetics and virologic response in HIV-1-infected children.

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Keywords

ABCB1; CYP2C19; children; nelfinavir; virologic response

Introduction

Protease inhibitors (PIs), which have been used widely as a component of highly active antiretroviral therapy (HAART) in children and adults are known to be a substrate of P-glycoprotein[1] and metabolized by hepatic cytochrome P450 (CYP), mainly by CYP3A4.[2] In particular, nelfinavir (NFV) is metabolized into the metabolite hydroxyl-tert-butylamide (M8) by the CYP2C19 enzyme, and subsequently NFV and M8 are metabolized by CYP3A4.[3] Several pharmacogenetic studies have shown that single nucleotide polymorphisms (SNPs) in adenosine triphosphate–binding cassette, subfamily B, member 1 *ABCB1* (previously called multidrug resistance 1, *MDR1*)[4] and *CYP*[5] can influence the activity and bioavailability of NFV.

We previously reported that genetic variants in *ABCB1* gene encoding for P-glycoprotein was responsible for variability in NFV pharmacokinetics (PKs) and virologic responses to HAART in children.[6] However, the study was limited because of the small number of subjects. In addition, our earlier results were in contrast to a study previously reported in adults.[4] Therefore, to expand the number of subjects, we examined children who received NFV as a component of HAART from 2 pediatric studies, Pediatric AIDS Clinical Trial Group (PACTG) 366[7] and 377,[8] to investigate the association between NFV PK and SNPs in *ABCB1* and *CYP* and virologic and immunologic responses.

Materials and Methods

Subjects

This was a retrospective study investigating 152 children who received NFV as a component of HAART regimens although participating in PACTG 366 (n = 75, 49%) and PACTG 377 (n = 77, 51%) (Table 1). Informed consent was obtained from study participants. This study followed the human experimentation guidelines of the US Department of Health and Human Services and the University of California, San Diego review board.

NFV PKs

Among the 152 patients, 106 children (70%) had intensive PK collected over 8 hours at week 4 of treatment and 46 subjects (30%) had sparse PK during HAART These results and detailed methods for the measurement of NFV and calculation of PK parameters have been previously reported from subjects participating in PACTG 377.[8,9] The M8 to NFV ratios were calculated based on each M8 and NFV value and averaged in subjects with the intensive PK data. When the M8 level was <50 ng/mL, such numbers were imputed to 50 ng/mL.[5]

Measurement of Plasma HIV-1 RNA and CD4$^+$ T Cells

Plasma HIV-1 RNA was quantified using the Roche Amplicor HIV-1 Monitor assay (Roche Molecular Systems, Alameda, CA) with a detection limit of 400 copies per milliliter. The numbers and percentages of CD4$^+$ T cells were determined in PACTG certified laboratories by flow cytometry.

Amplification and Detection of Polymorphisms in *ABCB1* and *CYP* Genes by Real-Time Polymerase Chain Reaction

Previously developed fluorescence assays and detection methods were used for analyzing the *ABCB1-3435C*>T (rs1045642) and *CYP3A4-392A>G* (rs2740574).[6] For the *ABCB1-2677G*>T (rs2032582),[10] *CYP2C19C*2*-681G>A (rs4244285), and *CYP2C19*3-636G*>A (rs28399504),[11] previously reported assays were used. In addition, novel fluorescent assays were developed to detect the *ABCB1*-1236C>T (rs1128503) and *ABCB1*-1199G>A (rs2229109) genotypes. The information regarding the sequences of custom designed primers and probes and polymerase chain reaction conditions are available upon request.

Statistical Analysis

Statistical analyses were performed using the SPSS 13.0 software (Chicago, IL). Comparisons among ordered 3 genotypes at each categorical group were performed using the Jonckheere-Terpstra (rank-based trend) test for continuous outcomes and Cochran-Armitage trend test for binomial outcomes. A multivariate analysis for NFV CL/F was performed to evaluate the contribution of covariates. The x^2 and Fisher exact tests were used to make pair-wise comparisons. All *P* values calculated were 2 sided and *P* value <0.05 was considered to be significant.

Results

Frequencies of *ABCB1* and *CYP* Gene Polymorphisms

The *ABCB1*-3435-T allelic frequency was 0.35 in the whole cohort; however, the frequencies were less common in black, non-Hispanic ($P = 0.006$). Similarly, there were significant differences in the frequencies of *ABCB1*-G2677T ($P < 0.001$) and *ABCB1*-C1236T genotypes ($P < 0.001$) in black, non-Hispanic, compared with others. The *CYP2C19*-681-A allelic frequency was 0.19 in whole cohort, and the frequencies were similar among race/ethnicity ($P = 0.35$) (Table 1). All patients had the *CYP2C19*-636-G/G genotype.

The *ABCB1*-3435C> T Genotype is Associated With NFV CL/F

NFV CL/F differed significantly among the *ABCB1*-3435C>T genotype ($P < 0.001$); children with the *ABCB1*-3435-C/C genotypes had higher median NFV CL/F [47.2 L/h/m^2, interquartile range (IQR): 32.7–68.7 $L \cdot h^{-1} \cdot m^{-2}$] compared with those with the C/T (36.1 L/h/m^2, IQR: 28.1– 56.7 $L \cdot h^{-1} \cdot m^{-2}$) and the T/T genotype (35.4 L/h/m^2, IQR: 17.8-61.3 $L \cdot h^{-1} \cdot m^{-2}$). NFV CL/F did not differ among subjects with the other *ABCB1* genotype ($P > 0.17$). There was no difference in the association when examined by each race/ethnic group ($P > 0.11$).

The *CYP2C19*-681G>A Polymorphism is Associated With NFV CL/F

NFV CL/F differed significantly among the *CYP2C19*-681G>A genotypes ($P < 0.001$). When the data were analyzed in each race/ethnicity (Fig. 1), a significant difference in NFV CL/F was observed in black, non-Hispanics ($P < 0.001$). The same trends were also observed for Hispanics ($P = 0.12$) and white, non-Hispanics ($P = 0.25$), but the differences did not achieve the level of significance.

Association Between the *CYP2C19*-681G>A Genotype and the M8 to NFV Ratio

Overall, the median M8 to NFV ratio was associated with the *CYP2C19*-681G>A genotype ($P < 0.001$); the ratio was 0.45 (IQR: 0.22–0.96) in those with the -G/G genotype compared with 0.26 (IQR: 0.13–0.47) in -G/A or 0.02 (IQR: 0.01–0.08) for the -A/A genotype. The

association between the *CYP2C9*-681G>A genotype was particularly strong for the black, non-Hispanic group ($P < 0.001$), but not for the Hispanic ($P = 0.56$), and white, non-Hispanic ($P = 0.30$) groups. No other genotypes were associated with the M8 to NFV ratio ($P = 0.29$–0.87).

Virologic and Immunologic Responses During HAART in Children With *ABCB1* and *CYP* Genotypes

The percentages in children who reached plasma HIV RNA <400 copies per milliliter at week 12 did not differ by the *CYP2C19*-681G>A genotypes ($P = 0.14$–1.00). However, at week 24, the percentage of subjects among the *CYP2C19*-681G>A genotype who reached plasma HIV RNA <400 copies per milliliter differed significantly ($P = 0.01$): 46% of subjects with the *CYP2C19*-681-G/G genotype achieved virologic suppression compared with 69% of those with the -G/A genotype, and 63% of those with the -A/A genotype. When examined by race/ethnicity, these differences were observed for the black, non-Hispanic group ($P = 0.02$) and the white, non-Hispanic group ($P = 0.03$), but not for Hispanics ($P = 0.84$). No differences were observed when the data were analyzed with the *ABCB1*-3435C>T genotype ($P = 0.06$) or the *CYP3A4*-392A>G genotype at week 24 ($P = 0.26$). Regarding immunologic recovery, changes in $CD4^+$ T-cell percentage from baseline to weeks 12 and 24 were not different among the 3 genotypes in *CYP2C19*-681G>A ($P = 0.50$, $P = 0.44$, respectively) or *ABCB1*-3435C>T ($P = 0.08$, $P = 0.21$, respectively).

