women, as well as multiparous women, for vaccination are needed in order to further reduce the incidence of influenza among pregnant Japanese women.

Introduction

Pregnant women are at an increased risk of severe influenza-related complications [1–9]. Evidence from several countries demonstrated increased hospitalization rates and disproportionately higher rates of mortality in pregnant women during the previous seasonal influenza and pandemic (H1N1) 2009 [1–9]. However, there were no mortalities among pregnant women during the pandemic (H1N1) 2009 in Japan [10, 11]. At that time of pandemic (H1N1) 2009, pregnant Japanese women had a high level of concern regarding pandemic influenza because the Japan Society of Obstetrics and Gynecology (JSOG) aggressively intensified outreach to pregnant women, policy-makers, and medical workers, including medical doctors, midwives, and nurses, to minimize the number of cases of infection among pregnant women [10, 12]. Indeed, more than 60 % of candidates were vaccinated within 1.5 months after the availability of vaccine against the pandemic (H1N1) 2009 virus and half of all women infected with influenza had taken prophylactic antiviral drugs after coming into close contact with an infected person in Hokkaido, the northernmost and second largest island of Japan [13].

However, to our knowledge, there have been no studies investigating how many pregnant Japanese women are vaccinated against seasonal influenza and how many pregnant Japanese women contracted seasonal influenza before or after the pandemic (H1N1) 2009. It may be important to determine the baseline level of concern regarding influenza among pregnant Japanese women in order to prepare for future avian influenza epidemics. Therefore, we conducted this multicenter study to determine vaccination coverage against seasonal influenza and the prevalence rate of influenza infection among pregnant Japanese women during the 2013–2014 influenza season.

Materials and methods

This multicenter observational study was conducted with the approval of the Institutional Review Boards of Hokkaido University Hospital and the following 11 hospitals widely dispersed throughout Japan that participated in this study: Nagasaki University Hospital, Rakuwakai Otowa Hospital, Toyama University Hospital, Mie Chuo Medical Center, Nippon Medical School Tama-Nagayama Hospital, National Center for Child Health and Development, University of Tsukuba Hospital, Jichi Medical University Hospital, Hakodate Central General Hospital, Sapporo Toho Hospital, and Hokkaido University Hospital.

Table 1 Questionnaire form given to women within 5 days after delivery during the study period (March 1, 2014, to July 31, 2014

Q1: When did you give birth?
□March, □April, □May, □June, □July in 2014
Q2: How old are you?
□≤19, □20–24, □25–29, □30–34, □35–39, □40–44, □≥ 45
Q3: Was this your first experience of birth?
□Yes, □No
Q4: Were you vaccinated against influenza on or after October 2013?
□Yes, □No
Q5: Did you contract influenza during the current pregnancy?
□Yes, □No
The following questions are for women with “Yes” in response to Q5
Q6: Were you hospitalized for treatment of influenza?
□Yes, □No
Q7: What was the type of influenza?
□A, □B, □Unknown
Q8: Did you receive antiviral agent for the treatment of influenza?
□Yes, □No
The following question is for women with “Yes” in response to Q8
Q9: What was the type of antiviral agent given?
□Oral tablet, □Inhalation drug

In Japan, women usually remain at obstetric facilities for 4 to 8 days after giving birth. We conducted an anonymous questionnaire study (Table 1) among all postpartum women who gave birth at and after gestational week 22 and within 5 days after delivery before leaving the obstetric facility during the study period from March 1, 2014, to July 31, 2014. Therefore, the majority of these postpartum women conceived in or before October 2013.

All data are presented as means±standard deviation (SD). For the statistical analysis of categorical data, χ^2 or Fisher's exact test was applied. The statistical software package StatView 5.0 for Macintosh (SAS Institute, Inc. Cary, NC) was used for the data analysis. In all analyses, $p<0.05$ was taken to indicate statistical significance.

Results

During the 5-month study period, 2,808 women gave birth on and after gestational week 22 at the 11 participating hospitals. Preterm birth (at<37 weeks of gestation) occurred in 373 (13 %) women. A total of 1,713 (61 %) of the 2,808 women participated in this study (Table 2). Although the response rate and vaccination coverage differed considerably between hospitals, the mean (SD) response rate was 62 % (22 %), and the mean vaccination rate was 53 % (13 %) for the 11

Table 2 Number of participants, response rates, and vaccination rates according to institution

Institution	No. of women		
	Candidates*	Participants	Vaccinated
NUH	132	112 (85 %)	55 [49 %]
ROH	136	97 (71 %)	31 [32 %]
TUH	131	60 (46 %)	36 [60 %]
MCMC	190	124 (65 %)	82 [66 %]
NMSTH	252	77 (31 %)	33 [43 %]
NCCHD**	318	139 (44 %)	97 [70 %]
UTH	392	382 (97 %)	166 [43 %]
JMUH	338	298 (88 %)	127 [43 %]
HCGH	259	140 (54 %)	81 [58 %]
STH	480	166 (35 %)	80 [48 %]
HUH	180	118 (66 %)	88 [75 %]
Overall	2,808	1,713 (61 %)	876 [51 %]
Overall (mean ± SD)	255±116	156±98 (62 %±22 %)	80±41 [53 %±13 %]

NUH Nagasaki University Hospital; *ROH* Rakuwakai Otowa Hospital; *TUH* Toyama University Hospital; *MCMC* Mie Chuo Medical Center; *NMSTH* Nippon Medical School Tama-Nagayama Hospital; *NCCHD* National Center for Child Health and Development; *UTH* University of Tsukuba Hospital; *JMUH* Jichi Medical University Hospital; *HCGH* Hakodate Central General Hospital; *STH* Sapporo Toho Hospital; *HUH* Hokkaido University Hospital

*Number of women who gave birth during the study period

**Study subjects were women who gave birth in March and April only

The percentage response rate (participants/candidates) to this questionnaire survey and vaccination rate (vaccinated/participants) are indicated in parentheses and square brackets, respectively

hospitals. The age-specific response rate was as follows: 54 % (124/229), 59 % (332/559), 62 % (571/927), 63 % (501/793), and 62 % (185/300) for women aged 24 years or less, 25–29, 30–34, 35–39, and 40 years or more, respectively. Of the 1,713 respondents, 876 (51 %) and 837 (49 %) women did and did not receive a vaccine against influenza in or after October 2013, respectively.

