Target SNPs were selected upon the satisfaction of the following two conditions: (1) the SNP is located in TLR-2, TLR-4, or TLR-9 and its minor genotype frequency is >5% in the databases for the Japanese general population to allow statistical analysis, and (2) association of the SNP with the severity of viral disease or antiviral responses has been reported previously. Only five SNPs in TLR-2, nine in TLR-4, and four in TLR-9 have minor genotypes at frequencies of more than 5% in the Japanese population. All of these 18 SNPs locate in the introns, in the untranslated regions, or as synonymous alterations in open reading frames (ORFs). We analyzed the following five SNPs: rs3804100 and rs1898830 in TLR-2, rs11536889 in TLR-4, and rs352139 and rs352140 in TLR-9, all of which have been reported to be associated with the severity of viral disease or antiviral responses.[24–28] The frequencies of genotypes of each SNP in the general Japanese population (group A, Figure 1, $n = 83–86$ for TLR-2 and TLR-9, $n = 42$ for TLR-4) were obtained from the National Center for Biotechnology Information dbSNP and HapMap databases (http://www.ncbi.nlm.nih.gov/projects/SNP/). Since individuals in the general Japanese population in the database were recruited in Tokyo, to where people move from most areas of Japan, the database can be considered to reflect the general Japanese population.

Polymorphic sites in each DNA specimen were analyzed by TaqMan allelic discrimination assay. Primers and probes listed in the TaqMan SNP Genotyping Assays (Applied Biosystems) were used for the analysis. Thermal cycling conditions were 2 min at 50 °C, 10 min at 95 °C, and 40 cycles each of 15 s at 92 °C and 1 min at 60 °C. Accuracy of genotyping was confirmed by inclusion of control plasmids. The control plasmids were prepared as follows. DNA fragments of 0.8–1.3 kb containing at least one of the targeted SNP sites were amplified by PCR of genomic DNA and cloned into pBluescriptSKII(+). The control plasmids containing minor alleles were produced by oligonucleotide-based mutagenesis using a commercial kit (Stratagene).

2.4. Statistical analyses

Hardy–Weinberg equilibrium, linkage disequilibrium analysis, and haplotype analysis were performed using SNPStats software (http://bioinfo.iconcologia.net/en/SNPStats_web). To analyze the associations between target gene SNPs and congenital CMV infection, we compared the genotypes of all cases in our cohort (group B) to those of the general Japanese population in the databases (group A). To analyze the associations of target gene SNPs with congenital CMV disease, we compared the genotypes between two groups in the following combinations: (1) group C vs. group D, (2) group E vs. group F, and (3) group G vs. group H (Figure 1). Genotype and allelic frequencies of all five SNPs were in Hardy–Weinberg equilibrium among each group (data not shown). Differences in the prevalence of genotyped polymorphisms between two groups were estimated by odds ratios (ORs), 95% confidence intervals (95% CI), and p-values. ORs and 95% CI were calculated for each group compared to the reference class (homozygous genotype for the prevalent allele). p-Values were derived from the Chi-square test for 2×2 or 2×3 tables. A p-value of <0.05 was considered to be

Table 1
Associations between target gene SNPs and congenital CMV infection

Gene	rs number[a] (region)	Genetic model	Genotype	General population (A[b]) or congenital CMV infection (B[b])			
				A (%)	B (%)	OR (95% CI)	p-Value[c]
TLR-2	rs1898830	Codominant	AA	19.7	34.5	1	NS
	(Intron 1)		AG	62.8	47.1	0.43 (0.21–0.88)	
			GG	17.4	18.4	0.60 (0.24–1.52)	
		Dominant	AA	19.7	34.5	1	0.030
			AG + GG	80.2	65.5	0.47 (0.23–0.93)	
		Recessive	AA + AG	82.6	81.6	1	NS
			GG	17.4	18.4	1.07 (0.49–2.32)	
	rs3804100	Codominant	TT	57.8	42.5	1	0.015[d]
	(Exon 3)		TC	41	47.1	1.56 (0.84–2.92)	
	No amino acid change		CC	1.2	10.3	11.7 (1.42–96.3)	
		Dominant	TT	57.8	42.5	1	0.046
			TC + CC	42.2	57.5	1.85 (1.01–3.41)	
		Recessive	TT + TC	98.8	89.7	1	0.011
			CC	1.2	10.3	9.46 (1.17–76.4)	
TLR-4	rs11536889	Codominant	GG	57.1	51.7	1	NS
	(3′ UTR)		GC	35.7	40.2	1.24 (0.57–2.72)	
			CC	7.1	8	1.24 (0.29–5.25)	
		Dominant	GG	57.1	51.7	1	NS
			GC + CC	42.9	48.3	1.24 (0.59–2.61)	
		Recessive	GG + GC	92.9	92	1	NS
			CC	7.1	8	1.14 (0.28–4.64)	
TLR-9	rs352139	Codominant	AA	29.1	25.3	1	NS
	(Intron 1)		AG	43	54	1.44 (0.70–2.96)	
			GG	27.9	20.7	0.85 (0.37–1.97)	
		Dominant	AA	29.1	25.3	1	NS
			AG + GG	70.9	74.7	1.21 (0.62–2.37)	
		Recessive	AA + AG	72.1	79.3	1	NS
			GG	27.9	20.7	0.67 (0.33–1.36)	
	rs352140	Codominant	GG	29.1	25.3	1	NS
	(Exon 2)		GA	43	54	1.44 (0.70–2.96)	
	No amino acid change		AA	27.9	20.7	0.85 (0.37–1.97)	
		Dominant	GG	29.1	25.3	1	NS
			GA + AA	70.9	74.7	1.21 (0.62–2.37)	
		Recessive	GG + GA	72.1	79.3	1	NS
			AA	27.9	20.7	0.67 (0.33–1.36)	

SNP, single nucleotide polymorphism; CMV, cytomegalovirus; OR, odds ratio; CI, confidence interval; NS, not significant; UTR, untranslated region.
[a] Public database (dbSNP) reference number.
[b] Characteristics of each group are shown in Figure 1.
[c] p-Values were derived from the Chi-square test for 2×2 or 2×3 tables. A p-value of <0.05 was considered to be statistically significant.
[d] $p = 0.045$ after Bonferroni multiple correction.

statistically significant. To account for multiple testing, Bonferroni multiple correction of *p*-values was applied.

3. Results

3.1. Associations between target gene SNPs and congenital CMV infection

The homozygous CC genotype of SNP rs3804100 in TLR-2 was identified in children with congenital CMV infection at a significantly higher frequency in all considered genetic models (OR 11.7, 95% CI 1.42–96.3, $p = 0.015$ in the codominant genetic model) (Table 1). The heterozygous AG genotype at the rs1898830 locus in TLR-2 tended to be identified in children with congenital CMV infection at a lower frequency, in other words the frequency of the homozygous AA genotype in children with congenital CMV infection was higher than that in the general population (OR 0.43, 95% CI 0.21–0.88 in the codominant genetic model; OR 0.47, 95% CI 0.23–0.93, $p = 0.030$ in the dominant genetic model). The analysis of these two SNPs in the

Table 2
Associations between target gene SNPs and congenital CMV disease

Gene	rs number[a] (region)	Genetic model	Genotype	Asymptomatic (C[b]) or symptomatic (D[b]) at birth			Asymptomatic (E[b]) or symptomatic (F[b])			Without (G[b]) or with (H[b]) late-onset symptoms		
				C (%)	D (%)	OR (95% CI)	E (%)	F (%)	OR (95% CI)	G (%)	H (%)	OR (95% CI)
TLR-2	rs1898830 (Intron 1)	Codominant	AA	31.6	40.0	Ref.	29.0	37.5	Ref.	33.3	28.6	Ref.
			AG	47.4	46.7	0.78 (0.29–2.06)	45.2	48.2	0.83 (0.30–2.28)	44.4	52.4	1.37 (0.40–4.77)
			GG	21.1	13.3	0.50 (0.13–1.92)	25.8	14.3	0.43 (0.12–1.50)	22.2	19.1	1.00 (0.21–4.71)
		Dominant	AA	31.6	40.0	Ref.	29.0	37.5	Ref.	33.3	28.6	Ref.
			AG+GG	68.4	60.0	0.69 (0.28–1.74)	71.0	62.5	0.68 (0.26–1.76)	66.7	71.4	1.25 (0.39–4.04)
		Recessive	AA+AG	79.0	86.7	Ref.	74.2	85.7	Ref.	77.8	81.0	Ref.
			GG	21.1	13.3	0.58 (0.17–1.97)	25.8	14.3	0.48 (0.16–1.44)	22.2	19.1	0.82 (0.21–3.15)
	rs3804100 (Exon 3)	Codominant	TT	36.8	53.3	Ref.	41.9	42.9	Ref.	36.1	38.1	Ref.
			TC	54.4	33.3	0.42 (0.16–1.11)	51.6	44.6	0.85 (0.34–2.13)	55.6	52.4	0.89 (0.28–2.82)
	No amino acid change		CC	8.8	13.3	1.05 (0.24–4.55)	6.5	12.5	1.90 (0.34–10.49)	8.3	9.5	1.08 (0.15–7.96)
		Dominant	TT	36.8	53.3	Ref.	41.9	42.9	Ref.	36.1	38.1	Ref.
			TC+CC	63.2	46.7	0.51 (0.21–1.25)	58.1	57.1	0.96 (0.40–2.34)	63.9	61.9	0.92 (0.30–2.79)
		Recessive	TT+TC	91.2	86.7	Ref.	93.5	87.5	Ref.	91.7	90.5	Ref.
			CC	8.8	13.3	1.60 (0.40–6.47)	6.5	12.5	2.07 (0.40–10.65)	8.3	9.5	1.16 (0.18–7.56)
TLR-4	rs11536889 (3′ UTR)	Codominant	GG	56.1	43.3	Ref.	64.5	44.6	Ref.	62.9	42.9	Ref.
			GC	35.1	50.0	1.85 (0.73–4.68)	25.8	48.2	2.70 (1.01–7.22)	25.7	52.4	2.99 (0.92–9.66)
			CC	8.8	6.7	0.98 (0.17–5.73)	9.7	7.1	1.07 (0.21–5.33)	11.4	4.8	0.61 (0.06–6.25)
		Dominant	GG	56.1	43.3	Ref.	64.5	44.6	Ref.	62.9	42.9	Ref.
			GC+CC	43.9	56.7	1.67 (0.69–4.08)	35.5	55.4	2.25 (0.91–5.57)	37.1	57.1	2.26 (0.75–6.80)
		Recessive	GG+GC	91.2	93.3	Ref.	90.3	92.9	Ref.	88.6	95.2	Ref.
			CC	8.8	6.7	0.74 (0.14–4.08)	9.7	7.1	0.72 (0.15–3.44)	11.4	4.8	0.39 (0.04–3.72)
TLR-9	rs352139 (Intron 1)	Codominant	AA	24.6	26.7	Ref.	19.4	28.6	Ref.	22.3	28.6	Ref.
			AG	54.4	53.3	0.90 (0.31–2.60)	61.3	50.0	0.55 (0.18–1.67)	61.6	42.9	0.55 (0.15–2.03)
			GG	21.1	20.0	0.88 (0.24–3.24)	19.4	21.4	0.75 (0.19–2.91)	16.7	28.6	1.33 (0.28–6.28)
		Dominant	AA	24.6	26.7	Ref.	19.4	28.6	Ref.	22.2	28.6	Ref.
			AG+GG	75.4	73.3	0.90 (0.33–2.46)	80.7	71.4	0.60 (0.21–1.74)	77.8	71.4	0.71 (0.21–2.44)
		Recessive	AA+AG	79.0	80.0	Ref.	80.7	78.6	Ref.	83.3	71.4	Ref.
			GG	21.1	20.0	0.94 (0.31–2.81)	19.4	21.4	1.14 (0.38–3.40)	16.7	28.6	2.00 (0.55–7.27)
	rs352140 (Exon 2)	Codominant	GG	24.6	26.7	Ref.	19.4	28.6	Ref.	23.1	27.8	Ref.
			GA	54.4	53.3	0.90 (0.31–2.60)	61.3	50.0	0.55 (0.18–1.67)	61.5	38.9	0.55 (0.15–2.03)
	No amino acid change		AA	21.1	20.0	0.88 (0.24–3.24)	19.4	21.4	0.75 (0.19–2.91)	15.4	33.3	1.33 (0.28–6.28)
		Dominant	GG	24.6	26.7	Ref.	19.4	28.6	Ref.	23.1	27.8	Ref.
			GA+AA	75.4	73.3	0.90 (0.33–2.46)	80.7	71.4	0.60 (0.21–1.74)	76.9	72.2	0.71 (0.21–2.44)
		Recessive	GG+GA	79.0	80.0	Ref.	80.7	78.6	Ref.	84.6	66.7	Ref.
			AA	21.1	20.0	0.94 (0.31–2.81)	19.4	21.4	1.14 (0.38–3.40)	15.4	33.3	2.00 (0.55–7.27)