Other Factors Contributing to NFV CL/F Concomitant Antiretrovirals

Because nevirapine induces hepatic CYP3A and decreases the levels of PIs[12] and ritonavir acts as a potent PK enhancer for CYP substrates,[13] we evaluated the association between NFV CL/F and concomitant use of nevirapine or ritonavir. NFV CL/F was not different between subjects who received nevirapine and those who did not receive nevirapine ($P = 0.70$). In contrast, ritonavir use decreased NFV CL/F significantly ($P = 0.002$); the median NFV CL/F in patients who received ritonavir (35.8 L/h/m^2, IQR: 24.7– 47.5 $L \cdot h^{-1} \cdot m^{-2}$) was lower compared with those who did not receive ritonavir (47.4 L/h/m^2, IQR: 32.5–70.6 $L \cdot h^{-1} \cdot m^{-2}$).

Association of Race/Ethnicity on NFV CL/F and Clinical Outcomes

Because race/ethnicity is an important determinant of these SNPs, we also analyzed the data based on their race/ethnicity. Black, non-Hispanics (43.4 $L \cdot h^{-1} \cdot m^{-2}$, IQR: 33.1–66.6 $L \cdot h^{-1} \cdot m^{-2}$) and Hispanics (45.2 $L \cdot h^{-1} \cdot m^{-2}$, IQR: 26.2–65.2 $L \cdot h^{-1} \cdot m^{-2}$) had higher median NFV CL/F compared with white, non-Hispanics (31.7 $L \cdot h^{-1} \cdot m^{-2}$, IQR: 27.3–53.3 $L \cdot h^{-1} \cdot m^{-2}$), but it did not reach a statistical significance ($P = 0.09$). M8 to NFV ratio was not associated with race/ethnicity ($P = 0.67$). Furthermore, clinical outcomes including percentages in children who reached plasma HIV RNA <400 copies per milliliter at week 12 ($P = 0.54$) or changes in $CD4^+$ T-cell percentage from baseline to weeks 12 ($P = 0.89$) was not associated with race/ethnicity.

A Multivariate Analysis for Predicting NFV CL/F

A multivariate analysis showed that the *CYP2C19*-681G>A variants ($P < 0.001$), concomitant use of ritonavir ($P < 0.001$), and age ($P = 0.03$) were independently associated with NFV CL/F. However, the *ABCB1-3435* variants ($P = 0.61$), *CYP3A4-392* homozygous variants ($P = 0.42$), and race/ethnicity (black, non-Hispanics) ($P = 0.07$) were no longer statistically significant. Thus, the *CYP2C19*-681G>A genotype remains an important pharmacogenetic determinant of NFV CL/F even after controlling for other factors.

Discussion

The data presented here demonstrate that *CYP2C19*-681G>A variants exert the greatest impact on NFV PK and virologic response. Controlling for various factors, only *CYP2C19*-681G>A genotype and concomitant PI usage continued to demonstrate a highly significant association with NFV CL/F.

Hepatic CYP2C19 is the critical enzyme responsible for conversion of NFV to its M8 metabolite.[14] Alteration in *CYP2C19*-681G>A in exon 5, which creates an aberrant splice site resulting in a truncated nonfunctional protein[15] is the SNP identified in *CYP2C19* most often associated with different clinical responses to pharmacologic agents for treating diseases (eg, treatment of peptic ulcer disease using proton pump inhibitors).[16] Previous reports have described the impact of this genotype on the NFV to M8 ratio in HIV-1–infected adult populations.[5,17,18] Notably, Haas et al[5] reported a trend toward decreased virologic failure associated with the *CYP2C19*-681G>A genotype. Our current data in children is in agreement with the Haas study and provide further support for an important role of *CYP2C19*-681G>A variants in NFV PK and virologic response. We cannot rule out that other *CYP2C19* SNPs might also alter the PK of NFV or other PIs.[19] In addition, we only investigated 7 SNPs which have been reported to be related to NFV PK. Furthermore, when we analyzed the data by each race/ethnicity, significant differences in NFV PK were only observed in black, non-Hispanics and virologic response in black, non-Hispanics and white, non-Hispanics. These apparent differences are likely related, in part, to the lower number of study participants in the white, non-Hispanic and Hispanic cohorts.

NFV was used extensively in years past but is now rarely used in clinical practice for children in developed countries for a few reasons; NFV has been replaced by more potent PIs (eg, lopinavir/ritonavir) with better PK profile and fewer incidences of adverse effects (eg, diarrhea) and recent manufacture problem with a contamination of ethyl methylate.[20] However, NFV continues to be used in developing countries. The information learned in this current study may be helpful to improve the clinical outcomes of children who receive NFV.

In conclusion, the *CYP2C19*-681G>A, age, and concomitant ritonavir are significantly associated with NFV PK in HIV-1–infected children. In addition, favorable virologic response was observed in children with the *CYP2C19*-681G>A variants associated with lower oral NFV CL/F. These findings suggest that *CYP2C19*-681G>A is the most important pharmacogenetic determinant for NFV PK and virologic responses in children who receive HAART-containing NFV.

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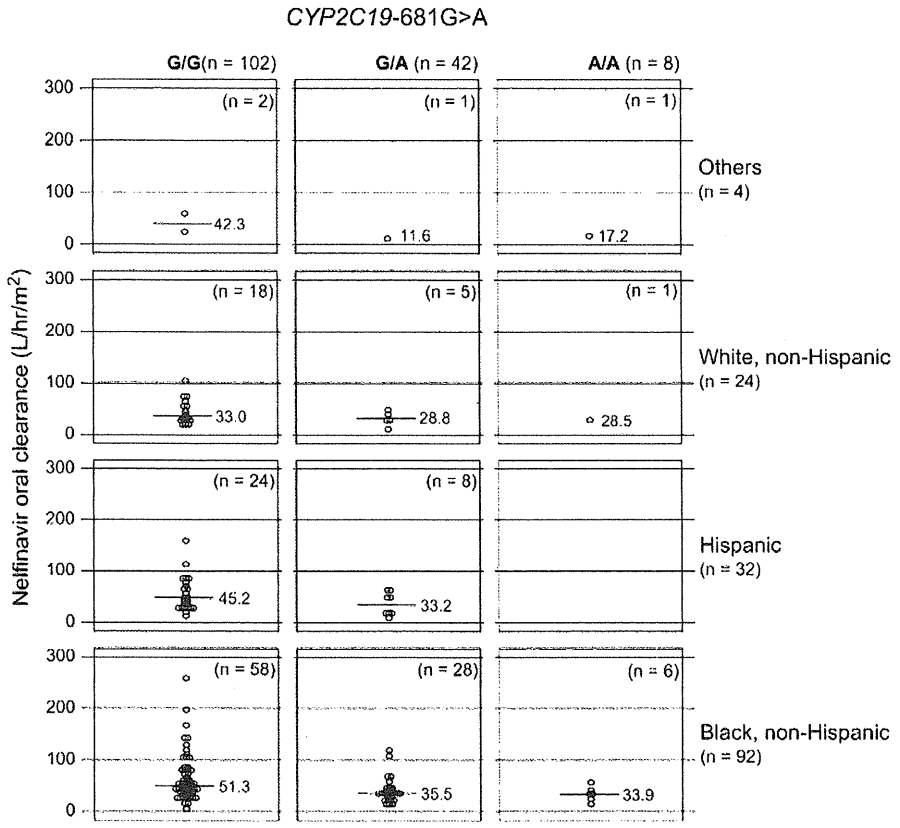

Figure 1.

Oral clearance rate (CL/F, L/h/m^2) for nelfinavir in children with the *CYP2C19*-681G>A genotypes in each race/ethnicity. Each circle represents nelfinavir CL/F in each subject with the *CYP2C19*-681-G/G (left), -G/A (heterozygous, middle), and -A/A (homozygous, right) in each race/ethnicity. The lines in the middle represent the median of CL/F for NFV.