Table 4 Comparison of women who did and did not contract influenza

	Infection with influenza		
	Yes	No	*p*-Value
No. of women	87	1,626	
Vaccinated	34 (39 %)	842 (52 %)	0.0210
Primiparous	26 (30 %)	877 (54 %)	0.0000
Maternal age (years)			
≤29	21 (24 %)	435 (27 %)	0.7088
30–35	34 (39 %)	537 (33 %)	0.2450
≥35	32 (37 %)	654 (40 %)	0.5235

Maternal age affected vaccination coverage: women aged 24 years or less received vaccination significantly less often than those in the other age categories (Table 3). However, experience of prior birth did not affect the overall vaccination coverage (50 % for primiparous vs. 53 % for multiparous women), although a significantly larger number of primiparous women aged 25–29 years received the vaccination compared to multiparous women in the same age group (57 % vs. 44 %, respectively). In contrast, among women 35 years old or more, multiparous women tended to have a greater vaccination rate than primiparous women.

Eighty-seven (5.1 %) and 1,626 (94.9 %) women did and did not contract influenza, respectively (Table 4). The prevalence of influenza did not differ markedly between hospitals, ranging from 3.4 % (10/298) in Jichi Medical University Hospital to 8.9 % (10/112) in Nagasaki University Hospital (mean±SD, 5.5 %±1.8 %). In the 1,626 women without influenza compared to the 87 women with influenza, the fraction sizes of women with vaccination and that of primiparous women were significantly greater (Table 4). As this suggested that the experience of childbirth was a risk factor for contracting influenza, we analyzed the differences in the prevalence rates of influenza between primiparous and multiparous women according to maternal age (Fig. 1). The infection rate was consistently higher for multiparous than

Table 3 Vaccination rates according to maternal age and experience of prior birth

Age (years)	Overall	Primiparous	Multiparous	*p*-Value*
≤24	39/124 (31 %)**	29/92 (32 %)**	10/32 (31 %)***	0.9772
25–29	171/332 (52 %)	112/198 (57 %)	59/134 (44 %)	0.0249
30–34	305/571 (53 %)	156/299 (52 %)	149/272 (55 %)	0.5330
35–39	260/501 (52 %)	106/222 (48 %)	154/279 (55 %)	0.0974
≥40	101/185 (55 %)	44/92 (48 %)	57/93 (61 %)	0.0659
Overall	876/1,713 (51 %)	447/903 (50 %)	429/810 (53 %)	0.1525

*Comparison between primiparous and multiparous women

**p<0.05 vs. any other age category

***p<0.05 vs. any other age category except women aged 25–29 years

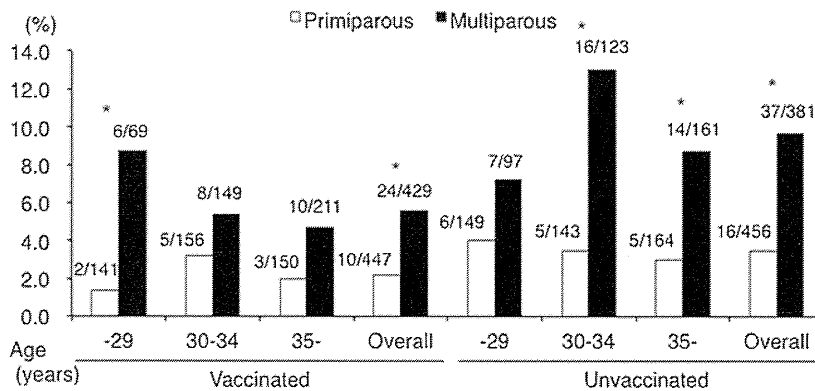

Fig. 1 Prevalence rate of influenza infection according to maternal age among primiparous and multiparous women. *$p<0.05$ vs. primiparous women. The figures at the top of the bars indicate actual numbers of women with influenza/designated population. The prevalence rate of influenza was consistently higher for multiparous (*black bars*) than primiparous (*white bars*) women in all age categories, irrespective of vaccination status. Overall, the infection rate was significantly higher in multiparous than in primiparous women for both vaccinated (5.6 % [24/429] vs. 2.2 % [10/427], $p=0.0216$) and unvaccinated women (9.7 % [37/381] vs. 3.5 % [16/456], $p=0.0003$). Among primiparous women, the infection rate did not differ between those with and without vaccination in any age category. Among multiparous women, the infection rate was significantly lower in those with vaccination and aged 30–34 years than in their counterparts (5.4 % [8/149] vs. 13.0 % [16/123], respectively; $p=0.0321$). Overall, the infection rate did not differ significantly between primiparous women with and without vaccination (2.2 % [10/447] vs. 3.5 % [16/456], respectively), whereas it was significantly lower in multiparous women with than without vaccination (5.6 % [24/429] vs. 9.7 % [37/381], respectively; $p=0.0324$)

primiparous women, irrespective of vaccination status. Maternal age was not associated with the infection rate (Fig. 1, Table 4).