SNP, single nucleotide polymorphism; CMV, cytomegalovirus; OR, odds ratio; CI, confidence interval; UTR, untranslated region.
Note: No *p*-values were significant (data not shown).
[a] Public database (dbSNP) reference number.
[b] Characteristics of each group are shown in Figure 1.

48 cases who were identified in our screening program resulted in similar findings (data not shown).

There were no significant differences in prevalence of genotypes in SNPs rs11536889 in TLR-4 and rs352139 and rs352140 in TLR-9 between groups A and B.

3.2. Absence of any association of SNPs with congenital CMV disease

As shown in Table 2, no statistically significant associations were observed for any SNPs between two groups in the following combinations: (1) group C vs. group D, (2) group E vs. group F, and (3) group G vs. group H. Inclusion of newborns with only IUGR or with any single, non-pathognomonic manifestation into groups D or F did not alter the overall results (data not shown). Further, no haplotypes were associated with congenital CMV disease.

4. Discussion

In this study, we found that the frequencies of SNPs in TLR-2, rs3804100 and rs1898830, in children with congenital CMV infection differed from those in the general Japanese population and that there were no significant associations between SNPs in the analyzed TLR genes and congenital CMV disease. Similar results in the 48 cases identified in our screening program exclude a possibility that selection bias due to enrichment with symptomatic infants affected the association, since the screening population can represent the Japanese general population.[4] A potential drawback of our study is that we could not analyze SNPs in the general population completely matched with the congenital cases, since the ethics committee restricted our genetic tests to congenital cases. However, it is unlikely that there is a particular bias in the genetic polymorphisms in the general Japanese population, since the population in the database is comprised of individuals living in Tokyo, the melting pot of Japan, and since the Japanese population is believed to be homogeneous already. It would be important to analyze more SNP loci in TLR-2 to confirm our findings. An additional limitation of our study includes the fact that the timing and procedures for the neuroimaging examinations were not uniform and thus we could not predict their effects on our analysis; this might affect the statistical analysis comparing group E to group F and group G to group H. Nevertheless, this limitation did not affect our findings with regard to the difference between groups A and B.

Maternal CMV infection does not always cause fetal infection; for example, the transmission rate is expected to be around 40% in mothers with primary infection,[3] although the rates in mothers with reinfection or with reactivation are unclear. Therefore, we hypothesize that polymorphisms in TLR-2 could be the risk factors for viral transmission at least from primary-infected mothers to the fetus. Although we were also interested in the polymorphisms in mothers, the material available for this purpose was insufficient to allow the design of a statistically meaningful study.

The frequency of the TT genotype of TLR-2 SNP rs3804100 in responders to hepatitis B vaccine was found to be significantly higher than that in non-responders.[24] The major alleles of SNP rs3804100 in TLR-2 have been associated with a higher antibody level in individuals with measles vaccine.[25] SNP rs1898830 appeared to be involved in viral shedding and the lesion rate in patients with genital herpes simplex virus type 2 infection.[26] Hence, these two SNPs might be associated with TLR-2 gene expression, which may affect the level of innate immunity against viral infections in some way, in spite of the absence of any changes in the amino acid sequences. There are many examples showing the associations of polymorphisms located outside of ORFs. Several studies have even reported that synonymous SNPs influence the splicing process,[29,30] post-transcriptional regulation,[31] mRNA stability,[32,33] translation efficiency, and protein folding.[34] However, it is difficult to exclude a possibility that the TLR-2 SNPs are simply genetic markers for other polymorphisms that link directly to the risk factors for transmission. Further studies are required to investigate the biological significance of the synonymous SNPs for which we identified the abovementioned differences.

A relationship between TLR-4 rs11536889 and protection from hepatitis B virus recurrence after liver transplantation has been reported.[27] Also some TLR-4 SNPs might be related to CMV disease in renal transplantation recipients.[15] The haplotypes of TLR-9 rs352139 and rs352140 may influence mother-to-child transmission of HIV,[28] and another SNP in TLR-9 was found to be highly predictive of susceptibility to CMV infection in allogeneic stem cell transplantation patients.[16] However, we did not find any significant associations between the SNPs in TLR-4 and TLR-9 and congenital CMV infection or disease.

The identification of high-risk groups might make treatment with antiviral agents, such as ganciclovir,[35] a more viable option in the treatment of asymptomatic infants. Further studies on polymorphisms in additional genes associated with the immune systems may provide a means of identifying high-risk groups. The analysis of polymorphisms not only in infected newborns but also in their mothers may be important for the identification of risk factors.

In conclusion, TLR-2 polymorphisms may have some association with congenital CMV infection, although the mechanism underlying this effect remains to be clarified.

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Short communication

Cytomegalovirus (CMV) glycoprotein H-based serological analysis in Japanese healthy pregnant women, and in neonates with congenital CMV infection and their mothers

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ABSTRACT

Background: Congenital cytomegalovirus (CMV) infection is caused by maternal primary infection as well as CMV reinfection or reactivation during pregnancy, although differences in the clinical impact between these modes of infection remain to be clarified.
Objectives: To investigate the latest prevalence and risk of multiple CMV infection in healthy pregnant women, as well as the types of maternal CMV infection associated with congenital CMV infection.
Study design: Seroprevalence against CMV and IgG subclasses were determined in 344 serum samples from healthy pregnant women in Japan. CMV genotype and serotype were also determined in 18 pairs of mothers and neonates with congenital CMV infection identified in our CMV screening program.
Results: Thirty-two percent of the pregnant women were seronegative, while 66% of CMV seropositive women had IgG3 antibodies against one epitope on glycoprotein H (gH) as the major subclass, and 52% had IgG1 antibodies against one epitope on glycoprotein B (gB). Only a single genotype determined by CMV gH neutralizing epitope was found in the urine from the 18 neonates with congenital CMV infection, even though one case possessed antibodies against multiple CMV strains. In that case, the antibodies against the strain not detected in the urine from the infant disappeared within one month after birth, whereas the antibodies against the infecting CMV strain continued to be detected at 12 months after birth.
Conclusions: Two (11%) of 18 cases of congenital CMV infection occurred via maternal CMV reinfection. Maternal humoral immunity did not prevent congenital CMV infection with another gH subtype.