Table 1

Baseline Characteristics of the 152 Children in PACTG 366 and 377 With the CYP2C19-G681A Genotypes

	All Subjects*	PACTG 366	PACTG 377		CYP2C19-G681A			
	n = 152 (%)	n = 75 (49)	n = 77 (51)	*P*	G/G n = 102 (%)	G/A n = 42 (%)	A/A n = 8 (%)	*P*
Sex, n (%)								
Male	79 (52)	46 (61)	33 (43)	0.01	53 (67)	22 (28)	4 (5)	0.99
Female	73 (48)	29 (39)	44 (57)		49 (67)	20 (27)	4 (6)	
Race/ethnicity								
Black, non-Hispanic	92 (61)	45 (60)	47 (61)	0.73	58 (63)	28 (30)	6 (7)	0.35
Hispanic	32 (21)	15 (20)	17 (22)		24 (75)	8 (25)	0 (0)	
White, non-Hispanic	24 (16)	12 (16)	12 (16)		18 (75)	5 (21)	1 (4)	
Others	4 (3)	3 (4)	1 (1)		2 (50)	1 (25.0)	1 (25.0)	
Age (yrs) [median, (IQR)]	7.1 (3.8-9.6)	6.9 (3.2-10.6)	6.8 (4.4-9.2)	0.42	7.1 (3.5-10.1)	7.1 (3.9-9.4)	7.9 (5.9-10.7)	0.82
Concomitant PI or NNRTI								
No	23 (15)	0	23 (30)		12 (52)	9 (39)	2 (9)	0.63
Ritonavir	40 (26)	40 (53)	0		29 (73)	9 (23)	2 (5)	
Nevirapine	60 (40)	6 (8)	54 (70)		43 (72)	14 (23)	3 (5)	
Ritonavir + nevirapine	29 (19)	29 (39)	0		18 (62)	10 (35)	1 (3)	
Baseline $CD4^+$ (%) median, (IQR)	24 (16–32)	19 (10–28)	27 (21–35)	<0.001	23 (16–32)	25 (19–31)	22 (6–40)	0.85
Mean HIV-1 RNA $\log_{10}$ copies/mL (SD)	4.56 (0.68)	4.71 (0.61)	4.41 (0.72)	0.003	4.54 (0.70)	4.62 (0.64)	4.44 (0.74)	0.65

*The subjects were selected from the whole study populations if they satisfied the following criteria: (1) received NFV as a component of HAART for >24 weeks with reported excellent compliance to their treatment regimen; (2) virologic and immunologic data were available at baseline, weeks 12 and 24; and (3) PK data for NFV were available at week 4.

NNRTI, nonnucleoside reverse transcriptase inhibitor.

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Bifidobacterium Septicemia Associated with Postoperative Probiotic Therapy in a Neonate with Omphalocele

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We report the one case of sepsis caused by *Bifidobacterium breve* administered as probiotic therapy. Probiotics can be a potential cause of an invasive disease and should be used with care in vulnerable patients. *(J Pediatr 2010;156:679-81)*

Probiotic therapy has been associated with reduced risk of necrotizing enetrocolitis[1-4] and methicillin-resistant *Staphylococcus aureus* colonization in the intestine of premature infants.[5] This approach has been adopted in neonatal intensive care units in many countries. At our center, we have been administering *Bifidobacterium breve* BBG-01 to all neonates admitted to neonatal intensive care units because of surgical problems or prematurity. The present case is the first in which we have encountered sepsis secondary to *Bifidobacterium breve* BBG-01 probiotic therapy.

Case Report

A fetus was diagnosed with omphalocele at 13 weeks' gestation. A female infant was delivered at 37 weeks and 2 days of gestation by scheduled cesarean delivery. The birth weight was 2060 g; Apgar scores at 1 and 5 minutes were 6 and 7, respectively. The liver and intestine were prolapsed, but the hernia sac was intact. There were no external malformations other than omphalocele and polydactyly of the right hand. The other anatomic examinations and laboratory tests on admission showed no abnormalities.

Four hours after birth, surgery for omphalocele was performed (**Figure 1**). On day 2, administration of *Bifidobacterium breve* BBG-01 was begun. On day 8, a peripheral arterial catheter was removed. On day 10, gastric fluid became bilious, and the laboratory findings were as follows: C-reactive protein, 1.2 mg/dL(normal range: <0.2 mg/dL); white blood cell count 3500/mm^3, with 18% bands and 26% neutrophils. A peripherally inserted central catheter was replaced. Peripheral aerobic and anaerobic blood cultures were obtained; ampicillin/sulbactam and amikacin were initiated empirically and enteral feedings were discontinued. Cerebrospinal fluid cultures were not obtained because the patient was not in critical condition and had no abnormal neurogical signs and symptoms. On day 12, C-reactive protein and white blood cell count increased to 8.2 mg/dL and 9520 /mm^3 (16% bands and 42% neutrophils), respectively. Antibiotics were changed from ampicillin/sulbactam to meropenem. On day 14, the blood cultures obtained on day 10 grew *Bifidobacterium* spp; oral *Bifidobacterium breve* BBG-01 therapy was discontinued. The patient continued to improve, and repeated blood cultures were negative. She recovered without any sequelae or complications. Isolated *Bifidobacterium* spp was susceptible in vitro to penicillin and ampicillin sulbactam (MICs 1 μg/mL) but not to meropenem (MIC >8 μg/mL) or amikacin (MIC ≥32 μg/mL).

The strain detected from the patient was therefore genetically identical to the probiotic *Bifidobacterium breve* BBG-01. Polymerase chain reaction analysis of *Bifidobacterium* spp isolated from the blood cultures showed positive results for *Bifidobacterium*, *Bifidobacterium breve*, and more specifically, *Bifidobacterium breve* BBG-01. Strain-specific identification by a randomly amplified polymorphic DNA analysis using strain-specific primers confirmed *Bifidobacterium breve* BBG-01 (**Figure 2**). The isolates were positive for a monoclonal antibody against *Bifidobacterium breve* BBG-01.

Discussion

"Probiotics" are defined by the Food and Agriculture Organization of the United Nations and the World Health Organization as "live microorganisms, which when administered in adequate amounts, confer a health benefit on the host."[6]

In our center, we use *Bifidobacterium breve* BBG-01 as the probiotic agent. *Bifidobacterium breve* BBG-01 is supplied as a freeze-dried powder in corn starch, containing about 10^9 CFU/g (Yakult Honsya Co Ltd, Tokyo, Japan). Before administering to infants, the nurses add 1 g of the powder in 1.5 mL of sterile water and obtain 0.5 mL of the supernatant after centrifugation in a sterile environment. The supernatant is given 2 times daily. This case report describes invasive disease attributed to *Bifidobacterium* used in a neonate. Although it is uncertain, we suspect that the systemic edema

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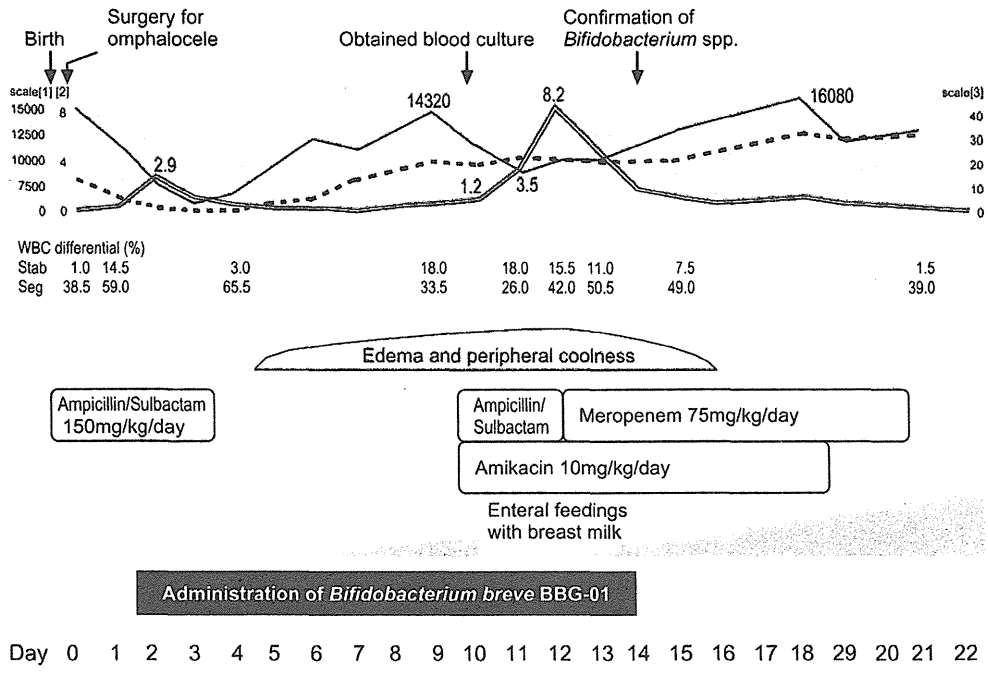

Figure 1. Clinical course in this case of *Bifidobacterium breve* septicemia.

and elevated inflammatory markers resulted from the *Bifidobacterium breve* BBG-01 sepsis for the following reasons: (1) the bacteria were detected from the blood when the increase in inflammatory markers was observed; (2) the intestinal inflammation from the surgical repair could have led to the translocation of *Bifidobacterium breve* BBG-01; and (3) no organisms were detected in other cultures except the blood cultures.