The effects of vaccination on the prevention of influenza were analyzed (Table 5). Two women (one was vaccinated and the other was unvaccinated) contracted both influenza A and B viruses. The number of women infected with influenza A virus did not differ significantly between the 876 and 837 women with and without vaccination (2.7 % vs. 3.2 %, respectively), while that of women with influenza B virus infection was significantly lower among women with than without vaccination (0.8 % vs. 1.9 %, respectively; $p=0.0455$). Overall, the 2013–2014 vaccination program against influenza reduced the risk of influenza infection by 35 % ([54 – 35]/54) among pregnant Japanese women.

Among the 87 women with influenza, 72 (83 %) reported having taken antiviral agents for the treatment of influenza. Antiviral agents administered for these 72 women were oral tablets in 34 (47 %) women and inhalation drugs for the remaining 38 (53 %) women. Two (2.3 %) women required hospitalization for treatment of the influenza; both were multiparous, one with vaccination contracted both influenza A and B viruses, and the other without vaccination contracted influenza A virus.

Table 5 Vaccination and infection with influenza A and B viruses

	Vaccinated	Unvaccinated	*p*-Value
No. of women	876	837	
Type of influenza virus			
A	24* (2.7 %)	27* (3.2 %)	0.5542
B	7 (0.8 %)	16 (1.9 %)	0.0455
Unknown	4 (0.5 %)	11 (1.3 %)	0.0569
Overall	34 (3.9 %)	53 (6.3 %)	0.0272

*One was also infected with influenza B virus

Discussion

To our knowledge, this is the first study focusing on the behavior of pregnant Japanese women with regard to seasonal influenza.

It may be important to determine the attitudes of pregnant women toward vaccination and antiviral drugs. A survey conducted in the 2006–2007 influenza season in the USA indicated that almost one-third of healthcare workers did not believe that vaccines are a safe and effective way to decrease infections, although the Advisory Committee on Immunization Practices (ACIP) began recommending routine influenza vaccination for healthy pregnant women during the influenza season in 2004 [14]. A meeting designed to integrate scientific evidence and expert opinion [13] in 2008 in the USA concluded that pregnant women should be considered a high-priority group for the receipt of vaccine and that increased seasonal influenza vaccine coverage may improve vaccine uptake in a pandemic [13]. Indeed, during the pandemic (H1N1) 2009, 7.8 % of 102 obstetric patients who required hospitalization for pandemic (H1N1) 2009 died in California [15]. In Australia and New Zealand, pregnant women accounted for 9.1 % of 722 patients who required treatment at an intensive care unit (ICU) [7] and 11 % of 64 pregnant women who required ICU treatment died [8]. Pregnant women accounted for 5 %, 7.5 %, and 8.3 % of hospitalized cases

in Canada, the UK, and Brazil, respectively [16], although pregnant women account for approximately 1.0 % of the total population.

Approximately half of pregnant Japanese women were vaccinated against seasonal influenza. As this figure was similar to those during and after the pandemic (H1N1) 2009 in the USA [17, 18], it may have been brought about by the pandemic (H1N1) 2009. Before the pandemic (H1N1) 2009, the estimated influenza vaccination coverage among pregnant women in the USA was consistently low (approximately 15 %) [18–20], although there is no significant increase in adverse reactions in mothers or neonates related to the vaccine and side effects are similar to those in the general population [19, 20]. Women offered influenza vaccination by a healthcare provider are more likely to be vaccinated and are more likely to have positive attitudes about vaccine effectiveness and safety [18]. The Healthy People 2020 initiative of the U.S. Department of Health and Human Services has set a goal of 80 % vaccine coverage among pregnant women in the USA [21]. As maternal influenza immunization is a highly cost-effective intervention to reduce disease rates and severity corresponding to both seasonal influenza epidemics and occasional pandemics [22], continued efforts are needed in order to encourage pregnant women to receive influenza vaccination.

Eighty-three percent of infected Japanese women reported having taken antiviral drugs. An early treatment of pregnant women with antiviral medications is associated with fewer ICU admissions and fewer maternal deaths [23]. No harmful effects of neuraminidase inhibitors, including oseltamivir, zanamivir, and laninamivir, on fetuses exposed in utero have been reported [24, 25], and they are believed to have contributed to the lack of maternal mortality during the pandemic (H1N1) 2009 in Japan [10–12].

Multiparous women had an approximately two-fold higher risk of influenza infection compared to primiparous women in this study. To our knowledge, this phenomenon has not been reported to date. The reason for this phenomenon is not yet clear. However, these observations may be explained by the greater number of cohabitants for multiparous than primiparous women, which may be associated with a higher risk of infection.

In conclusion, although it was difficult to verify that respondents answered questions correctly due to the nature of this questionnaire study, our results suggested that influenza vaccine coverage was approximately 50 % among pregnant women in Japan and approximately 1 in 20 pregnant women contracted influenza in the 2013–2014 influenza season. These observations indicated that the higher vaccination level achieved during the pandemic (H1N1) 2009 was sustained. However, the vaccination coverage was insufficient in younger pregnant women, and multiparous women had an approximately two-fold higher risk of infection compared to primiparous women. Continued efforts are needed in order to encourage pregnant women, especially those less than 25 years old and multiparous women, to receive the vaccination to further reduce the number of pregnant women with influenza in Japan.

Conflict of interest All authors declare that they have no financial relationships with biotechnology manufacturers, pharmaceutical companies, or other commercial entities with an interest in the subject matter or materials discussed in this manuscript.