1. Background

Cytomegalovirus (CMV) primary infection during pregnancy can lead to congenital CMV infection to fetuses [1], result in severe clinical complications [2] or defects like hearing loss [3–7]. Congenital CMV infection is also caused by multiple CMV infections during pregnancy [8]. Multiple strains of CMV are known to infect humans [9–17]. One reason is that CMV evades from CD8+ T cells [18]. Another reason is that the neutralizing antibodies against the primary infection are not sufficient to protect against infection with another CMV strain [19,20]. CMV can be classified into at least two serotypes, AD169 type (AD) and Towne type (To), based on polymorphisms in the glycoprotein H (gH) neutralizing epitope, useful for the identification of the history of CMV infection in

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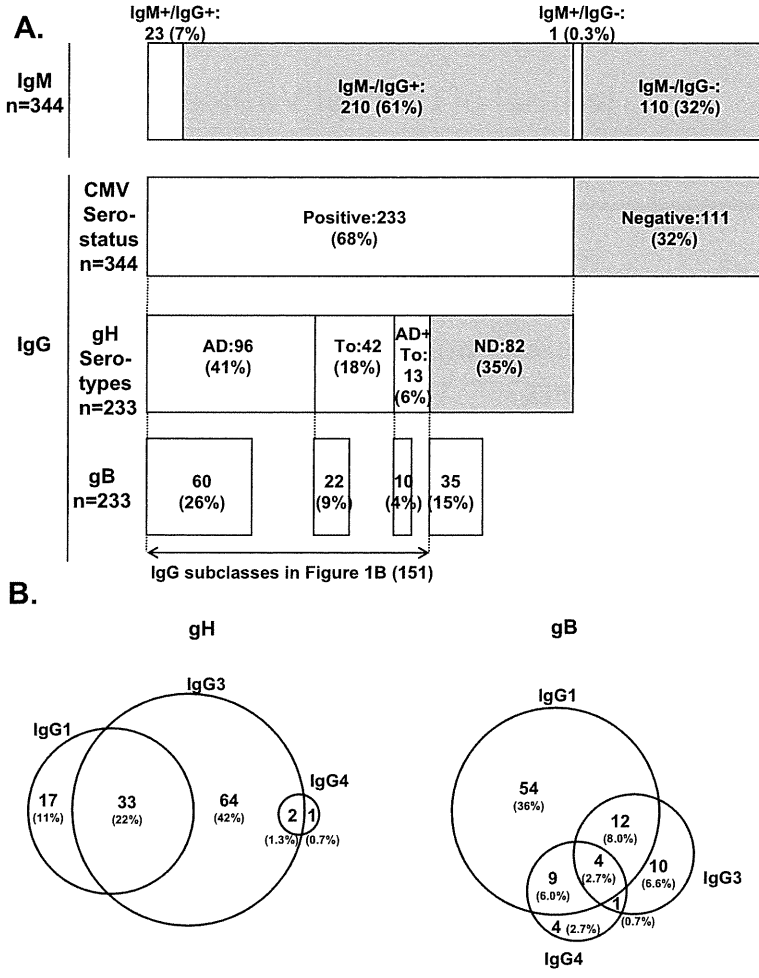

Fig. 1. Seroprevalence of CMV in the middle stage of pregnancy in healthy women. (A) CMV antibodies were screened using serum samples from women in the 2nd trimester of pregnancy. CMV-specific IgG or IgM antibodies were detected using commercial kits. The CMV serotype was determined by serotype-specific ELISA analysis targeting the CMV gH region. AD, AD169 strain; To, Towne strain; ND, not determined; Open, positive or determined; close, negative or undetermined. (B) Distribution of CMV IgG subclasses was determined by ELISA analysis targeting the CMV gH or gB regions.

transplantation patients [21] as well as in congenital CMV cases [2,22]. In this study, we analyzed the serological status of healthy pregnant women, and neonates with congenital CMV infection and their mothers to better understand the mode of transmission in cases of fetal CMV infection.

2. Objective

To investigate the prevalence and risk of maternal CMV primary infection and reinfection during pregnancy, based on serological analyses of pregnant healthy women, and pairs of neonates with congenital CMV infection and their mothers.

3. Study design

3.1. Specimens

Sera from 344 healthy women in the 2nd trimester of pregnancy were collected at the National Center for Child Health and Development. Sera from 18 cases of congenital CMV infection, identified in the screening program [23], were also collected within a month after identification. Case #20026 presented with hearing loss and was treated with oral valganciclovir for 6 weeks from 5 months of age [24]. Case #19389 had normal hearing capability at 6 months, but hearing loss was observed at one year. DNA was isolated from urine specimens using a QIAamp DNA mini kit (Qiagen, Valencia, CA) according to the manufacturer's protocol.

3.2. CMV-specific serological status

CMV serostatus was determined using commercial ELISA kits (Enzygnost Anti-CMV IgM and IgG, Siemens, Munich, Germany) and ELISA using the gB epitope [21]. For serotype-specific anti-CMV IgG, gH epitopes from the AD and To strains were used for our previously established ELISA method [21]. We modified the serotype-specific ELISA by using HRP-conjugated anti-human IgG1, IgG2, IgG3 and IgG4 antibodies (Beckman Coulter Inc., Fullerton, CA) to determine the subclasses of anti-CMV IgG for gH and gB.

3.3. Determination of the CMV gH genotype by DNA analysis

The nucleotide sequences of the CMV gH gene from neonates were determined by fluorescent dye-terminator sequencing and CMV genotype-specific real-time quantitative PCR (qPCR) as described previously [22,25].

Table 1
CMV serotypes and genotypes in neonates with congenital CMV infection and their mothers.

ID		IgG			IgM	AI (%)	CMV in urine
		CMV	AD	To	CMV		
13518	mother	+	+	–	+	47.7 (M)	
	neonate	+	+	–	–		AD
19389[a]	mother	+	+	–	+	85.7 (H)	
	neonate	+	+	–	+		AD
20040	mother	+	+	–	+	56.1 (H)	
	neonate	+	+	–	–		AD
31694	Mother	+	+	–	+	22.8 (L)	
	Neonate	+	+	–	+/–		AD
20026[a]	mother	+	+	–	+/–	81.4 (H)	
	neonate	+	+	–	+		AD
82718	mother	+	+	–	+/–	77.6 (H)	
	neonate	+	+	–	–		AD
83641	mother	+	+	–	+/–	80.2 (H)	
	neonate	+	+	–	–		AD
20270	mother	+	+	–	–	56.6 (H)	
	neonate	+	+	–	+		AD
21558	mother	+	+	–	–	74.1 (H)	
	neonate	+	+	–	–		AD
22986	mother	+	+	–	+/–	41.2 (M)	
	neonate	+	+	–	+		AD
72119	mother	+	–	–	–	60.2 (H)	
	neonate	+	–	–	–		AD
80654	mother	+	+	–	–	63.0 (H)	
	neonate	+	–	–	–		AD
22383	mother	+	+	+	+/–	88.9 (H)	
	neonate	+	+	+	–		AD
71306	mother	+	+	+	–	72.4 (H)	
	neonate	+	–	+	–		To
19382	mother	+	–	+	+/–	63.5 (M)	
	neonate	+	–	+	+/–		To
20117	mother	+	–	+	–	73.8 (H)	
	neonate	+	–	+	–		To
18189	mother	+	–	+	–	68.9 (H)	
	neonate	+	–	+	+/–		To
72072	mother	+	–	+	–	70.7 (H)	
	neonate	+	–	+	+		To

The CMV serotype was determined by serotype-specific ELISA analysis. The genotypes of CMV DNA in the urine were determined by direct sequencing and CMV genotype-specific qPCR. All analyses targeted the CMV gH region. AD, AD169 strain; To, Towne strain in CMV genotypes. All samples were diagnosed in reference #23. The clinical details of case #20026 were shown in reference #24. CMV genotypes in the urine from case #20040, 20026, 20270, 22383, 19382, 20117 and 18189 were shown in reference #22. AI, IgG avidity index; L, low (less than 30%); M, mid-range (30–50%); H, high (more than 50%) in AI; according to reference #26.

[a] Symptomatic infection.

3.4. CMV IgG avidity index (AI) test

The antibody avidity index test was performed using a commercial CMV ELISA kit (Enzygnost Anti-CMV IgG, Siemens) by the urea denaturation procedure as described previously [26].

4. Results

4.1. Seroprevalence of CMV and distribution of IgG subclasses against CMV gH or gB epitopes in healthy pregnant women in Japan

To investigate the risk of congenital CMV infection, seroprevalence among pregnant Japanese women was determined. CMV IgM and IgG antibodies were detected in 7% and 68% of the 344 subjects, respectively. None of the 24 cases with IgM had multiple infections. To avoid false-seronegative results among individuals, we also detected antibodies against CMV glycoprotein B (gB), were detected in 43% of serotype-undetermined 82 individuals (Fig. 1A).

Maternal CMV antibodies acquired prior to conception can decrease the frequency of transplacental CMV transmission [27]. The ratio of serum levels of each subclass (IgG1:IgG2:IgG3:IgG4) was 9:3:1:0.5 [28]. However, both IgG1 and IgG3 against CMV had a major neutralizing capacity [29,30], and showed an epitope-specific distribution of IgG subclasses against the antigenic domains on CMV [31]. IgG1 and 3 were the major subclasses against the gB and gH epitope in 151 women, respectively (Fig. 1B).

4.2. CMV serotypes in neonates with congenital CMV infection and their mothers

Identical serotypes in the mother and neonate were observed in 14 pairs of mothers and their babies (Table 1). The genotypes of CMV obtained from urine specimens of those 14 neonates were consistent with the serological results. One mother and her neonate had antibodies against both serotypes, although the neonate excreted AD-genotype CMV in its urine (#22383). Only the mother had antibodies against both serotypes (#71306).

4.3. Disappearance of maternal CMV antibodies in neonates with congenital CMV infection

The To-type antibodies disappeared within one month in case #22383, without To genotype CMV in the urine specimens at any time (data not shown). CMV IgG against the AD type remained at detectable levels in case #20026 until 12 months after birth (Table 2).

Table 2
Time course of AD/To CMV antibodies and virus copy numbers in urine. The CMV serotype-specific antibodies were titrated by ELISA at different periods. The CMV copy numbers were determined by qPCR analysis (copies/μl urine).

#22383						
Months after birth		Mother	Neonate			
		0	0	1	6	12
IgG	AD gH	+	+	+	+	+
	To gH	+	+	–	–	–
DNA	CMV	NT	7.7×10^4	1.4×10^5	1.9×10^4	1.6×10^4
#20026						
Months after birth		Mother	Neonate			
		0	0	1	6[a]	12
IgG	AD gH	+	+	+	+	+
	To gH	–	–	–	–	–
DNA	CMV	NT	4.1×10^4	4.1×10^4	2.2×10^2	3.5×10^1

AD, AD169 strain; To, Towne strain; NT, not tested.
[a] Valganciclovir-treatment [24].

4.4. A mid-range or high AI was detected in most mothers of neonates with congenital CMV infection

The AI test was performed using serum samples from 18 mothers of neonates with congenital CMV infection (Table 1). One (#31694), 3 and 14 case(s) had low, mid-range and high AI, respectively.