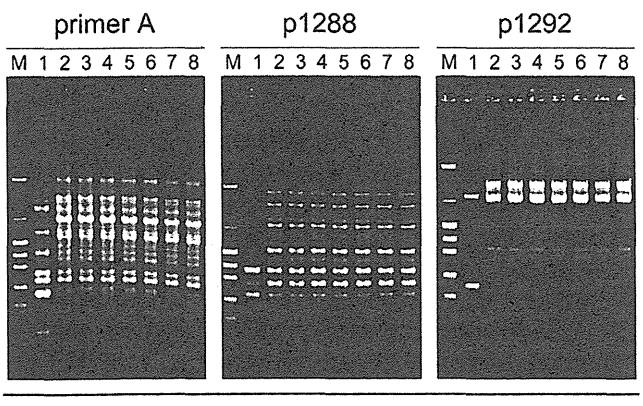

Figure 2. Strain-specific identification of *Bifidobacterium breve* BBG-01 by randomly amplified polymorphic DNA analysis. Three strain-specific primers were used: primer A (-CCGCAGCCAA-), p1288 (-GGGGTTGACC-), and P1292 (-CCCGTCAGCA-). Lane M, pHY DNA size marker; lane 1, other strain of *Bifidobacterium breve*; lanes 2 through 7, *Bifidobacterium* spp detected from the patient; lane 8, *Bifidobacterium breve* BBG-01.

The benefits of probiotics therapy for preterm infants are indicated in many studies; it is shown to reduce the incidence of necrotizing enterocolitis[1-4] and to prevent infections and other diseases.[7] Moreover, contribution of *Bifidobacterium breve* to the intestinal function of premature infants and their prognosis has been suggested.[8] Recently, Kanamori et al[9] reported the beneficial effects of *Bifidobacterium breve* for patients with short bowel syndrome.

Use of probiotic therapy has the fundamental dilemma that patients with the greatest potential benefit from therapy, such as neonates who have immature immune systems or require surgery for gastrointestinal abnormalities, are those who are prone to sepsis from common microorganisms. On the other hand, invasive diseases caused by well-characterized, less pathogenic organisms might not be life-threatening and might be eradicated easily or even be self-limiting.

Some authors have questioned the safety of probiotic therapy. Guarner et al[10] reported that the risk of infection, unrestricted stimulation of the immune system, and gain of antibiotic resistance to virulent microorganisms should be evaluated while administering antibiotic therapy. Some reports demonstrate *Lactobacillus* infection during probiotic therapy.[11] It is also possible that sepsis related to *Bifidobacterium* is under-diagnosed because the anaerobic microorganism is undetected under regular aerobic conditions. In this case, the culture became positive within 40 hours of incubation. Subculture onto several agar plates yielded only a few colonies on an ABHK agar plate (Nissui Pharmatheuticals, Tokyo, Japan) incubated under anaerobic conditions.

Although the clinical course of this case was not life-threatening, one should be aware that the introduction of a new

living microorganism as a therapeutic agent can be harmful, especially in patients who are predisposed to invasive disease. Further studies should aim for better understanding of the appropriate method, strains or combination of strains administered, dosage, and period of administration. Moreover, it is necessary to demonstrate whether the method is cost-effective and safe when performed in neonates.

This report should serve as a reminder that probiotic agents have potential benefits and risks of invasive disease under certain physiological conditions. ■

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Is a 6-Week Course of Ganciclovir Therapy Effective for Chorioretinitis in Infants with Congenital Cytomegalovirus Infection?

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Effective treatment for chorioretinitis caused by congenital cytomegalovirus (CMV) infection remains unknown. We report an infant with congenital CMV infection, who required a 6-month course of antiviral therapy to control his chorioretinitis. Long-term treatment may be necessary for managing congenital CMV-associated chorioretinitis. *(J Pediatr 2010;157:331-3)*

Cytomegalovirus (CMV) is the most common cause of congenital infection in humans.[1] It has been shown that a 6-week course of intravenous ganciclovir (GCV) therapy prevents hearing deterioration in infants with symptomatic congenital CMV disease involving the central nervous system.[2] However, safe and effective treatment for chorioretinitis caused by congenital CMV infection is not yet established.[3] We recently experienced a case of congenital CMV-associated chorioretinitis, which required a 6-month course of antiviral therapy to be controlled.

See editorial, p 179 and related article, p 191

Case report

A 28-year-old mother was admitted to our neonatal intensive care unit because of intrauterine growth retardation of the fetus at 31 weeks of gestation. With fetal magnetic resonance imaging, enlargement of ventricles bilaterally and periventricular calcifications were demonstrated. Antenatal serologic testing at 32 weeks' gestational age had no remarkable findings, except for the presence of CMV-specific immunoglobulin (Ig) G.

The male infant was born at 38 weeks of gestational age. His weight, length, and head circumference were 2270 g (<10th percentile), 44 cm (<10th percentile), and 31 cm (10th percentile), respectively. Physical examination results were remarkable for petechiae on the entire body and hepatosplenomegaly. Complete blood count was within reference ranges except for thrombocytopenia (30 600/mm^3). Liver enzymes and kidney test results were within reference ranges. On the basis of these physical and laboratory findings, congenital infection was strongly suspected, and further evaluation was performed. With computed tomography of the head, enlargement of the ventricles bilaterally and remarkable bilateral periventricular calcifications were revealed. Although CMV-specific IgM was undetectable, CMV DNA was found in his blood and urine specimens collected on the fourth day of life with the real-time polymerase chain reaction assay described previously,[4] and CMV was isolated in human fibroblast cells from a urine specimen, confirming congenital CMV infection.

Ophthalmoscopic examination showed bilateral active chorioretinitis on the seventh day of life, which triggered treatment with intravenous GCV (12 mg/kg/day every 12 hours). His hospital course is summarized in the **Figure**. The patient tolerated a 6-week course of the GCV therapy well, and ophthalmoscopy indicated improvement of chorioretinitis without an active lesion. However, 2 weeks after discontinuation of the therapy, his chorioretinitis partially recurred, and GCV therapy with the same dose was reinitiated.

After restarting therapy, chorioretinitis improved gradually. Although active lesions of chorioretinitis were diminished, the presence of some exudates suggested incomplete stabilization. As a result, intravenous GCV was administrated for a total of 9 weeks and subsequently switched to oral valganciclovir (Val-GCV; 32 mg/kg/day every 12 hours) for the duration of therapy. The peak concentrations of GCV in serum specimens before and after changing to the Val-GCV therapy were 3.9 μg/dL and 6.6 μg/dL, respectively. Oral Val-GCV treatment was continued for 7 weeks. After the completion of the therapies, ophthalmoscopy did not identify any signs of recurrence of chorioretinitis.

In addition to chorioretinitis, the patient had hearing impairment (ABR measurements of the left and right ears were 80 dB and 105 dB, respectively) at the beginning of initial GCV treatment, and significant recovery of hearing of the

CMV	Cytomegalovirus
GCV	Ganciclovir
Ig	Immunoglobulin
Val-GCV	Valganciclovir

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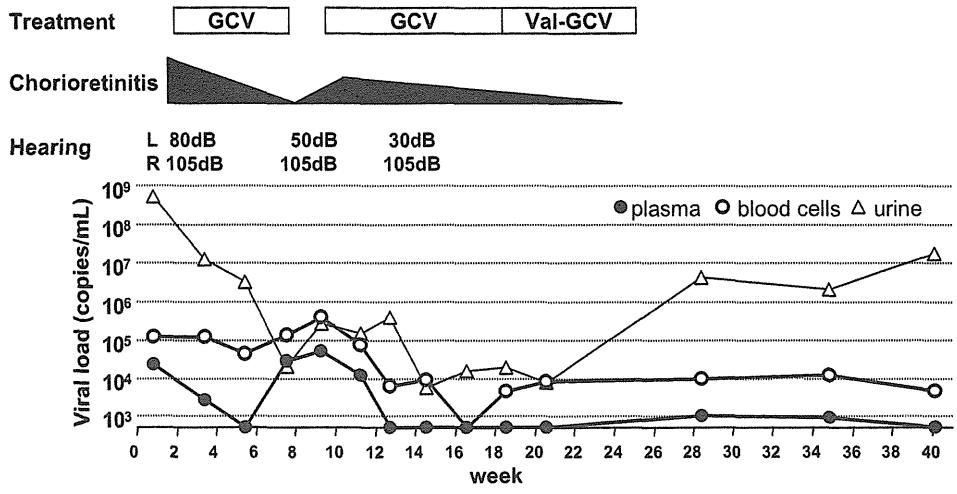