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Intestinal Microbiota is Different in Women with Preterm Birth: Results from Terminal Restriction Fragment Length Polymorphism Analysis

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Abstract

Preterm birth is a leading cause of perinatal morbidity and mortality. Studies using a cultivation method or molecular identification have shown that bacterial vaginosis is one of the risk factors for preterm birth. However, an association between preterm birth and intestinal microbiota has not been reported using molecular techniques, although the vaginal microbiota changes during pregnancy. Our aim here was to clarify the difference in intestinal and vaginal microbiota between women with preterm birth and women without preterm labor. 16S ribosomal ribonucleic acid genes were amplified from fecal and vaginal DNA by polymerase chain reaction. Using terminal restriction fragment length polymorphism (T-RFLP), we compared the levels of operational taxonomic units of both intestinal and vaginal flora among three groups: pregnant women who delivered term babies without preterm labor (non-PTL group) (n = 20), those who had preterm labor but delivered term babies (PTL group) (n = 11), and those who had preterm birth (PTB group) (n = 10). Significantly low levels of *Clostridium* subcluster XVIII, *Clostridium* cluster IV, *Clostridium* subcluster XIVa, and *Bacteroides*, and a significantly high level of *Lactobacillales* were observed in the intestinal microbiota in the PTB group compared with those in the non-PTL group. The levels of *Clostridium* subcluster XVIII and *Clostridium* subcluster XIVa in the PTB group were significantly lower than those in the PTL group, and these levels in the PTL group were significantly lower than those in non-PTL group. However, there were no significant differences in vaginal microbiota among the three groups. Intestinal microbiota in the PTB group was found to differ from that in the non-PTL group using the T-RFLP method.

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Introduction

We harbor more than 100 trillion microbes in and on our body and these microbes constitute our microbiota [1,2]. Although only a small part of this microbiota can be cultured in a medium, culture-independent analyses, like 16S ribosomal ribonucleic acid (rRNA)-based genomic analysis [3,4] and metagenomic analysis [5], have recently revolutionized our understanding of the microbiota in our body. The microbiota in our intestine plays a major role in health and disease [1] in our body. The gut microbiota interacts with the immune system, providing signals to promote the maturation of immune cells and the normal development of immune functions [6,7,8].

Preterm birth (PTB) is the leading cause of perinatal morbidity and mortality in developing and developed countries [9]. The frequency of preterm births is about 12 to 13% in the USA and 5 to 9% in many other developed countries including Japan. Spontaneous PTB is regarded as a syndrome resulting from multiple causes, including intrauterine infection or inflammation, stress, socioeconomic environment, and uterine over-distension. Risk factors for PTB in Western countries include a previous preterm birth, black ethnicity, periodontal disease, low maternal body mass index, short cervical length, and an elevated cervical-vaginal fetal fibronectin concentration, while multiple pregnancy, short cervical length, part-time worker, steroid use for asthma or collagen disease, low educational level, and male fetus were shown to be risk factors for PTB in Japan [10]. Although antibiotic treatment can eradicate bacterial vaginosis (BV) in pregnancy, the overall risk of PTB in pregnant women with BV was found not to be significantly reduced by antibiotic treatment [11].

An association between preterm birth and intestinal microbiota has not been reported using molecular techniques, although the vaginal microbiota changes during pregnancy. Aagaard et al. [12] showed that the vaginal microbiome signature in pregnancy was distinct from that in non-pregnant women with variation of taxa across vaginal subsites and depending on gestational age. Romero et al. reported that, in a longitudinal study, *Lactobacillus* spp. were the predominant members of the microbial community in normal pregnancy [13], and they did not detect a difference in human vaginal microbiota between women who subsequently had a spontaneous preterm delivery and those who delivered at term [14]. Koren et al. described dramatic remodeling of the gut microbiota over the course of pregnancy [15]. These changes of intestinal microbiota induced insulin resistance during pregnancy. Although abnormal vaginal microbiota such as BV was studied using 16S rDNA-based genomic analysis [16], there are no reports on a link between PTB and gut microbiota as determined by genomic analysis.

Terminal restriction fragment length polymorphism (T-RFLP) analysis has used non-targeted approaches to identify differences and similarities in microbial communities, but it does not provide direct sequence information. Because of its relative simplicity, T-RFLP analysis has been applied to the analysis of bacterial 16S rRNA genes and provides a facile means to assess changes in microbial communities [17]. In this prospective and cross-sectional study using T-RFLP analysis, we examined bacterium-derived 16S rRNA genes in feces and vaginal discharge to determine whether the microbiota differs among three groups as follows: pregnant women who delivered term babies without preterm labor (non-PTL group), those who had preterm labor but delivered term babies (PTL group), and those who had preterm birth (PTB group).

Materials and Methods

This study is a prospective and cross-sectional study. The study protocol and informed consent documents were reviewed and approved by the University of Toyama Institutional Review Board. Written informed consent was obtained from all subjects prior to participation in the study. From 2011 to 2013, the participants were categorized into three groups as follows: (1) pregnant women who delivered term babies without preterm labor during pregnancy (non-PTL group) (n = 20), (2) those who had regular uterine contraction and received tocolytic agents but delivered term babies (PTL group) (n = 11), and (3) those who had regular uterine contractions, received tocolytic agents, and finally delivered preterm babies (PTB group) (n = 10). Women who were at earlier than 22 weeks of gestation and those who took any antibiotics, tocolytics and steroids during pregnancy were excluded. Clinical characteristics of all participants are shown in Table 1. Vaginal samples were obtained from the posterior vaginal fornix using a swab. Swabs were immersed in vaginal discharge for 10 seconds, then immediately placed into 3 mL of a buffer medium (100 mM Tris-HCl, pH 9.0, 40 mM Tris-EDTA, pH 8.0, and 4 M guanidine thiocyanate), and stored in a freezer at −20°C. Fecal samples were collected at home or our hospital by the participants. Briefly, the toilet was covered with a sterile sheet of paper, which was temporarily waterproof but would dissolve in the water of the toilet within a few minutes and could be flushed away with the remaining stool. The defecated fecal samples were quickly collected using a sterile spoon or a swab, immediately placed in 3 mL of buffer medium, and stored in a freezer at −20°C.