5. Discussion

The 32% of pregnant Japanese women have no antibodies against CMV (Fig. 1A), similar to previous reports [32–36]. They face the risk of primary CMV infection during pregnancy. The antibodies against the limited epitope regions of CMV gB and gH showed a preference for IgG1 and IgG3, respectively (Fig. 1B). IgG1 and IgG3 antibodies against CMV have a neutralization capacity [29,30,37], and can be transferred through the placenta to protect the fetus.

The CMV AD and To serotypes were determined by polymorphisms in the CMV gH neutralizing region [8,37,38]. We found that 6% of women had both serotypes. Infection with multiple genotypes of CMV can occur in immunocompetent individuals [10–16], result of reinfection [17]. Primary infection with mixed CMV strains must be infrequent event [39]. CMV reinfection can occur because the neutralizing antibodies are basically serotype-specific [8,19]. Thus, the maternal CMV antibody is not always able to protect the fetus, particularly in the case of reinfection with different CMV serotypes. CMV intrauterine infection is not a rare event in a highly cytomegalovirus-immune population [40–45]. We identified 2 cases (#22383 and #71306, 11%) of multiple serotypes in 18 neonates with congenital CMV infection. Low avidity, indicating primary infection, was confirmed only in case #31694 (Table 1). For the cases with mid-range or high avidity, we could not rule out the possibility of primary infection at the early phase of pregnancy. In case #22383, the antibodies against To disappeared at 1 month after birth (Table 2). The maternal antibody titer against the primary infecting serotype was much lower than that for recently reinfecting serotype that induced the congenital infection. CMV subtype, distinguished by the gH epitope, plays a sero-immunological role in CMV infection. Our gH genotype-specific quantitative real-time PCR, that is able to detect even 100-fold lower minor CMV [22], could find only a single CMV gH genotype in the urine samples. It is likely that infection with the other CMV gH genotype, to which the mother had never been exposed (i.e., AD in case #22383), occurred during pregnancy.

6. Conclusion

The maternal antibodies against CMV are not sufficient to protect the fetus against CMV infection in cases of multiple infections with different CMV strains. The maternal antibodies against the strain that was not detected in the urine from the infant of congenital CMV infection disappeared within one month after birth. We also found that the antibodies against the CMV gH neutralizing epitope were mainly from the IgG3 subclass, which can be transmitted through the placenta to the fetus. Research and development of a CMV vaccine targeting neutralizing regions, such as gH, might be possible not only for seronegative but also for seropositive women of reproductive age to allow active immunization with the aim of preventing congenital defects caused by primary CMV infection or reinfection during pregnancy.

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Competing interests

The authors have no competing interests.

Ethical approval

The appropriate Ethical Committees approved the protocol and study documents and written informed consent was obtained from all participants. All authors participated in the design, implementation, analysis and/or interpretation of the study. The corresponding author had final responsibility to submit for publication.

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ORIGINAL ARTICLE

Awareness of and knowledge about mother-to-child infections in Japanese pregnant women

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ABSTRACT **To reduce the incidence of infants with congenital infections, women should be aware of and know prevention measures against maternal infection with mother-to-child infections during pregnancy. Our objective was to assess the awareness of and knowledge about mother-to-child infections in Japanese pregnant women. A survey of 343 Japanese pregnant women was completed. Awareness of 13 pathogens capable of mother-to-child transmission was surveyed. Knowledge about the transmission route, the most susceptible time of infection that may cause severe fetal disease during pregnancy, and methods to prevent maternal infection were investigated for four major pathogens (cytomegalovirus, rubella virus, *Toxoplasma gondii*, and parvovirus B19) and results were compared between these pathogens. The proportion of women aware of pathogens concerning TORCH syndrome was the following: rubella virus 76%, *Treponema pallidum* 69%, *Toxoplasma gondii* 58%, parvovirus B19 28%, herpes simplex virus 27%, and cytomegalovirus 18%. Only 8% knew how cytomegalovirus is transmitted, and only 12% knew how parvovirus B19 is transmitted; both were significantly lower than those who knew transmission routes for rubella virus or *Toxoplasma gondii*. The proportion of women who knew the most susceptible time for severe fetal infection by maternal acquisition of cytomegalovirus, *Toxoplasma gondii*, or parvovirus B19 was significantly lower than that for rubella virus. The vast majority of surveyed women were not aware of methods to prevent maternal infection with cytomegalovirus or parvovirus B19. In conclusion, current awareness of and knowledge about cytomegalovirus and parvovirus B19 infection are low in Japanese pregnant women.**

Key Words: cytomegalovirus, parvovirus B19, pregnant women, rubella virus, *Toxoplasma gondii*

INTRODUCTION

Mother-to-child infections, which occur when pathogens are transmitted from mother to child during pregnancy or the perinatal period, can lead to spontaneous abortion, fetal death, intrauterine growth restriction, severe congenital neonatal diseases including anomalies, or childhood and adulthood diseases. Although vaccination before pregnancy is the most effective prevention method for mother-to-child infection, vaccinations against some pathogens (such as cytomegalovirus [CMV], *Toxoplasma gondii*, and parvovirus B19) are not currently available. Therefore, to reduce the incidence of infants with congenital infections, it is essential for pregnant women and females of childbearing age to be aware of and know methods to prevent maternal infection with pathogens capable of mother-to-child transmission (Lazzarotto and Lanari 2011).

Several studies have reported pregnant women's awareness of and knowledge about CMV infection (Jeon et al. 2006; Cordier et al. 2012a; Lim et al. 2012). However, no report has evaluated the awareness of and knowledge about CMV infection in Japanese women, and no report has investigated detailed knowledge of other pathogens that may cause severe congenital diseases or anomalies by vertical transmission in Japanese women.

In this study, 13 pathogens that affect the fetus or newborns by maternal infections were selected and awareness of them was assessed. Then, four major pathogens that can cause severe congenital diseases or anomalies in infants by primary, recurrent or chronic maternal infections during pregnancy were selected for detailed analyses: CMV, rubella virus, *Toxoplasma gondii*, and parvovirus B19. The reasons for selecting these pathogens were as follows: CMV causes congenital CMV infection that can result in major neurological sequelae, including sensorineural hearing loss and developmental disabilities (Koyano et al. 2011; Lazzarotto and Lanari 2011; The Japanese Congenital Cytomegalovirus Infection Immunoglobulin Fetal Therapy Study Group 2012). Treatments for fetal and newborn CMV infections, as well as vaccinations against CMV, are currently underdeveloped (Nassetta et al. 2009; Koyano et al. 2011; Lazzarotto and Lanari 2011; The Japanese Congenital Cytomegalovirus Infection Immunoglobulin Fetal Therapy Study Group 2012). Rubella virus can cause congenital rubella syndrome (Ueda 2009), which has still occurred in Japan and other countries. *Toxoplasma gondii* can cause congenital toxoplasmosis, and while maternal medication against *Toxoplasma gondii* is available, it does not always prevent the development of congenital toxoplasmosis (Yamada et al. 2011). Parvovirus B19 can cause hydrops fetalis and fetal anemia, which can result in miscarriage, fetal or neonatal death, or severe disability in infected infants, and there is currently no effective treatment or vaccination against parvovirus B19 (de Jong et al. 2006).

The objective of this study was to assess Japanese pregnant women's awareness of and knowledge about mother-to-child infections, especially CMV, rubella virus, *Toxoplasma gondii*, and parvovirus B19, and to compare awareness and knowledge of the four pathogens. Women should be aware of these four pathogens, and should know prevention methods to reduce congenital handicaps in infants. This report can provide fundamental data to show

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the necessity of counseling and education about mother-to-child infections in Japanese pregnant women.

MATERIALS AND METHODS

Subjects

We conducted a survey at Kobe University Hospital between June 2011 and September 2012 to evaluate Japanese pregnant women's awareness of and knowledge about mother-to-child infections with the approval from the Ethical Committee of Kobe University Graduate School of Medicine (#1264). During the study period, women at any stage of pregnancy who provided written informed consent to participate were invited to complete a written questionnaire on mother-to-child infections at their first visit to the hospital. The inclusion criteria of this study required subjects to be pregnant and aged 18 years or older. Participation was voluntary and anonymous.

Methods

A multiple-choice questionnaire assessed participants' awareness of the following 13 pathogens that affect the fetus or newborns by maternal infection: CMV, *Toxoplasma gondii*, hepatitis B virus, rubella virus, herpes simplex virus, parvovirus B19, hepatitis C virus, human immunodeficiency virus, human T cell leukemia virus type 1, measles virus, varicella-zoster virus, *Chlamydia trachomatis*, and *Treponema pallidum*. For four selected pathogens (CMV, rubella virus, *Toxoplasma gondii*, and parvovirus B19), the questionnaire also assessed participants' knowledge of transmission routes, the most susceptible time of infection that may cause severe fetal disease during pregnancy, the maximum frequency of fetal infection in cases of maternal infection, and methods to prevent maternal infection. Accurate actions to reduce the risk of transmission for each pathogen were chosen (Choices of actions are shown in Table S1, Cunningham et al. 2010). Participants' background characteristics, including age, occupation, history of childbirth and spontaneous abortion, and gestational age at the time of survey completion, were also collected.

Correct answers for accurate knowledge

CMV is transmitted by "contact with children's urine and saliva, or semen," rubella virus is transmitted by "droplet," *Toxoplasma gondii* is transmitted by "cat feces or eating undercooked meat," and parvovirus B19 is transmitted by "droplet." For all four pathogens, the most susceptible time of infection that may cause severe fetal disease is the first trimester. The maximum frequency of fetal infection when being maternal infection is 10–50% for CMV and parvovirus B19, ≥80% for rubella virus, and 50–80% for *Toxoplasma gondii*. "I know" or "I have ever heard" was taken to mean that women knew methods to prevent maternal infection (Cunningham et al. 2010).

Statistical analyses

Data were expressed as the number (%) or median (range) of subjects. χ^2 tests were performed for 2×2 or $2 \times n$ tables to compare the background characteristics of pregnant women who are aware of four pathogens that can cause mother-to-child infections. χ^2 tests were also performed to compare the knowledge of transmission routes, the most susceptible times of infection during pregnancy, the maximum frequencies of fetal infection, and methods to prevent maternal infection with four pathogens. Differences were deemed statistically significant when $P < 0.05$.