Figure. Summary of clinical manifestations and treatment are depicted: visual and hearing impairment, antiviral treatments, and longitudinal changes in cytomegalovirus viral loads in the urine, blood cells, and plasma specimens measured with the real-time polymerase chain reaction assay.

better ear (left, 30 dB; right, 105 dB) was observed after the initial GCV therapy; hearing level remained the same 6 month after the cessation of Val-GCV therapy. There were no adverse effects because of GCV and Val-GCV treatments in the patient; absolute neutrophil counts were examined at least once a week during the 6 months of antiviral therapy and remained within the reference range. Neurodevelopment was delayed, and he required anti-epileptic medication to control seizures; however, his eye and ear abnormalities were improved during long-term antiviral treatment. The patient was discharged from our hospital at 7 months of life. The last examination 1 year after the completion of antiviral therapy demonstrated that his ophthalmologic condition remained stable without active lesions.

Discussion

A 6-week course of GCV therapy has been used for a symptomatic infant with congenital CMV to prevent progression of hearing loss.[2] A clinical trial to compare a 6-month course with a 6-week course of Val-GCV therapy is under way to assess safety and efficacy characteristics (ClinicalTrials.gov NCT00466817).[5] Appropriate treatment for congenital CMV-associated chorioretinitis has not been well established.[3] To our knowledge, there are only a few reports in the literatures describing antiviral therapies for active chorioretinitis in patients with congenital CMV (Table).[2,6-12] Initial therapies to control CMV chorioretinitis ranged from 2 to 7 weeks. Some reports showed treatment benefits for CMV retinitis, but other reports demonstrated no treatment benefit.[2,14] A few reports demonstrated that longer duration of therapy up to 3 months was necessary to control chorioretinitis.[6] Clinical outcomes of patients differ significantly in different reports. Factors influencing clinical outcomes of patients may include: time to diagnosis, time to initiate GCV therapy, host factors to control CMV retinitis, and possibly the length of GCV therapy.

For long-term GCV therapy of congenital CMV, it is important to consider the most effective and least invasive toxic antiviral regimen. Kimberlin et al reported that a 16-mg/kg/dose of oral Val-GCV solution administered twice daily provided GCV exposure comparable with that of 6 mg/kg/dose of GCV intravenously.[13] Applying these novel data, we changed treatment from GCV intravenously (12 mg/kg/day, divided every 12 hours) to Val-GCV orally (32 mg/kg/day, dividied every 12 hours). Our pharmacokinetic data also confirmed that oral Val-GCV dose was sufficient to achieve the GCV level equivalent to that of the intravenous GCV administration.

The major toxicity in patients receiving GCV is hematologic abnormalities, notably neutropenia.[14] However, the incidence of neutropenia in congenital CMV infected infants varies significantly. For example, in the clinical trial conducted by Kimberlin et al, neutropenia developed in 63% of infants with congenital CMV infection who received GCV during the first 6 weeks of treatment.[2] For the GCV-induced neutropenia, it has been demonstrated that granulocyte colony stimulation factor could be used to increase the absolute neutrophil count, while continuing long-term GCV therapy.[2] However, Tanaka-Kitajima et al reported that neutropenia did not develop in any of 6 Japanese cases of congenital CMV during GCV treatment in a small and uncontrolled cohort study.[11] Similarly, Nigro et al found neutropenia only in 1 of 12 cases of congenital CMV treated with GCV.[6] In our case, neutropenia did not develop in the patient during the entire course of therapy, which allowed long-term administration of the drugs. It would be useful to identify any indicator, especially host factors, predictive of neutropenia in GCV therapy. GCV is known to cause gonadal toxicity and carcinogenicity in animal models,[15] and long-term

Table. Reports on treatment and outcome of chorioretinitis caused by congenital cytomegalovirus infection

Authors	Number of cases	Antiviral regimen, dosage and frequency	Duration	Clinical outcome of chorioretinitis	References
Kimberlin et al	8	GCV 6 mg/kg/dose X2/day or no treatment	6 weeks	no change	2
Nigro et al	3	GCV 5 mg/kg/dose X2/day	2 weeks	no change	6
	2	GCV 7.5 mg/kg/dose X2/day	2 weeks	resolution	
		GCV 10 mg/kg/dose X3/wk	3 months		
Coats et al	1	GCV (dose unknown)	Unknown	resolution > stabilization	7
Baumal et al	1	GCV 5 mg/kg/dose X2/day	4 weeks	complete regression of active retinitis	8
Barampouti et al	1	GCV 5 mg/kg/day	3 weeks	resolution	9
Weng et al	1	GCV 5 mg/kg/dose X3/day + Anti-CMV immunoglobulin 400 mg/kg/dose every other day	3 weeks 20 days	resolution	10
Tanaka-Kitajima et al	5	GCV 2.5-6 mg/kg/dose X2/day	2-7 weeks*	4/5 (80%) resolution 1/5 (20%) stabilization	11
Whitley et al.	14	GCV 4 or 6 mg/kg/dose X2/day	6 weeks	8/14 (57%) resolution 6/14 (43%) consequences 3: retinal detachment, 2: optic atrophy, 1: retinal hemorrhage	12

*One patient required 3 additional series of GCV therapy intravenously.

effects in humans are not established. Thus, further follow-up is necessary for such infants who required long-term GCV or Val-GCV therapy. ■

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Typing of *Clostridium difficile* isolates endemic in Japan by sequencing of *slpA* and its application to direct typing

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A typing system for *Clostridium difficile* using sequencing of the surface-layer protein A encoding gene (*slpA*) was evaluated and used to analyse clinical isolates in Japan. A total of 160 stool specimens from symptomatic patients in Japan was examined and 87 *C. difficile* isolates were recovered. *slpA* sequence typing was found to have reliable typability and discriminatory power in comparison with PCR ribotyping, and the typing results were highly reproducible and comparable. *slpA* sequence typing was used to type *C. difficile* in DNA extracted directly from stool specimens. Among the 90 stool specimens in which direct typing results were obtained, 77 specimens were positive for *C. difficile* culture, and typing results from isolated strains agreed with those from direct typing in all 77 specimens. The *slpA* sequence type smz was dominant at all four hospitals examined, and this endemic type was detected by culture and/or direct typing in 61 (62 %) of 99 stool specimens positive for toxic culture and/or direct *slpA* sequence typing. Comparison of epidemic strains reported throughout the world revealed one isolate identified as *slpA* sequence type gc8, which was found to correspond to PCR ribotype 027 (BI/NAP1/027), whereas no isolates were found with the *slpA* gene identical to that of PCR ribotype 078 strain. *slpA* sequence typing is valuable for comparison of *C. difficile* strains epidemic in diverse areas because the typing results are reproducible and can easily be shared. In addition, *slpA* sequence typing could be applied to direct typing without culture.

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INTRODUCTION

Clostridium difficile is one of the important organisms causing healthcare-associated infections. Specific strains have been documented to cause multiple outbreaks (Kato *et al.*, 2001; McDonald *et al.*, 2005; Samore *et al.*, 1997), and patients infected with particular strains are more likely to develop severe disease (Barbut *et al.*, 2007; Goorhuis *et al.*, 2007, 2008), suggesting that strain differences play some role in the pathogenicity of this organism. Recent reports documented that a variant strain, characterized as restriction endonuclease analysis (REA) type BI, PFGE type NAP1 and PCR ribotype 027, caused a large number of outbreaks in North America and Europe (Kuijper *et al.*, 2008; McDonald *et al.*, 2005; Warny *et al.*, 2005). In addition, PCR ribotype 078 has been noted as another hypervirulent strain, and has been recovered not only from calves and pigs (Keel *et al.*, 2007) but also from humans (Goorhuis *et al.*, 2008). Among numerous schemes for typing *C. difficile*, PCR ribotyping and PFGE typing are widely used in Europe and North America to identify epidemic strains. However, it is not easy to share typing results by these schemes, which depend on banding-pattern

Abbreviations: CDI, *Clostridium difficile* infection; CDT, *Clostridium difficile* binary toxin; REA, restriction endonuclease analysis.