Terminal restriction fragment length polymorphism (T-RFLP) analysis

In order to investigate the microbiota of the fecal and vaginal samples obtained from all the subjects, terminal restriction fragment length polymorphism (T-RFLP) analysis was performed, as previously reported [18].

Fecal and vaginal samples were suspended in a solution containing 100 mM Tris-HCl, pH 9.0, 40 mM Tris-EDTA, pH 8.0, and 4 M guanidine thiocyanate, and kept at −20°C until deoxyribonucleic acid (DNA) extraction. An aliquot of 0.8 mL of the suspension was homogenized with zirconia beads in a 2.0 mL screw cap tube using a FastPrep FP120A Instrument (MP Biomedicals, Irvine, CA) and placed on ice. After centrifugation (at 5000×g, for 1 min), the supernatant was transferred to the automated nucleic acid isolation system 12GC (Precision System Science, Chiba, Japan). Thereafter, DNA was extracted from the bead-treated suspension using the Magtration-MagaZorb DNA Common Kit 200 N (Precision System Science, Chiba, Japan).

The 16S rDNA was amplified from human fecal DNA using fluorescent-labeled 516f primer (5′-(6-FAM)-TGCCAG-CAGCCGCGGTA-3′) and 1492r primer (5′-GGTTACCTTGT-TACGACTT-3′) and from human vaginal DNA using fluorescent-labeled 27f (5′-(6-FAM)-AGAGTTTGATCCTGGCTCAG-3′) and 1492r primer (5′-GGTTACCTTGTTACGACTT-3′), with Hot-starTaq DNA polymerase using Gene Amp PCR system 9600 (Applied Biosystems, CA, USA). The amplification program used was as follows: preheating at 95°C for 15 minutes; 30 cycles of denaturation at 95°C for 30 seconds, annealing at 50°C for 30 seconds, and extension at 72°C for 1 minute; and finally terminal extension at 72°C for 10 minutes. The amplified DNA was purified using a MultiScreen^{R} PCRμ96 Filter Plate (Millipore, MA, USA) and verified by electrophoresis.

The restriction enzymes were selected according to Nagashima *et al.* [19]. In brief, the PCR product purified from fecal samples was digested with 10 U of *Bsl* I (New England BioLabs, Inc., Ipswich, USA) at 37°C for 3 hours or that from vaginal samples with 10 U of *Msp* I (TAKARA, Shiga, Japan) at 37°C for 3 hours. The resultant DNA fragments, namely, fluorescent-labeled terminal restriction fragments (T-RFs), were analyzed using an ABI PRISM 3130xl genetic analyzer, and their length and peak area were determined using the genotype software GeneMapper (Applied Biosystems).

According to the methods described by Nagashima *et al.* [19], the T-RFs were divided into 29 operational taxonomic units (OTUs) for fecal samples or 22 OTUs for vaginal samples. The OTUs were quantified as the percentage values of individual OTU per total OTU areas, which were expressed as the percent of the area under the curve (%AUC). The bacteria predicted for each classification unit OTU were identified with reference to Human Fecal Microbiota T-RFLP profiling (http://www.tecsrg.co.jp/t-rflp/). To minimize inter- and intra-observer coefficient of variations of the OTU, 1 author (TM) conducted all T-RFLP analyses.

For objective interpretation of the difference in T-RF patterns, cluster analyses were performed using the software SPSS (IBM Statistics, ver. 20.0, NY, USA). T-RF patterns produced by digestion with restriction enzymes (*Bsl* I or *Msp* I) were quantified as the proportion of the total peak area of all T-RFs. The levels of similarity among fecal and vaginal samples were calculated as correlation coefficients, and represented graphically by a scatter plot using principal component analysis.

Table 1. Clinical characteristics of all participants.

Characteristics	Non-PTL (N = 20)	PTL (N = 11)	PTB (N = 10)	Analysis of variance Bonferroni's multiple comparison
Maternal age (year), median (range)	34.0 (27–41)	30.5 (22–37)	33.4 (22–41)	$P = 0.2134$
Gestational weeks at sample collection, average (range)	28.6 (23–34)	28.5 (22–33)	28.0 (22–34)	$P = 0.9351$
Gestational weeks at birth, average (range)	39.2 (37–41)	37.9 (37–40)	33.4 (27–36)	$P = 0.0000$
Previous PTB, percent (n/N)	10.0 (2/20)	9.1 (1/11)	10.0 (1/10)	$P = 0.7897$
Smoking during pregnancy, n/N	0/20	0/11	0/10	-
Parity number, average (range)	0.9 (0–2)	1.1 (0–2)	0.4 (0–2)	$P = 0.1651$
Nulliparous, percent (n/N)	40.0 (8/20)	27.3 (3/11)	70.0 (7/10)	$P = 0.3169$
BMI at sample collection, average (range)	23.3 (17.6–29.1)	23.2 (18.9–29.4)	21.7 (17.0–27.2)	$P = 0.3263$

BMI: Body mass index (kg/m^2).
non-PTL: non-preterm labor.
PTL: preterm labor.
PTB: preterm birth.
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Statistical analysis

All values of OTU are expressed as the mean ± standard error (SE). Comparisons of quantitative data among the three groups were carried out by a multiple comparison technique (Bonferroni). False discovery rates were calculated using Benjamini-Hochberg method. Differences were considered significant at P values of less than 0.05. For multivariate analysis of the data, principal component analysis and cluster analysis were used. The principal component analysis was performed unsupervised.

Results

Of 21 women with preterm labor (the PTL group plus the PTB group), 10 women (47.6%) finally delivered their babies before 37 weeks of gestation.