RESULTS

Characteristics of participants

Three hundred and forty-three Japanese pregnant women were included and analyzed in this study. No pregnant women refused to participate during the study period. The median age of surveyed women was 34 years (range: 19 to 45 years). Of 343 women, 209 (61%) were primipara and 93 (27%) had previously experienced a spontaneous abortion. Median gestational age at the time of survey was 15 weeks of gestation (range: 4 to 40). Fifteen women (4%) were working as healthcare professionals (two nurses, one physiotherapist, one dental hygienist) or care workers (six adult care workers, and five child care workers).

Awareness of pathogens that can infect the fetus

The proportion of pregnant women who were aware of pathogens that can infect the fetus is shown in Figure 1. Human immunodeficiency virus was the most known pathogen (298/343, 87%), followed by *Chlamydia trachomatis* (77%). The proportion of women aware of pathogens concerning TORCH syndrome was the follow-

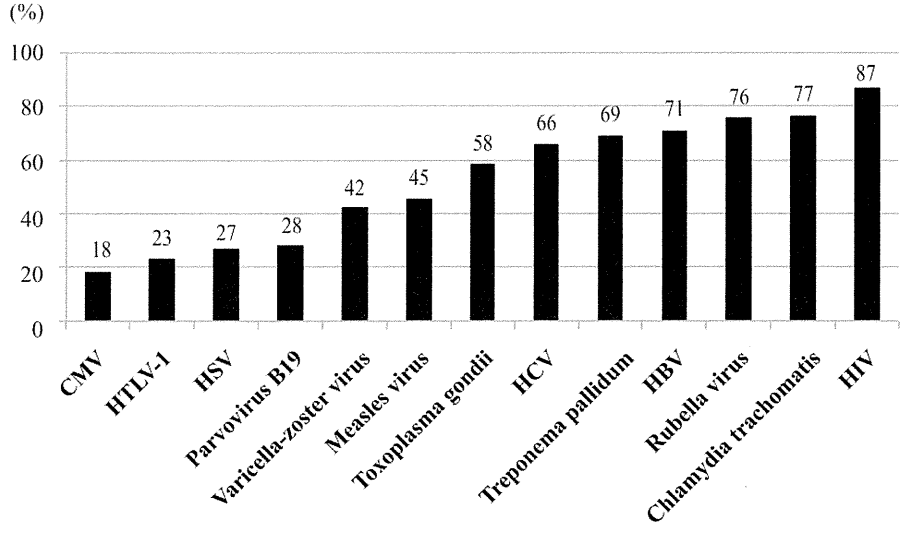

Fig. 1 The proportion of pregnant women who are aware of various pathogens that can infect the fetus. Data are expressed as percentage. CMV, cytomegalovirus; HTLV-1, human T cell leukemia virus type 1; HSV, herpes simplex virus; HCV, hepatitis C virus; HBV, hepatitis B virus; HIV, human immunodeficiency virus.

ing: rubella virus 76%, *Treponema pallidum* 69%, *Toxoplasma gondii* 58%, parvovirus B19 28%, and herpes simplex virus 27%. Only 18% of women were aware of mother-to-child transmission of CMV.

Awareness of rubella virus, *Toxoplasma gondii*, parvovirus B19, and CMV, stratified by participants' background characteristics

We stratified the awareness of rubella virus, *Toxoplasma gondii*, parvovirus B19, and CMV by age category, history of childbirth, history of spontaneous abortion, gestational age category at the time of survey, and employment as a healthcare professional or care workers (Table S2). The only significant difference ($P = 0.022$) we observed was higher awareness of *Toxoplasma gondii* in women working as healthcare professionals or care workers (87%, 13/15), compared to women not working in these positions (57%, 187/328).

Knowledge of transmission route, most susceptible time of infection that may cause severe fetal disease, maximum frequency of fetal infection when being maternal infection, and methods to prevent maternal infection

Fifty-two percent of women knew that rubella virus is transmitted by droplets and 43% knew that *Toxoplasma gondii* is transmitted by cat feces or eating undercooked meat, but 77% and 72% of women did not know the transmission routes for CMV and parvovirus B19 (Fig. 2). The most susceptible time of mother-to-fetus infection that may cause severe disease for all four pathogens is the first trimester. Forty percent of women knew this for rubella virus and 30% knew this for *Toxoplasma gondii*, but only 11% knew this for CMV and 8% knew this for parvovirus B19 (Fig. 3A). For each of the four pathogens, the majority of women did not know the maximum frequency of fetal infection when being maternal infection (Fig. 3B).

Nine percent of women knew methods to prevent maternal infection with rubella virus, and 35% had ever heard of them. Similarly, 12% of women knew prevention methods for *Toxoplasma gondii*, and 24% had ever heard of them. However, 85% and 92% of women did not know methods to prevent maternal infection with CMV and parvovirus B19 (Fig. 4).

We found that, depending on the pathogen, women had different levels of knowledge about methods to prevent maternal infection (Table 1). The percentages of women with accurate knowledge of transmission routes, the most susceptible time of infection that may cause severe fetal disease during pregnancy, and preventive methods for CMV and parvovirus B19 were significantly lower than those for rubella virus or *Toxoplasma gondii*. The percentages of women with accurate knowledge of transmission routes, the most susceptible time of infection, and preventive methods for *Toxoplasma gondii* were significantly lower than those for rubella virus (Table 1). Table 2 shows the proportion of pregnant women who knew each preventive method that can reduce risk of maternal infection during pregnancy.

DISCUSSION

This was the first study on awareness of and knowledge about 13 pathogens capable of mother-to-child infections in Japan. We found that human immunodeficiency virus was the most known pathogen (87%), followed by *Chlamydia trachomatis* (77%), rubella virus (76%), hepatitis B virus (71%), *Treponema pallidum* (69%), and hepatitis C virus (66%). The percentages of awareness of other pathogens for TORCH syndrome were *Toxoplasma gondii* 58%, parvovirus B19 28%, herpes simplex virus 27%, and only 18% for CMV.

The percentage of pregnant women who were aware of CMV (18%) was the lowest among the 13 pathogens surveyed. The low percentages of awareness for CMV were also demonstrated in France (34%, Cordier et al. 2012a), the USA (22%, Jeon et al. 2006), and Singapore (20%, Lim et al. 2012). In the present study, the questionnaire also assessed knowledge of transmission routes, the most susceptible time of infection that may cause severe fetal disease during pregnancy, the maximum frequency of fetal infection when being maternal infection, methods to prevent maternal

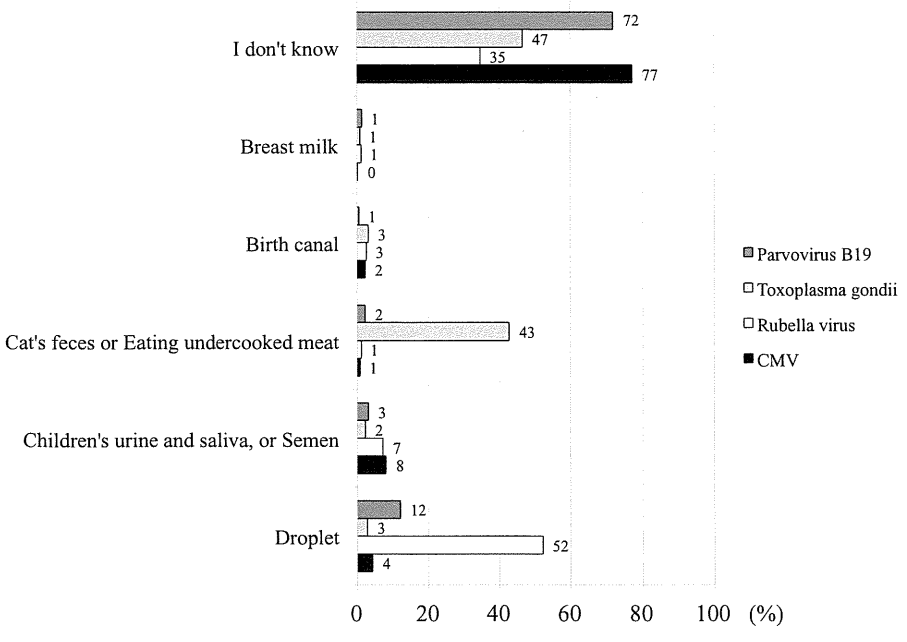

Fig. 2 The proportion of pregnant women who have knowledge about the transmission route of four pathogens. ■, parvovirus B19; ▨, *Toxoplasma gondii*; □, rubella virus; ■, cytomegalovirus (CMV).

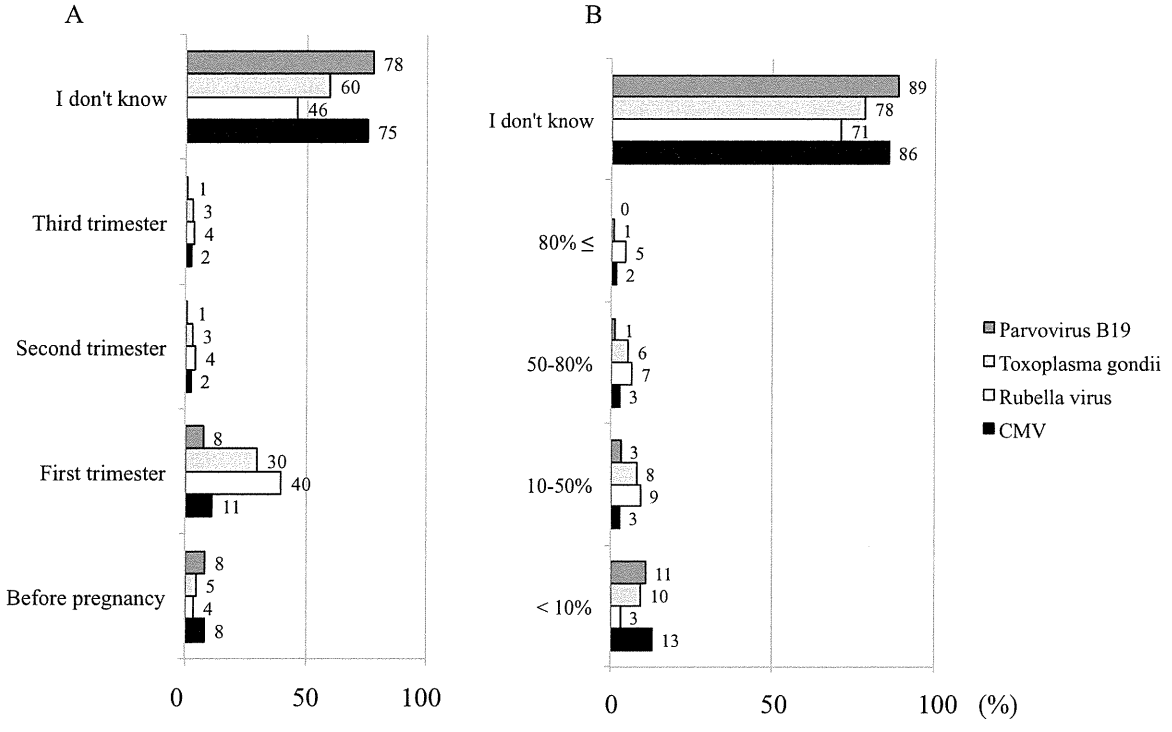

Fig. 3 The proportion of pregnant women who have knowledge of the most susceptible time of infection that may cause severe fetal disease (A) and the maximum frequency of fetal infection in cases of maternal infection (B).