The GenBank/EMBL/DDBJ accession numbers (*slpA* sequence type) for the *slpA* genes reported in this study are AB259785 (ar-02), AB258978 (cr-01), AB258979 (cr-02), AB258980 (cr-03), AB236153 (fr-01), AB249984 (gr-01), AB231583 (hr-01), AB258982 (kr-02), AB258981 (kr-03), AB239686 (xr-01), AB239685 (xr-02), AB261625 (xr-03), AB180242 (smz-01), AB181350 (smz-02), AB256018 (smz-04), AB240196 (yok-01), AB257283 (yok-02), AB236725 (hj2-01), AB236726 (j41-01), AB258983 (gc11-01), AB259787 (og39-01), AB538230 (y05-01), AB259786 (t25-01), AB269265 (g13-01), AB249986 (gc8-01) and AB470267 (078-01).

analysis among multiple laboratories. In the present study, a typing method involving the sequencing of the gene encoding the surface-layer protein A (*slpA*) was evaluated. Previous reports have documented that the low-molecular-mass peptide of the surface-layer protein varies among *C. difficile* isolates (Calabi & Fairweather, 2002; Eidhin *et al.*, 2006), and a variation of the gene has been used for typing *C. difficile* (Karjalainen *et al.*, 2002; Kato *et al.*, 2005a). In this study, clinical isolates from Japan were analysed by sequencing of *slpA* and the method was applied to type *C. difficile* from DNA extracted directly from stool specimens.

METHODS

Bacterial strains and stool specimens. The reference strains of F (ATCC 43598), G (ATCC 43599), H (ATCC 43600) (Delmee *et al.*, 1986) were obtained from the ATCC. The GAI 97660 strain was used as a reference strain for the PCR ribotype smz, *slp*A sequence type smz and serogroup JP (Kato *et al.*, 2001, 2005a). Included in the present study were strains US36 (REA type J/PCR ribotype 001), NL8 (REA type Y/PCR ribotype 014), US37 (REA type G/PCR ribotype 002), US42 (REA type BI/PFGE type NAP1/PCR ribotype 027; Killgore *et al.*, 2008) and UMCG12(3) (PCR ribotype 078; Goorhuis *et al.*, 2008). Stool specimens were obtained with the informed consent of patients who were hospitalized from 2003 to 2007 with a diagnosis of antibiotic-associated diarrhoea or colitis. A total of 147 stool specimens from patients admitted to four hospitals (A, B, C and D) and 13 specimens from sporadic cases from six other hospitals in Japan were tested. Hospitals A and D were located in different cities of the same prefecture (Gifu), whilst hospitals B and C were in different prefectures, Aichi and Nagano, respectively. The stool specimens were frozen at −80 °C until transportation, and were tested at the National Institute of Infectious Diseases, Tokyo, Japan.

Culture. *C. difficile* was isolated on cycloserine–cefoxitin–mannitol agar (Nissui Pharmaceutical) from stool specimens, which were treated with alcohol for spore selection, and identified as described previously (Kato *et al.*, 1998). The presence of the non-repeating sequences of the toxin B gene (*tcdB*) and the repeating sequences of the toxin A gene (*tcdA*) was examined by PCR as described previously (Kato *et al.*, 1998, 1999). PCR detection of the gene encoding the binding component of C. *difficile* binary toxin (CDT) was performed as described by Stubbs *et al.* (2000).

DNA extraction. DNA extraction from cultured isolates for PCR ribotyping and *slpA* sequence typing was performed using a High Pure PCR template preparation kit (Roche Diagnostics) according to the manufacturer's instructions. DNA was extracted directly from stool specimens using a QIAamp DNA stool mini kit (Qiagen) according to the manufacturer's instructions (Kato *et al.*, 2005a, b).

Typing of isolates. Typing of isolates by sequencing *slpA* was carried out with the primer set slpAcom19/slpAcom22, as described previously (Kato *et al.*, 2005a). Both strands of the amplified products were sequenced. Isolates were assigned to different major types when they had 20 or more amino acid differences, and to subtypes (01, 02, 03 and 04) when they had fewer than 20 such differences. PCR ribotyping of isolates was performed using the modified methods described by Stubbs *et al.* (1999). Briefly, the reaction volume for PCR was scaled down to 30 µl, and 1 µl DNA extracted by the method described above was used. The thermal profile was 35 cycles comprising 95 °C for 20 s and 55 °C for 120 s, followed by incubation at 75 °C for 5 min, and the resultant PCR products were separated in 2.5 % agarose gel at a constant voltage of 125 V for 3.5 h. A new PCR ribotype was identified when a banding pattern showed two or more band differences from previously identified patterns.

Detection of *tcdB* by nested PCR of DNA from stool specimens. The *tcdB* gene was detected by nested PCR on DNA extracted from stool specimens as described previously (Kato *et al.*, 2005b).

Direct typing by sequencing *slpA* of DNA from stool specimens. Amplification of *slpA* by a nested PCR was performed on DNA extracted from stool specimens that were PCR positive for *tcdB* by the nested PCR. The primer pair used for the first PCR was slpAcom19/slpAcom22 (Table 1), which was used for the typing of isolates. The *slpA* sequences of the 17 isolates representing different *slpA* sequence types were compared, and 5 forward primers (slpAcom33, slpAy32-1, slpAxr-1, slpAyok-9 and slpAog39-3) and 2 reverse primers (slpAcom30 and slpAog39-6) were selected for the second PCR for direct typing (Table 1). The primer pair slpAcom33/slpAcom30 was selected from the *slpA* sequences of eight isolates (GenBank accession nos AB180242, AB258978, AB258979, AB236153, AB249984, AB231583, AB236725 and AB236726); slpAy32-1 was from those of two isolates (AB258981 and AB258983); slpAxr-1 was from two isolates (AB239686 and AB239685); slpAyok-9 was from two isolates (AB240196 and AB257283); and slpAog39-3 and slpAog39-6 were from three isolates (AB259787, AB538230 and AF458880). The *slpA* sequences (AF458880) of ATCC 43597 (the reference strain of serogroup D) were available from the GenBank database. The thermal profiles were 35 cycles comprising 95 °C for 20 s and 55 °C for 180 s, followed by incubation at 75 °C for 5 min for the first PCR, and 35 cycles comprising 95 °C for 20 s and 55 °C for 120 s, followed by incubation at 75 °C for 5 min for the second PCR. After the first PCR with the primer pair slpAcom19/slpAcom22, a second PCR was performed by the primer pair slpAcom33/slpAcom30, which was designated primer set A. When no amplification was produced by the PCR with primer set A, the second PCR was performed separately by primer set B, consisting of primers slpAy32-1, slpAxr-1, slpAyok-9 and slpAcom30, and set C, consisting of primers slpAog39-3 and slpAog39-6. The PCR product was purified and sequenced with the same primers used for the second PCR in the same manner described for the *slpA* sequence typing on DNA extracted from isolates. Both strands of the amplified products were sequenced.

RESULTS

Typing of the reference strains

Typing analysis was performed on some epidemic strains that have been reported from around the world. ATCC 43598 strain, which was previously characterized as toxin A negative and toxin B positive (A^-B^+) and as serogroup F/PCR ribotype 017 (Delmee *et al.*, 1986; Stubbs *et al.*, 1999), was typed as *slpA* sequence type fr-01 (GenBank accession no. AB236153). The US36 strain (REA type J/PCR ribotype 001) (Killgore *et al.*, 2008) and ATCC 43599 strain had the same *slpA* sequences of type gr-01 (GenBank accession no. AB249984); NL8 strain (REA type Y/PCR ribotype 014) (Killgore *et al.*, 2008) and ATCC 43600 strain were identical by both *slpA* sequence typing (type hr-01, GenBank accession no. AB231583) and PCR ribotyping. The *slpA* sequences of the US37 strain (REA type G/PCR ribotype 002), US42 strain (REA type BI/PFGE type NAP1/PCR ribotype 027) (Killgore *et al.*, 2008) and UMCG12(3) strain (PCR ribotype 078) (Goorhuis *et al.*, 2008) were