Principal component analysis (PCA)

Fecal samples. PCA was performed based on the relative abundance of OTUs in fecal samples. The first two principal component scores, which accounted for 19.7% and 10.4% of the total variation, were calculated. Hierarchical clustering of fecal samples on the basis of their first two principal component scores separated the fecal samples into two primary clusters (Figure 1A). One cluster (labeled as 'cluster 1′, thick dotted line, left in Figure 1A) included all 10 cases of the PTB group, as well as 2/20 of the non-PTL group and 7/11 of the PTL group. The other cluster (labeled as 'cluster 2′, thick solid line, right in Figure 1A) was dominated by 90% of the non-PTL group (18/20) and 36.4% of the PTL group (4/11). No PTB cases were included in cluster 2 (0/10). The components of the PTL group occupied an intermediate position between the non-PTL group and the PTB group.

Vaginal samples. In vaginal samples, the first two principal component scores were calculated (the first and second principal components accounted for 18.5% and 15.8% of the total variation, respectively) (Figure 1B). The PCA showed that there was similarity among these three groups.

Terminal restriction fragment length polymorphism profile

The percentages of OTU (percentage values of individual OTU area per total OTU) of representative OTUs for fecal samples after *Bsl* I digestion are shown in Table 2 and those for vaginal samples after *Msp* I digestion in Table 3.

Fecal samples. When comparing respective OTUs of fecal samples between the non-PTL group and the PTB group, the peak areas of the OTU 650, OTU 657, OTU 749, OTU 853, and OTU 955 (digested with *Bsl* I) were significantly different (Table 2). The amounts of *Clostridium* cluster XVIII (OTU 650), *Clostridium* cluster IV (OTU 749), *Bacteroides* (OTU 853), and *Clostridium* subcluster XIVa (OTU 955) were significantly lower in the PTB group than those in the non-PTL group ($P = 0.0125$, $P = 0.0289$, $P = 0.0348$, and $P = 0.0005$, respectively). The amounts of *Clostridium* cluster XVIII and *Clostridium* subcluster XIVa in the PTL group were significantly higher than those in the non-PTL group (P = 0.0449 and P = 0.0125, respectively).

When comparing respective OTUs between the PTL group and the PTB group, the amount of *Clostridium* cluster XI and *Clostridium* subcluster XIVa (OTU 919) was significantly lower in the PTB group than that in the PTL group ($P = 0.0285$).

Vaginal samples. In contrast, the % OTU for vaginal samples after *Msp* I digestion from the non-PTL group was quite similar to that from the PTL or the PTB group (Table 3).

Discussion

In this study, we first showed that the fecal microbiota in the PTL group was substantially different from that in the non-PTL group using T-RFLP analysis. Average scores of OTU 650, OTU 749, OTU 853, and OTU 955 were significantly lower in the fecal microbiota from the PTB group than those from the non-PTL group. Nagashima *et al.* [19] reported that OTU 749 and OTU 955 most likely represent *Clostridium* cluster IV species and *Clostridium* subcluster XIVa species, respectively, as determined by the 16S rDNA clone library method. Taking these findings together, it was revealed that the levels of *Clostridium* cluster XVIII, *Clostridium* cluster IV, *Clostridium* subcluster XIVa, and *Bacteroides* were significantly reduced in the fecal microbiota from the PTB group. Our result first showed that there was a significant difference in the average OTU scores of *Clostridium* cluster XVIII, *Clostridium* cluster IV, and *Clostridium* subcluster XIVa between mothers with non-PTL and those with PTB, while mothers with PTL had intermediate scores between them. This finding does not mean that the intestinal microbiota accidentally