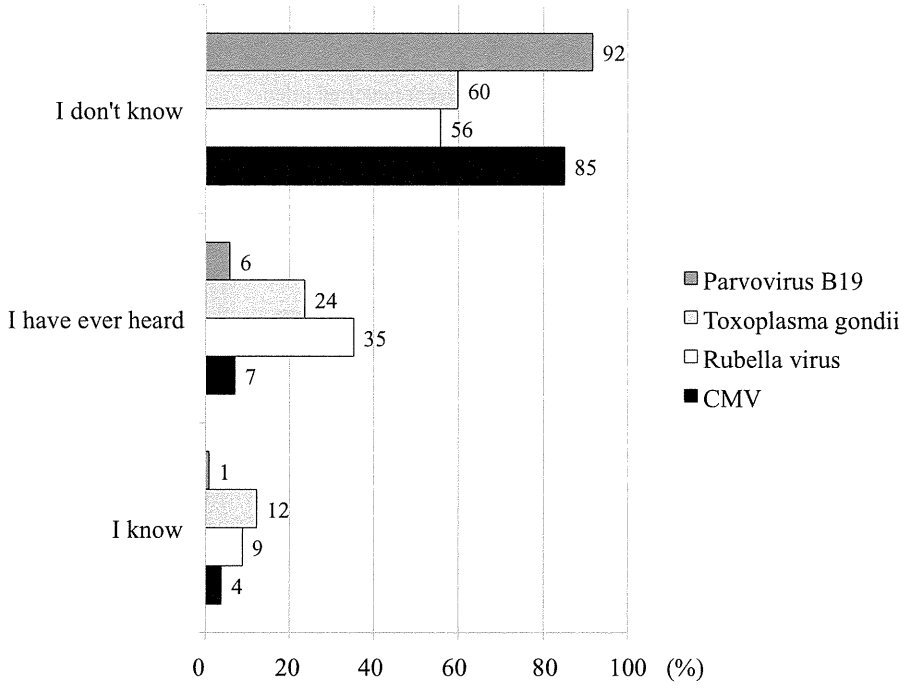

Fig. 4 The proportion of pregnant women who have knowledge about methods to prevent maternal infection.

infection and detailed actions to reduce the risk of maternal infection among four pathogens including CMV, rubella virus, *Toxoplasma gondii*, and parvovirus B19. Approximately 10% of pregnant women knew transmission routes, the most susceptible time of infection during pregnancy, and the methods to prevent maternal infection for CMV or parvovirus B19, and the percentage was much lower than that for rubella virus or *Toxoplasma gondii*. The detailed actions to reduce the risk of maternal infection for

Table 1 Proportion of pregnant women who had knowledge about infection during pregnancy and of methods to prevent maternal infection with four pathogens, $n = 343$

	Rubella virus	*Toxoplasma gondii*	CMV	Parvovirus B19	*P*-value
Transmission route	179 (52%)	147 (42%)*	28 (8%)**†	42 (12%)**†	<0.01
The most susceptible time of severe fetal infection	137 (40%)	103 (30%)**	39 (11%)**†	26 (8%)**†	<0.01
The maximum frequency of fetal infection	16 (5%)	19 (6%)	10 (3%)	11 (3%)	0.26
Methods to prevent maternal infection	151 (44%)	123 (36%)*	37 (11%)**†	23 (7%)**†	<0.01

P-values are shown for comparison among the four pathogens. *$P < 0.05$, **$P < 0.01$ compared to that of rubella virus, †$P < 0.01$ compared to that of *Toxoplasma gondii*.

Table 2 Proportion of pregnant women who knew of actions that can reduce risk of maternal infection during pregnancy

	Number of women who know of each preventive method (%) $n = 343$
CMV	
Wash hands after diaper changing	21 (6%)
Avoid kissing a child	16 (5%)
Do not share food, drinks, or eating utensils used by children	17 (5%)
Avoid taking care of children under 2.5 years old if you are working in a day-care center	4 (1%)
Use a condom during sexual intercourse	9 (3%)
Rubella virus	
Wear a mask	117 (34%)
Keep away from crowded places	127 (37%)
Keep away from people with fever or rash	124 (36%)
Wash hands and gargle	122 (36%)
Be vaccinated before pregnancy	58 (17%)
Toxoplasma gondii	
Cook meat to a safe temperature	75 (22%)
Wear gloves during any contact with sand	49 (14%)
Avoid trips to Europe during pregnancy	11 (3%)
Keep away from cats	87 (25%)
Parvovirus B19	
Wear a mask	18 (5%)
Keep away from crowded places	19 (6%)
Keep away from people with fever or rash	26 (8%)
Wash hands and gargle	28 (8%)

CMV, cytomegalovirus.

CMV in the questionnaire included "Wash hands after diaper changing", "Avoid kissing a child", and "Do not share food, drinks, or eating utensils used by children". The percentages of pregnant women who knew these preventive actions were found to be only 5–6%.

A recent study demonstrated that 0.31% of 21 272 infants were congenitally infected with CMV in Japan (Koyano et al. 2011). The number of infants with congenital CMV infection may increase in Japan, because the prevalence of serum anti-CMV antibodies in Japanese pregnant women recently decreased to approximately 70% (Azuma et al. 2010). Until a CMV vaccine becomes available, behavioral and educational interventions provide the best options to prevent maternal CMV infection (Lazzarotto and Lanari 2011). The results of the present study strongly suggested that Japanese women should receive counseling and more education about CMV transmission and infection prior to pregnancy or early in pregnancy. Adler et al. have demonstrated by a randomized controlled trial that behavioral prevention approaches (frequent hand washing, wearing latex gloves when changing diapers, and avoiding intimate contact with the children) effectively reduced child-to-mother transmission of CMV (Adler et al. 1996). Therefore, the education of protective behaviors and risky behaviors to avoid for pregnant women is very important to prevent maternal CMV infection during pregnancy.

We also found that a low proportion of Japanese pregnant women (28%) was aware of and had knowledge about parvovirus B19 infection during pregnancy. The percentages of awareness for parvovirus B19 were similar in France (24%, Cordier et al. 2012a) and the US (32%, Jeon et al. 2006). Parvovirus B19 infection is very common and usually presents with self-limited mild symptoms or no symptoms in children. Most maternal infections with parvovirus B19 occur through contact with infected children at home (de Jong et al. 2006). We recommend that pregnant women who have older children should be aware of a high risk of parvovirus B19 infection due to family transmission.

Rubella virus as a cause of congenital rubella syndrome was relatively well known in Japan (76% in the present study) as well as in France (97%, Cordier et al. 2012a), Brazil (74%, Vieira et al. 2011), and the US (53%, Jeon et al. 2006). The reason for a high proportion of women who knew about rubella might be the presence of the vaccine program to prevent rubella infection, which had been in effect since the 1970s in Japan (Ueda 2009).

The percentage of pregnant women who were aware of *Toxoplasma gondii* was 58% in the present study, and was reported as 48% in the US (Jones et al. 2003) and 98% in France (Cordier et al. 2012a). Differences in the percentages were possibly due to incidences of congenital toxoplasmosis in each country (Japan: 1.26, the US: 1.0, France: 3.3 per 10 000 births, Guerina et al. 1994; Villena et al. 2010; Yamada et al. 2011). The detailed actions to reduce risk of *Toxoplasma gondii* infection including "do not eat

uncooked meat" and "wear gloves during any contact with soil" should be informed to women prior to pregnancy or early in gestation.

Healthcare professionals generally have more awareness of and knowledge about mother-to-child infections during pregnancy (Ross et al. 2009; Cordier et al. 2012b). In subjects of the present survey, there were no physicians, and only four healthcare professionals and 11 care workers, who had more awareness of *Toxoplasma gondii* as mother-to-child infection.

In conclusion, current awareness of and knowledge about CMV and parvovirus B19 infections were found to be low in Japanese pregnant women. Counseling and education for women of childbearing age to prevent maternal CMV and parvovirus B19 infection should be urgently developed to reduce the incidence of these mother-to child infections.

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SUPPORTING INFORMATION

Additional Supporting Information may be found in the online version of this article at the publisher's web-site:

Table S1 Survey choices of actions that can reduce the risk for maternal infection during pregnancy.

Table S2 Background characteristics in pregnant women who are aware of four mother-to-child infections.

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Original article

Quantitative evaluation of ventricular dilatation using computed tomography in infants with congenital cytomegalovirus infection

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Abstract

Background: Infants with congenital cytomegalovirus infection (CCMVI) may develop brain abnormalities such as ventricular dilatation, which may potentially associate with sensorineural hearing loss. There is currently no recognized method for quantitative evaluation of ventricle size in infants with CCMVI. Our objectives were to establish a method for quantitative evaluation of ventricle size using computed tomography (CT) in infants with CCMVI, and determine a cut-off value associated with abnormal auditory brainstem response (ABR) early in life.