Table 1. Primers for *slpA* sequence typing

Primer	Direction	Sequence (5′→3′)	Nt position of primer for 2nd PCR (GenBank accession no. of *slpA* of the representative isolate)
1st PCR			
slpAcom19	Forward	GTTGGGAGGAATTTAAGRAATG	
slpAcom22	Reverse	GCWGTYTCTATTCTATCDTYWCC	
2nd PCR			
Set A			
slpAcom33	Forward	TAGGYGATGGDRAWTAYGTWG	311–331 (AB180242)
slpAcom30	Reverse	CATAWBBYTYAGCTAAAKHTTBWGC	832–856 (AB180242)
Set B			
slpAy32-1	Forward	TAGGYGATGGAAAATAYGTTC	287–307 (AB258981)
slpAxr-1	Forward	TAGGTGATGGAGATTTAGTATC	299–320 (AB239686)
slpAyok-9	Forward	GTTGCAGATGGTGTAACAGGC	157–177 (AB240196)
slpAcom30	Reverse	CATAWBBYTYAGCTAAAKHTTBWGC	853–877 (AB258981)
Set C			
slpAog39-3	Forward	GTTGYWRATRHARAKTATGTTG	364–385 (AB259787)
slpAog39-6	Reverse	TTAWWSCATCAKARTCWGTTGC	955–976 (AB259787)

examined and registered as type yok-01 (GenBank accession no. AB240196), type gc8-01 (GenBank accession no. AB249986) and type 078-01 (GenBank accession no. AB470267), respectively.

Typing analysis of recovered isolates

A total of 160 stool specimens was examined and 87 *C. difficile* isolates were recovered. Of the 87 isolates, 75 were A^+B^+ and 12 were A^-B^+. Three of the A^+B^+ isolates were positive for PCR detecting the binary toxin gene ($A^+B^+CDT^+$). By *slpA* sequence typing, the 87 isolates were typed into 14 major types and further divided into 18 subtypes (Table 2). In one isolate, DJNS 0403, the *slpA* gene could not be amplified by the primer set slpAcom19/slpAcom22, but was sequenced using slpAcom19 and a reverse primer (5′-GCTGTTTGTATTCTGTCATCACC-3′). This isolate was typed as *slpA* sequence type ar-02 (Table 2). Among the 87 isolates, 18 different PCR ribotypes were identified (Fig. 1). Typing results by *slpA* sequence typing were found to be concordant with those by PCR ribotyping (Table 2). A total of 51 isolates belonging to PCR ribotype smz was classified into three *slpA* sequence subtypes (smz-01, smz-02 and smz-04), and the sequences of subtypes smz-02 and smz-04 differed by one and two deduced amino acids from that of smz-01. Two isolates showed the same PCR ribotype pattern (PCR ribotype og39), but their *slpA* amino acid sequences shared only 23 % homology. All of the 12 A^-B^+ isolates examined in the present study had the same *slpA* gene (type fr-01) but were typed into three different PCR ribotypes, 017, trf and sgf.

Direct typing by sequencing of *slpA*

A nested PCR detecting *tcdB* was performed on DNA extracted from the 160 stool specimens, and 109 (68 %) were found to be positive for *tcdB* by the nested PCR, and *tcdB*-positive *C. difficile* was detected in 86 (79 %) of the 109 stool specimens by culture (Table 3). No specimens negative for PCR detecting *tcdB* but positive for toxic culture were found. The 109 specimens that were PCR positive for *tcdB* were examined by a PCR detecting *slpA*. *slpA* was amplified by a nested PCR and sequenced in 90 (83 %) of the 109 stool specimens tested; of these 90 specimens, *slpA* typing results were obtained in 83 and 7 specimens by primer sets A and B, respectively (Tables 2 and 3). Of the 90 stool specimens in which direct typing results were obtained, 77 specimens were positive for *C. difficile* culture, and typing results from isolated strains agreed with those from direct typing in 76 specimens. In one stool specimen, the typing result on the recovered isolate (*slpA* sequence type og39-01) differed from that by direct typing with primer set A (*slpA* sequence type smz-01). *C. difficile* culture was repeated in the stool specimen, and ten colonies were selected randomly and tested. Of the ten colonies examined for PCR ribotyping, eight and two were typed as PCR ribotype smz and og39, respectively. The *slpA* genes of two of the eight smz isolates and both og39 isolates obtained were sequenced, and PCR ribotype smz and og39 isolates were typed as *slpA* sequence types smz-01 and og39-01, respectively. Second PCRs with primer sets B and C were performed on the PCR product from the first PCR from this stool specimen; *slpA* sequence type og39-01 was obtained by PCR with primer set C. In 13 stool specimens, *C. difficile* was not cultured, but typing results were obtained by direct *slpA* sequence typing (Table 3). Of the 13 stool specimens, 6 were collected after vancomycin treatment had started. No amplification product was produced by PCRs for *slpA* in nine stool specimens that were positive for *C. difficile* culture.

Table 2. Typing results of recovered isolates and direct typing results obtained from 160 stool specimens

slpA sequence type*	GenBank accession no.	PCR ribotype	Toxin production	No. of isolates/no. of stool specimens typed directly						Second primer set for direct typing
				Recovered at hospital:				Other (13†)	Total (160†)	
				A (36†)	B (44†)	C (26†)	D (41†)			
ar-02	AB259785	km0403	$A^+B^+CDT^+$	1/0					1/0	NA‡
cr-02	AB258979	km0429	$A^+B^+CDT^-$	1/1					1/1	A
cr-03	AB258980	g9376	$A^+B^+CDT^+$				1/1		I /1	A
fr-01	AB236153	017§	$A^-B^+CDT^-$					1/1	1/1	A
		trf	$A^-B^+CDT^-$				8/9	2/3	10/12	A
		sgf	$A^-B^+CDT^-$					1/1	1/1	A
gr-01	AB249984	001§	$A^+B^+CDT^-$			2/1	2/2		4/3	A
hr-01	AB231583	014§	$A^+B^+CDT^-$		1/1		3/1		4/2	A
		gc0637	$A^+B^+CDT^-$				2/1		2/1	A
kr-02	AB258982	nt0442	$A^+B^+CDT^-$					1/1	1/1	B
xr-03	AB261625	gc0577	$A^+B^+CDT^-$				1/1		1/1	B
smz-01	AB180242	smz	$A^+B^+CDT^-$	13/14	8/12		9/9‖	1/1	31/36‖	A
smz-02	AB181350	smz	$A^+B^+CDT^-$	9 /13	5/5	1/1			15/19	A
smz-04	AB256018	smz	$A^+B^+CDT^-$			5/5			5/5	A
yok-01	AB240196	002§	$A^+B^+CDT^-$		1/0			2/2	3/2	B
yok-02	AB257283	tk0437	$A^+B^+CDT^-$		1/1				1/1	B
og39-01	AB259787	og39	$A^+B^+CDT^-$				1/1‖		1/1‖	C
t25-01	AB259786	og39	$A^+B^+CDT^-$		1/0				1/0	NA‡
gc11-01	AB258983	gc0578	$A^+B^+CDT^-$				1/1		1/1	B
gc13-01	AB269265	gc0636	$A^+B^+CDT^-$				1/1		1/1	B
gc8-01	AB249986	027§	$A^+B^+CDT^+$				1/1		1/1	A
Total no. isolates/ total no. specimens typed				24/28	17/19	8/7	30/27‖	8/9	87/90‖	

*Isolates were assigned to different *slpA* sequence major types when they had 20 or more amino acid differences, and to subtypes (01, 02, 03 and 04) when they had fewer than 20 such differences.

†No. of stool specimens examined.

‡NA, No PCR products were amplified by any of the second primer sets used.

§The nomenclature of Stubbs *et al.* (1999) was used for PCR ribotypes 017, 001, 014, 002 and 027.

‖In one specimen, two *slpA* sequence types, smz-01 and og39-01, were identified.