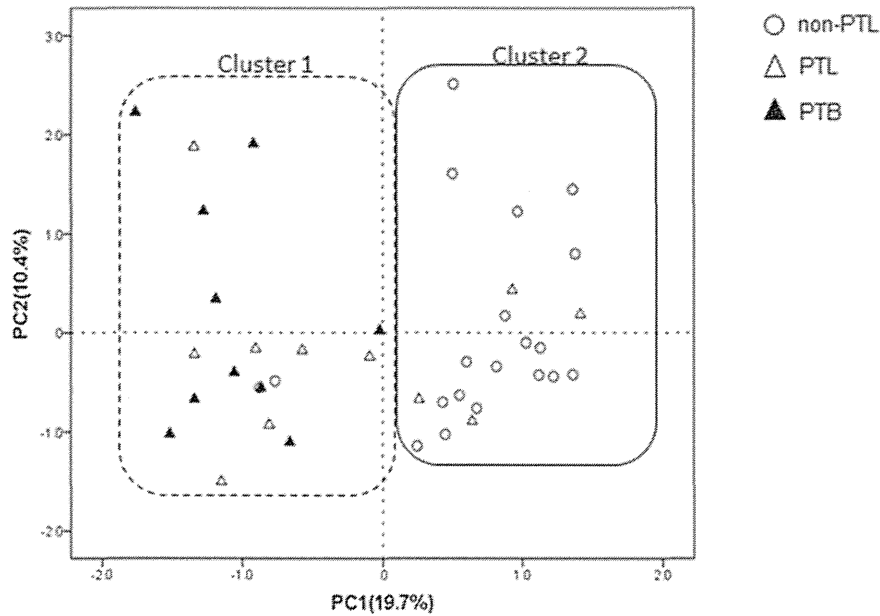

Figure 1B. Principal component analysis for vaginal microbiota

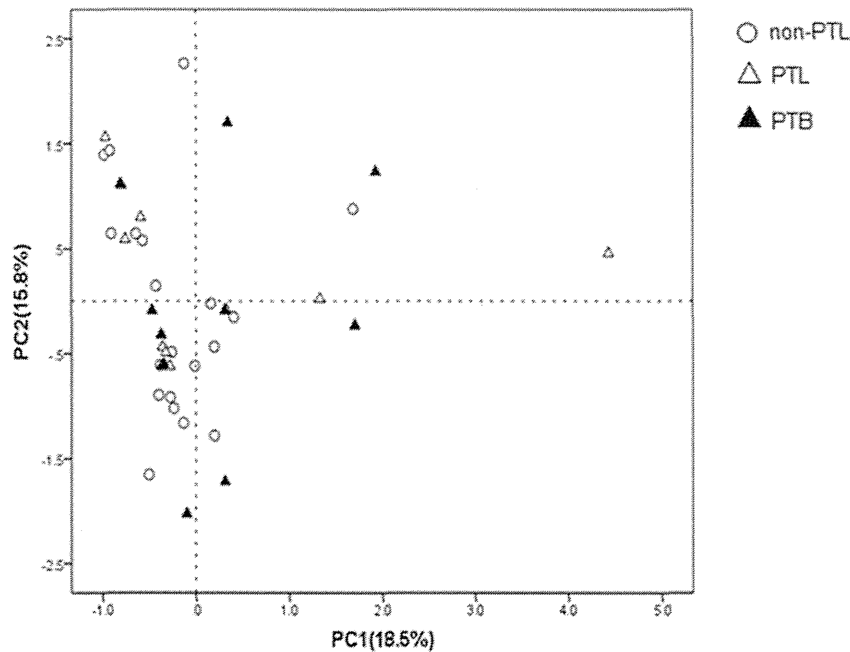

Figure 1. A. Principal component analysis of fecal microbiota. Principal component analysis scores are plotted based on the relative abundance of OTUs of vaginal microbiota. The percentage of variation explained by the principal coordinates is indicated on the axis. Open circles (○) represent the non-PTL group, open triangles (△) the PTL group, and closed triangles (▲) the PTB group. A dotted line, on the left in Figure 1A, shows 'cluster 1', which contains all 10 cases of the PTB group, as well as 2/20 of the non-PTL group and 7/11 of the PTL group. A solid circle, on the right in Figure 1A, shows 90% of the non-PTL group (18/20) and 36.4% of the PTL group (4/11). The PTL group occupied an intermediate position between the non-PTL group and the PTB group. B. Principal component analysis of vaginal microbiota. Principal component analysis scores are plotted based on the relative abundance of OTUs of vaginal microbiota. The percentage of variation explained by the principal coordinates is indicated on the axis. Open circles (○) represent the non-PTL group, open triangles (△) the PTL group, and closed triangles (▲) the PTB group.
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changed in the case of the PTB group, but rather it suggests that some alterations in intestinal microbiota are associated with the clinical findings of mothers with PTB.

Our result is compatible with the report by Romero, who found no differences in human vaginal microbiota between women who subsequently had a spontaneous preterm delivery and those who

Table 2. Average OTU sores in fecal samples.

OTU	Bacteria predicted by T-RF length	Non-PTL (N=20)	PTL (N=11)	PTB (N=10)	Bonferroni's adjustment *P* value	False Discovery Rate *P* value
106	*Clostridium* subcluster XIVa	1.85±0.75	0.33±0.27	0.19±0.13	NS	NS
110	*Clostridium* cluster IV	1.25±0.42	3.89±2.99	3.03±1.30	NS	NS
124	*Bifidobacterium*	11.19±1.98	14.10±4.08	5.91±2.61	NS	NS
168	*Clostridium* cluster IV	0.52±0.15	0.18±0.18	0.08±0.08	NS	NS
317	*Prevotella*	4.58±2.79	0.31±0.16	0.99±0.60	NS	NS
332	*Lactobacillares*	1.29±0.34	1.91±0.60	0.87±0.42	NS	NS
338	*Clostridium* cluster IV	0.57±0.16	0.37±0.19	0.00±0.00	NS	NS
366	*Bacteroides*	3.56±1.13	4.62±1.96	5.71±2.38	NS	NS
369	*Clostridium* cluster IV	0.27±0.18	0.89±0.86	2.76±1.32	NS	NS
469	*Bacteroides*	37.10±3.33	30.44±5.66	22.76±4.44	NS	NS
494	*Clostridium* subcluster XIVa	5.90±1.51	10.49±4.46	10.51±5.04	NS	NS
520	*Lactobacillares*	0.79±0.33	0.18±0.12	0.00±0.00	NS	NS
650	*Clostridium* cluster XVIII	2.12±0.34	0.70±0.35	0.26±0.21	Non-PTL vs. PTB, *P*=0.0016; non-PTL vs. PTL, *P*=0.0150	Non-PTL vs. PTB, *P*=0.0125; non-PTL vs. PTL, *P*=0.0449
657	*Lactobacillares*	5.18±2.54	17.63±8.29	24.24±7.17	Non-PTL vs. PTB, *P*=0.0497	NS (*P*=0.1089)
749	*Clostridium* cluster IV	6.41±1.15	2.90±1.22	0.80±0.80	Non-PTL vs. PTB, *P*=0.0060	Non-PTL vs. PTB, *P*=0.0289
754	*Clostridium* subcluster XIVa	1.70±0.28	1.63±1.03	0.00±0.00	NS	NS
853	*Bacteroides*	0.84±0.13	0.60±0.27	0.07±0.07	Non-PTL vs. PTB, *P*=0.0087	Non-PTL vs. PTB, *P*=0.0348
919	*Clostridium* cluster XI, *Clostridium* subcluster XIVa	1.30±0.23	0.36±0.16	2.06±0.55	PTL vs. PTB, *P*=0.0047	PTL vs. PTB, *P*=0.0285
940	*Clostridium* subcluster XIVa, *Enterobacteriales*	3.79±0.68	3.62±1.10	11.43±3.72	NS	NS
955	*Clostridium* subcluster XIVa	5.37±0.64	1.72±0.80	0.34±0.34	non-PTL vs. PTB, *P*=0.0000; non-PTL vs. PTL, *P*=0.0012	non-PTL vs. PTB, *P*=0.0005; non-PTL vs. PTL, *P*=0.0125
990	*Clostridium* subcluster XIVa	3.61±0.69	2.87±0.86	7.98±6.66	NS	NS
Others		0.83±0.42	0.26±0.20	0.00±0.00	NS	NS