Design/Subjects: This study enrolled 19 infants with CCMVI and 21 non-infected newborn infants as a control group. Infants with CCMVI were divided into two subgroups according to ABR at the time of initial examination: normal ABR (11 infants) or abnormal ABR (8 infants). Ventricle size was assessed by calculating Evans' index (EI) and lateral ventricle width/hemispheric width (LVW/HW) ratio on brain CT images, and was compared among groups. A cut-off ventricle size associated with abnormal ABR was determined.

Results: EI and LVW/HW ratio were significantly higher in the CCMVI with abnormal ABR group than the control and CCMVI with normal ABR groups. Cut-off values of 0.26 for EI and 0.28 for LVW/HW ratio had a sensitivity of 100% and 100%, respectively, and a specificity of 73% and 91%, respectively, for association with abnormal ABR.

Conclusions: We established a method for quantitative evaluation of ventricle size using EI and LVW/HW ratio on brain CT images in infants with CCMVI. LVW/HW ratio had a more association with abnormal ABR in the early postnatal period than EI.

Keywords: Auditory brainstem response; Cytomegalovirus infection; Evans' index; Lateral ventricle width/hemispheric width ratio; Ventricle

1. Introduction

Cytomegalovirus (CMV) is the main pathogen causing congenital infection in developed countries [1] and

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affects 0.31% of live newborn infants in Japan [2]. Approximately 10–15% of infants with congenital CMV infection (CCMVI) have clinical manifestations at birth such as jaundice, hepatosplenomegaly with or without liver dysfunction, thrombocytopenic purpura, chorioretinitis, and abnormalities of the central nervous system. Of such symptomatic infants, approximately 80–90% develop major neurological sequelae including sensorineural hearing loss (SNHL) and developmental disabilities [1].

Previous studies have shown that brain abnormalities, including intracranial calcification and ventricular dilatation (VD), are associated with the development of SNHL [3–5] and can be used to predict SNHL in infants with CCMVI [4,5]. Computed tomography (CT) images are still considered in infants with suspected CCMVI in the early postnatal period to rule out calcifications or VD of their brains, although magnetic resonance imaging (MRI) and ultrasound examinations have widely spread in Japan. VD has been generally assessed qualitatively (presence or absence of VD) or semi-quantitatively based on CT images (mild, moderate, or severe VD) [3–5]. However, because such assessment has yielded inconsistent results among pediatric radiologists [3], it is critical to establish a method for quantitative evaluation of ventricle size that can provide a more accurate marker of SNHL in infants with CCMVI.

Evans' index (EI), the ratio of the maximum width of the frontal horns of the lateral ventricles to the greatest internal diameter of the skull, is the most well-known index for quantitative evaluation of ventricle size on CT images [6]. International guidelines for the diagnosis of hydrocephalus define VD as EI > 0.3 [7,8]. The lateral ventricle width/hemispheric width (LVW/HW) ratio is the standard index used for evaluation of fetal VD on ultrasound examination [9–12]. No reported studies to date have used either EI or LVW/HW ratio to assess VD on CT images in infants with CCMVI.

The aims of this study were to establish a method for quantitative evaluation of ventricle size using CT images to obtain EI and LVW/HW ratio, and to determine cut-off values for EI and LVW/HW ratio associated with abnormal auditory brainstem response (ABR) in infants with CCMVI early in life.

2. Methods

2.1. Study design

This study was conducted from April 2009 to March 2012 at Kobe University Hospital. The collections and uses of human materials for this study were approved by the Ethical Committee of Kobe University Graduate School of Medicine. Written informed consent was obtained from the parents of the enrolled infants.

Infants of mothers who had confirmed or suspected primary CMV infection were enrolled in this study. All of them underwent blood testing, CMV-DNA analysis, brain CT, ABR evaluation, and ophthalmologic examination. CMV-DNA analysis was used to allocate infants to the CCMVI or control groups. The CCMVI group was divided into two subgroups according to ABR: CCMVI with normal ABR and CCMVI with abnormal ABR. The clinical background characteristics of all enrolled infants were recorded, including gestational age, birth weight, gender, initial physical examination findings, and postconceptional age at the time of brain CT and ABR evaluation. EI and LVW/HW ratio were obtained as shown in Fig. 1. Clinical background characteristics, EI, and LVW/HW ratio were compared among the groups (control, CCMVI, CCMVI with normal ABR, and CCMVI with abnormal ABR). Finally, cut-off values for EI and LVW/HW ratio associated with abnormal ABR were determined.

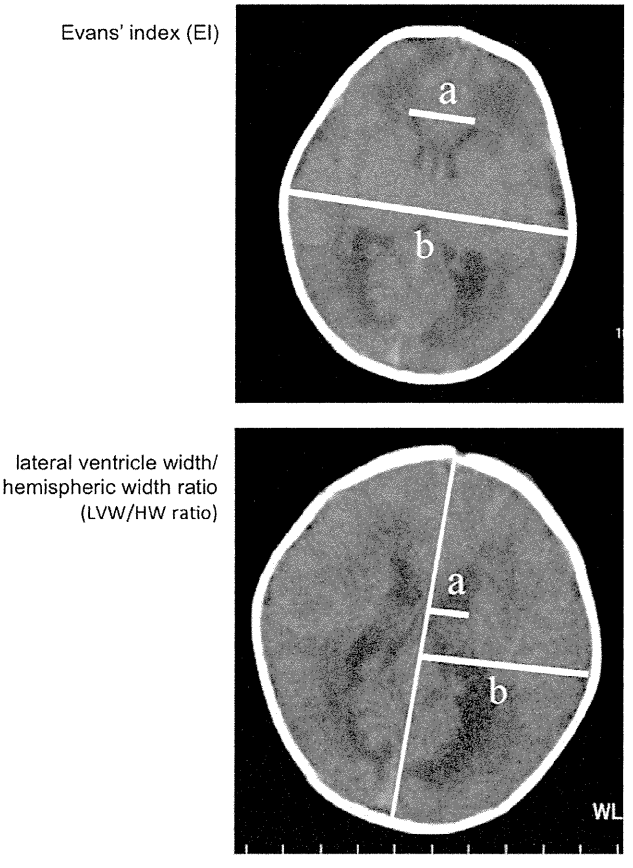


Fig. 1. Quantitative evaluations of ventricle size: Evans' index (EI) and lateral ventricle width/hemispheric width (LVW/HW) ratio. EI = maximum width of the frontal horns of the lateral ventricles (a)/internal diameter of the skull (b), LVW/HW ratio = maximum lateral ventricle width (a)/hemispheric width (b).

2.2. Diagnosis of CCMVI

Once pregnant mothers were confirmed or suspected primary CMV infection based on positive CMV-IgM and/or a low CMV-IgG avidity index (<45%) during pregnancy [13], the presence of CMV-DNA in urine specimens of their newborn infants within 1 week of birth was tested by the urine filter-based quantitative polymerase chain reaction (PCR) assay, and then was confirmed by the standard quantitative real-time PCR assay as previously described [2,14]. CCMVI of infants with more than 3 weeks of age was diagnosed if CMV-DNA was detected in a dried umbilical cord specimen using the real-time PCR assay as previously described [14,15].

2.3. Definitions of manifestations of CCMVI

Hepatosplenomegaly was confirmed by ultrasound examination and/or abdominal X-ray. Hepatitis was defined as serum alanine aminotransferase level >100 U/L, thrombocytopenia as platelet count $<1 \times 10^5\ \mu L^{-1}$, and jaundice as serum direct bilirubin level >2 mg/dL [4,16]. Chorioretinitis was diagnosed by a pediatric ophthalmologist. Intracranial calcification on brain CT images was diagnosed by a radiologist. ABR abnormality was diagnosed by using a Neuropack S1 (Nihon Kohden Co., Tokyo, Japan) according to the manufacturer's recommended protocol. A non-response to 40 dB for infants with a postconceptional age of $\geqslant$37 weeks and 50 dB for infants with a postconceptional age of 34–36 weeks was defined as abnormal, either unilaterally or bilaterally [17,18].

2.4. Brain CT images

All brain CT examinations were performed using a 64-slice multidetector CT scanner (Aquilion, Toshiba Medical Systems, Tokyo, Japan). The following technical parameters were used for CT scanning: 120 kV peak energy, 120 mA current with automated radiation exposure control, and 4-mm reconstruction thickness. CT images parallel to the orbitomeatal line were analyzed.

2.5. Quantitative evaluations of ventricle size

EI and LVW/HW ratio were used to quantitatively evaluate ventricle size (Fig. 1). An expert radiologist, who was blinded to detailed clinical findings, reviewed all CT scans. EI was calculated as the ratio of the maximum width of the frontal horns of the lateral ventricles to the largest internal diameter of the skull. The maximum width of the frontal horns was measured on the slice with the largest width, and the largest internal diameter of the skull was measured on the same slice [6]. LVW/HW ratio was calculated as the ratio of the maximum lateral ventricle width to the maximum hemispheric width on the left side. The maximum lateral ventricle width was measured on the slice with the largest width, and the maximum hemispheric width was measured on the same slice [9,10].

2.6. Statistical analysis

Statistical analyses were performed using the Mann–Whitney nonparametric rank test for comparison of two independent data sets. Differences were deemed statistically significant at $p < 0.05$. Cut-off values for EI and LVW/HW ratio associated with abnormal ABR were determined using receiver operating characteristic curve (ROC) analyses [19]. Sensitivity, specificity, negative predictive value, positive predictive value, and likelihood ratio for positive and negative results were calculated.

3. Results

3.1. Clinical background characteristics of enrolled infants

This study enrolled 19 infants in the CCMVI group and 21 infants in the control group. Of the 19 CCMVI infants, 18 were diagnosed by CMV-DNA detection in urine specimens and one was diagnosed by CMV-DNA detection in a dried umbilical cord specimen. Ten infants with CCMVI were asymptomatic and nine had some manifestations at the time of initial examination as follows: eight had abnormal ABR, four had hepatosplenomegaly, three had hepatitis, one had jaundice, two had thrombocytopenia with petechiae, two had chorioretinitis, and five had intracranial calcifications. Baseline characteristics of infants in the CCMVI and control groups are shown in Table 1. There were no significant differences in gestational age, birth weight, gender, or postconceptional age at the time of brain CT or ABR evaluation between these two groups. There were also no significant differences in gestational age, birth weight, gender, or postconceptional age at the time of brain CT or ABR evaluation between the CCMVI with normal ABR group ($n = 11$) and the CCMVI with abnormal ABR group ($n = 8$; five with bilateral and three with unilateral abnormality). No infants with abnormal ABR were found in the control group.