Endemic and sporadic types at hospitals in Japan

slpA sequence type smz (subtypes smz-01, smz-02 and smz-04)/PCR ribotype smz isolates were identified in 51 (59 %) of 86 stool specimens from which *tcdB*-positive *C. difficile* was cultured, and *slpA* of type smz was detected in 60 (67 %) of 90 stool specimens in which direct typing results could be obtained. In total, *slpA* sequence type smz was detected by culture and/or direct typing in 61 (62 %) of 99 stool specimens positive for toxic culture and/or direct *slpA* sequence typing. Type smz was found most frequently at each of the four hospitals examined here; two subtypes (smz-01 and smz-02) were predominant at hospitals A and B, and only smz-01 was found at hospital D. *slpA* sequence subtype smz-04 was found to be unique to hospital C. In the present study, eight patients suffered from *C. difficile* infection (CDI) caused by A^-B^+ isolates at hospital D, which were all typed into *slpA* sequence type fr-01/PCR ribotype trf. Among the A^-B^+ isolates examined in this study, one isolate of PCR ribotype 017 and one isolate of PCR ribotype sgf were recovered from patients admitted to the same hospital in 2004 and 2007, respectively. Both patients suffered from CDI with severe complications; the patient with the PCR ribotype 017 isolate died of CDI and the patient with PCR ribotype sgf survived after emergency colectomy. *slpA* sequence types gr, hr and yok were recovered from sporadic cases but were not predominant at any of the hospitals examined in this study. One $A^+B^+CDT^+$ isolate was found to be *slpA* sequence type gc8-01/PCR ribotype 027 in this study (Kato *et al.*, 2007). No isolates with the *slpA* gene identical to that of PCR ribotype 078 (Goorhuis *et al.*, 2008) were found among the 87 isolates tested.

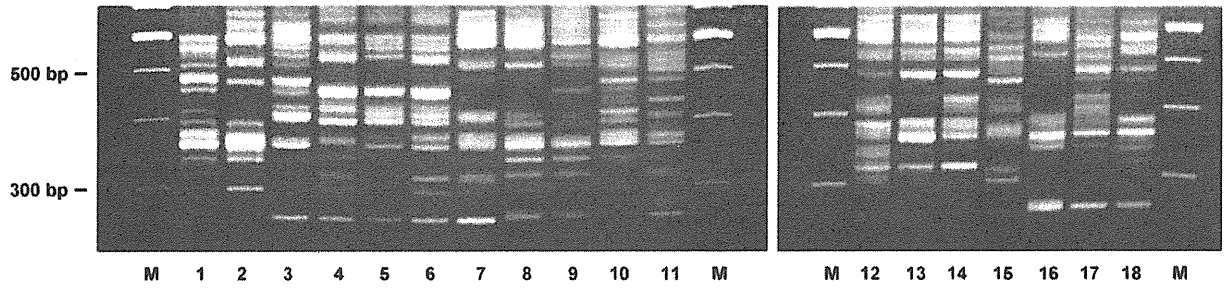

Fig. 1. PCR ribotype patterns of *C. difficile* isolates representing 18 different ribotypes. Lanes: 1, PCR ribotype km0403; 2, km0429; 3, g9376; 4, 017; 5, trf; 6, sgf; 7, 001; 8, 014; 9, gc0637; 10, nt0442; 11, gc0577; 12, smz; 13, 002; 14, tk0437; 15, 027; 16, gc0578; 17, gc0636; 18, og39; M, 100 bp ladder.

DISCUSSION

slpA sequence typing was found to have reliable typability and discriminatory power in comparison with PCR ribotyping. To date, 24 different *slpA* sequence major types and 56 subtypes have been found among *tcdB*-positive isolates (data not shown). Typing results by *slpA* sequence typing exhibited a high concordance with those by PCR ribotyping with the exception of two isolates, which belonged to the same PCR ribotype but were classified as different major types, suggesting a high variability of the *slpA* gene in this PCR ribotype. However, the limitations of this study include the low number of strains examined for each *slpA* sequence type and PCR ribotype except for types smz and fr, so further analysis using more clinical isolates is warranted to compare the typing results of the two systems.

A definite advantage of sequence-based typing techniques is the transportability of typing results from laboratory to laboratory. When an increase in the incidence of CDI or in the severity of CDI cases is perceived, or one type is found to spread to multiple patients in a healthcare facility or an area, it is prudent to ascertain whether the epidemic or endemic strain corresponds to one of the hypervirulent strains already reported worldwide (Barbut *et al.*, 2007; Goorhuis *et al.*, 2008; McDonald *et al.*, 2005; Warny *et al.*, 2005). Especially in areas or countries where there is no experience of isolating the above-mentioned strains, identification of specific strains by typing systems, which depend on banding pattern analysis such as PCR ribotyping or PFGE analysis, is difficult without obtaining previously characterized reference strains. However, if the sequence results have been registered previously in the database, obtaining reference strains for comparison is unnecessary. Although *slpA* sequence typing requires sequencing steps, once the typing results are obtained, it is easier and more objective to compare typing results of tested strains with those typed previously without repeating experiments. On this point, typing by sequencing analysis is not always time-consuming.

Table 3. Comparison of results of *tcdB*$^+$ *C. difficile* culture and direct PCR for the detection of *tcdB* and *slpA* in 160 stool specimens

tcdB$^+$ *C. difficile* culture	Direct PCR for:		No. of stool specimens (n=160)
	tcdB	*slpA*	
+	+	+	77
+	+	−	9
−	+	+	13
−	+	−	10
−	−	ND	51

ND, Not done.

slpA sequence typing was applied successfully to direct typing. Of 13 stool specimens from which *C. difficile* was not cultured but typing results were obtained by direct *slpA* sequence typing, six were collected after vancomycin treatment had started; vancomycin in the stool specimens might inhibit the growth of *C. difficile*. *slpA* sequence typing could be used to analyse *C. difficile* in some stool specimens that are inadequate for culture, such as those obtained after starting treatment with vancomycin or metronidazole. Direct typing by sequencing of *slpA* at present has the limitation of the tedious steps required. As this typing method depends on the variability of the *slpA* gene, three sets of primers for the second PCR are needed to amplify the variable region of the gene, which complicates the procedure. Although the procedure must be simplified for practical use, the method could be valuable for detecting epidemiologically important strains.

Toxin B-positive *C. difficile* was recovered from 86 (54%) of 160 stool specimens tested. The stool specimens tested in this study were obtained from patients who had symptoms that were considered severe and so the samples were sent to the National Institute of Infectious Diseases for further analysis. This is the major reason for the high prevalence.

The type smz strain has been documented to cause healthcare-associated infection in many hospitals in Japan

(Kato *et al.*, 2001, 2005a; Sawabe *et al.*, 2007). Killgore *et al.* (2008) tested 42 *C. difficile* isolates from four countries in North America and Europe using seven techniques including *slpA* sequence typing, and none was identified as *slpA* sequence type smz. In another report, only 3 of 33 isolates recovered from an outbreak in the USA were type smz (Kato *et al.*, 2001, 2005a). Joost *et al.* (2009) examined the *slpA* sequences of their *C. difficile* isolates and compared the sequence results with those registered in the database; they found type smz in only 3 % of isolates recovered from CDI patients at a university hospital in German. Whilst these limited studies suggest that type smz is not frequently isolated in North America or Europe, the real prevalence is unknown. *slpA* sequence typing could be useful for studying the distribution of the smz strain worldwide.

A^-B^+ *C. difficile* has been reported to cause nosocomial outbreaks (Komatsu *et al.*, 2003; Kuijper *et al.*, 2001) and to be predominant in Ireland, Poland (Barbut *et al.*, 2007; Pituch *et al.*, 2007) and Korea (Kim *et al.*, 2008), as well as in Japan (Rupnik *et al.*, 2003), indicating the emergence of CDI caused by A^-B^+ *C. difficile*. As far as we know, A^-B^+ isolates that have 1.8 kbp deletions in the repeating sequences of *tcdA* (Kato *et al.*, 1999), including the ATCC 43598 strain, are all typed into the *slpA* sequence major type fr together with exceptional isolates that belonged to serogroup X (data not shown). Notably, A^-B^+ *C. difficile* was isolated from two patients representing two sporadic cases with severe complications, although no specific characteristics were found in these isolates. In the present study, 1 isolate was identified as PCR ribotype 027; none of the 87 isolates tested was PCR ribotype 078. To date, we have found only sporadic CDI cases caused by *slpA* sequence type gc8/PCR ribotype 027 isolates in Japan, which were historic isolates (Kato *et al.*, 2007; Sawabe *et al.*, 2007). It has been documented that PCR ribotype 078 more frequently causes community-associated diseases (Goorhuis *et al.*, 2008). All patients examined in this study had healthcare-associated infection, which might be one of the reasons why CDI caused by PCR ribotype 078 was not found. Typing by sequencing of *slpA* could be a reliable tool for discovering CDI cases and outbreaks due to these epidemic strains, which may have been overlooked.

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