3.2. EI and LVW/HW ratio in infants without and with CCMVI

In infants without CCMVI, the median EI was 0.23 and the median LVW/HW ratio was 0.19 (Fig. 2).

Fig. 2 shows comparisons of EI and LVW/HW ratio between the CCMVI and control groups. EI and LVW/HW ratio were significantly higher in the CCMVI group

Table 1
Clinical background characteristics of enrolled infants.

	Control, $n = 21$	CCMVI		
		Total, $n = 19$	Normal ABR, $n = 11$	Abnormal ABR, $n = 8$
Gestational age (weeks)	38 (36–41)	38 (31–41)	38 (35–41)	37 (31–39)
Birth weight (g)	2822 (2218–3688)	2868 (1378–3840)	3074 (2362–3840)	2334 (1378–3160)
Male/female	9/12	6/13	3/8	3/5
Postconceptional age at the time of brain CT (weeks)	38 (36–42)	40 (34–52)	42 (38–45)	38 (34–52)
Postconceptional age at the time of ABR evaluation (weeks)	39 (36–42)	40 (34–48)	42 (39–45)	38 (34–48)

Data are expressed as median (range) or number. CCMVI = congenital cytomegalovirus infection; ABR = auditory brainstem response; CT = computed tomography.

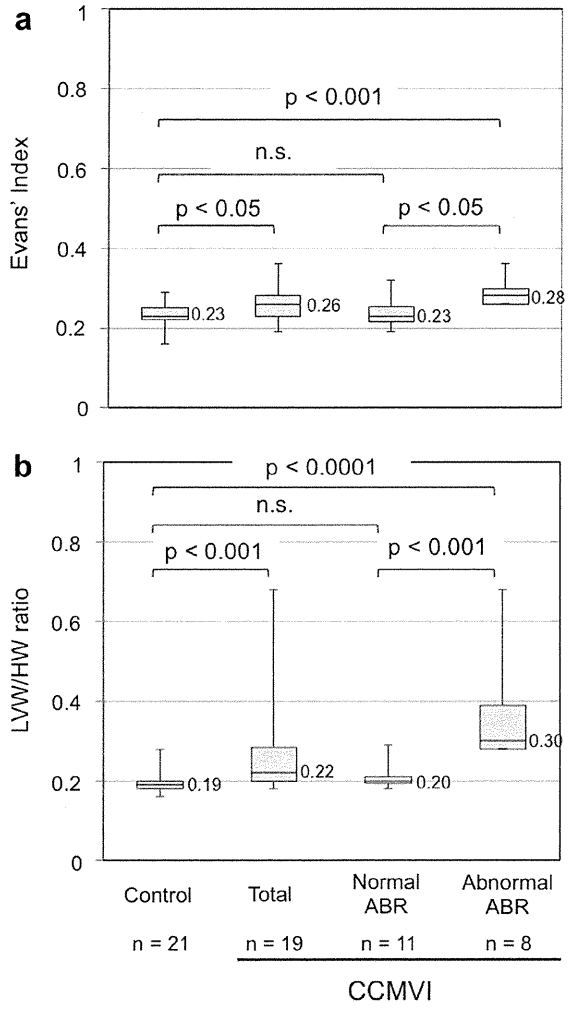


Fig. 2. Comparisons of Evans' index (a) and lateral ventricle width/hemispheric width (LVW/HW) ratio (b) between the congenital cytomegalovirus infection (CCMVI) and control groups. Data are expressed as median and range. ABR = auditory brainstem response. p-Values between any two groups are shown. n.s. = not significant.

than the control group ($p < 0.05$ for EI and $p < 0.001$ for LVW/HW ratio). EI and LVW/HW ratio were significantly higher in the CCMVI with abnormal ABR group than the control and CCMVI with normal ABR groups. However, there were no significant differences in EI or LVW/HW ratio between the control and CCMVI with normal ABR groups.

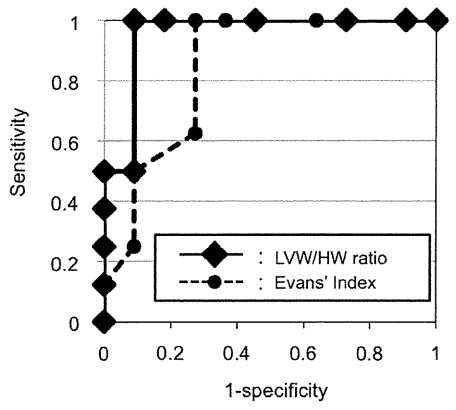
3.3. Cut-off values associated with abnormal ABR

EI and LVW/HW ratio in infants with CCMVI with normal or abnormal ABR were analyzed by ROC analyses (Fig. 3). Area under curve for EI and LVW/HW ratio was 0.847 and 0.955, respectively. The cut-off values associated with abnormal ABR were 0.26 for EI and 0.28 for LVW/HW ratio. The test performance characteristics were as follows. A cut-off value of 0.26 for EI had sensitivity of 100%, specificity of 72.7%, negative predictive value of 100%, positive predictive value of 72.7%, and likelihood ratio for positive and negative results of 3.67 and 0. A cut-off value of 0.28 for LVW/HW ratio had sensitivity of 100%, specificity of 90.9%, negative predictive value of 100%, positive predictive value of 88.9%, and likelihood ratio for positive and negative results of 11.0 and 0.

4. Discussion

This is the first study to report a method for quantitative evaluation of ventricle size using EI and LVW/HW ratio on brain CT in infants with CCMVI. We found that VD was associated with abnormal ABR in infants with CCMVI early in life. The cut-off values associated with abnormal ABR in infants with CCMVI were 0.26 for EI and 0.28 for LVW/HW ratio. Interestingly, LVW/HW ratio had a more association with abnormal ABR than EI.

We selected EI and LVW/HW ratio for quantitative evaluation of ventricle size, because these are the most popular indexes of VD in adult patients with hydrocephalus [6–8] and in fetuses [9–12]. Infants with and without CCMVI sometimes have asymmetrical ventricle sizes. We calculated LVW/HW ratios on the right and left sides in the CCMVI group, but found no significant dif-



ABR

Evans' Index

	Abnormal	Normal	
≥ 0.26	8	3	11
< 0.26	0	8	8
	8	11	19

LVW/HW ratio

	Abnormal	Normal	
≥ 0.28	8	1	9
< 0.28	0	10	10
	8	11	19

Fig. 3. Receiver operating characteristic curve analyses of Evans' index (EI) and lateral ventricle width/hemispheric width (LVW/HW) ratio for associating with abnormal auditory brainstem response (ABR) in infants with congenital cytomegalovirus infection (CCMVI). The cut-off values were 0.26 for EI and 0.28 for LVW/HW ratio. LVW/HW ratio had a more association with abnormal ABR in infants with CCMVI than EI.

ference between sides (data not shown). LVW/HW ratio was therefore calculated only for the left side in this study.

To our knowledge, this is the first study reporting EI and LVW/HW ratio on brain CT in newborn infants with and without CCMVI. We suggest that EI and LVW/HW ratio can be used for quantitative evaluation of ventricle size, and for assessment of changes in ventricle size after treatment, in infants with CCMVI or other conditions causing VD such as intraventricular hemorrhage, congenital brain anomaly, or tumor. EI and LVW/HW ratio were significantly higher in infants with CCMVI with abnormal ABR than infants with CCMVI with normal ABR early in life. These data suggest that VD was associated with abnormal ABR in infants with CCMVI. Previous studies have reported that calcifications, cysts, white matter changes, or VD on CT or ultrasound examination predicted SNHL in infants with CCMVI [3–5]. CMV infection *in utero* may cause injury to the brain and inner ears, resulting in auditory nerve injury in infants with VD, although we have not known the mechanisms of their associations. On the other hand, Rivera et al. reported that the presence of microcephaly and other neurologic abnormalities was not predictive of SNHL including late-onset SNHL [20]. A reason for the difference between our and their results may be whether the study subjects include late-onset SNHL (30% and 0% of enrolled CCMVI patients in Rivera's and our reports) [20]. Further studies involving larger numbers of infants with CCMVI and VD are needed to confirm this association. The mechanisms for simultaneous incidence of VD and abnormal ABR also should be clarified.

We found that LVW/HW ratio had a more association with abnormal ABR in infants with CCMVI than EI. Neural stem cells are the predominant cell type in the fetal brain, and are located predominantly in the subventricular and subgranular zones of the hippocampus. These cells are damaged by CMV infection during fetal development [21]. In Alzheimer's disease, the hippocampus is one of the first regions of the brain to show damage. The size of the body of the lateral ventricle and LVW/HW ratio were found to be better predictors of Alzheimer's disease than the width between the frontal horns of the lateral ventricles or EI [22]. This study indicates that in infants with CCMVI, using EI as a marker of abnormal ABR is more likely to give false-positive results than using LVW/HW ratio.

CT scanning requires the additional risk and expense of transporting a seriously ill infant. It is already known that detection of VD is correlated between CT and ultrasound examinations [23]. Fetal ultrasound examination is routinely performed during pregnancy [9–12], and it has been reported that detection of VD using LVW/HW ratio in the fetus can assist with diagnosis of myelomeningocele or meningitis due to intrauterine infection in the early postnatal period [12]. We suggest that ultrasound evaluation of ventricle size using LVW/HW ratio in fetuses with CCMVI may be used as an early predictor of hearing impairment in the early postnatal period. Further studies are needed to investigate the association between ventricle size in fetuses with CCMVI and the development of abnormal ABR after birth.

The limitations of our study are as follows. First, abnormal ABR has not been proved to cause by VD alone in the previous reports [3–5]. Correlations between VD and other neurological symptoms, such as microcephaly, intracranial calcification and cortical dysplasia should be analyzed. However, we could not correctly assess their correlations as this study enrolled only a small number of infants with these neurological symptoms. Second, we investigated the association between VD on brain CT images and abnormal ABR in the early period after birth in this study. There are some reports that patients who were infected CMV during late pregnancy did not present VD, but showed progressive SNHL [24,25]. Because asymptomatic CCMVI infants