OTU: operational taxonomic unit,
T-RF: terminal restriction fragment.
non-PTL: non-preterm labor.
PTL: preterm labor.
PTB: preterm birth.
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delivered at term. In addition, more recent studies have shown that bacteria from the oral cavity are most often found in the amniotic fluid of patients with preterm labor, demonstrating that periodontal pathogens/byproducts may reach the placenta and spread to the fetal circulation and amniotic fluid [20]. Taken together, these findings may suggest that oral and/or intestinal, not vaginal, microbiota could induce pathogens and the secretion of elevated levels of inflammatory mediators, which in turn may cause premature birth or suggest that dysbiosis caused by oral and/or intestinal, not vaginal, microbiota may render the uterus and/or the placenta susceptible to infection.

Recently, bacterial vaginosis-associated bacteria (BVAB) in the order *Clostridiales:* BVAB1, BVAB2, and BVAB3, were identified in association with bacterial vaginosis [21]. Subsequently, Foxman et al. reported that, in vaginal fluids, one of the Clostridia-like bacteria, BVAB-3, was consistently associated with a reduction in the risk of preterm birth for all ethnic groups (risk ratio, 0.55; 95% confidence interval, 0.39–0.78) [22]. Their result is compatible with our findings in that the level of *Clostridium* was significantly lower in preterm birth, although its level in our studies was lower in the samples not from vaginal fluid but from feces.

Among the microbiota indigenous to the murine and human colon, the genus *Clostridium* belonging to clusters XIVa and IV is reported to be an outstanding inducer of colonic $CD4^+$ $CD25^+$ $Foxp3^+$ regulatory T (Treg) cells [7,8]. *Bacteroides fragilis* has also been shown to induce Treg cells in mouse [23]. It was suggested that polysaccharide A (PSA) produced by *Bacteroides fragilis* potentiates the suppressor activity of Treg cells. The balance between beneficial and potentially harmful species in the commensal microbial community, known as dysbiosis, has often been linked to the development of inflammatory bowel disease (IBD) in humans and analogous intestinal inflammation in mice [18]. Importantly, intestinal Treg cells play a key role in regulating inflammation by the production of IL-10 [8,23,24,25] and decreased intestinal Treg cells were observed in IBD patients. Preterm labor may be viewed as inflammation caused by

Table 3. Average OTU scores in vaginal samples.

OTU	Bacteria predicted by T-RF length	non-PTL (N = 20)	PTL (N = 11)	PTB (N = 10)	*P* value
57	Unknown	8.78±1.77	12.58±4.12	10.12±2.61	NS
74	*Enterococcus*	0.47±0.27	0.36±0.31	0.72±0.49	NS
93	*Chitinophagaceae*	1.20±0.53	4.25±2.74	4.16±2.41	NS
98	*Prevotella*	0.54±0.24	2.39±2.19	5.45±2.30	NS
134	*Bifidobacterium*	0.89±0.86	0.05±0.05	2.23±1.78	NS
147	*Firmicutes*	2.37±1.56	0.28±0.13	3.94±3.55	NS
163	*Actinobacteria*	3.17±1.67	0.01±0.01	5.78±4.93	NS
170	*Firmicutes*	0.33±0.17	0.74±0.35	0.53±0.28	NS
179	*Lactobacillus*	27.42±8.09	34.34±12.45	25.40±8.72	NS
189	*Lactobacillus*	43.41±8.38	39.68±13.11	29.01±10.90	NS
230	*Firmicutes*	0.41±0.15	0.59±0.38	0.37±0.25	NS
281	*Actinobacteria*	6.34±4.12	1.16±1.04	2.95±2.95	NS
300	*Clostridiales*	1.03±0.59	0.08±0.08	5.73±4.40	NS
563	*Streptococcus*	0.18±0.15	0.78±0.78	0.92±0.41	NS
573	*Lactobacillus*	3.24±1.29	2.39±1.35	2.62±0.91	NS
Others		0.20±0.15	0.33±0.33	0.05±0.05	NS

OTU: operational taxonomic unit.
T-RF: terminal restriction fragment.
non-PTL: non-preterm labor.
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inappropriately expanded components of the normal bacterial community. In IL-10 knockout mice, a very small amount of lipopolysaccharide (LPS) induces preterm delivery [26]. These findings suggest that decreased intestinal *Clostridium* cannot induce an adequate volume of Treg cells, resulting in susceptibility to inflammation. Indeed, decreased Treg cell volume and function in the peripheral blood have been reported in preterm labor [27,28,29,30,31], although the level of intestinal Treg cells is unclear in these papers and this study. Further studies are needed to clarify the relationship between intestinal microbiota and peripheral Treg cells or intestinal Treg cells.

The peak area of the 919-bp OTU (*Clostridium* cluster XI, *Clostridium* subcluster XIVa) in the PTB group was significantly larger than that in the PTL group ($P = 0.0285$). This is a limitation of the T-RFLP method. We should thus reevaluate the bacterial species using metagenomic analysis.

Several limitations were identified in this study. Because of the small number of cases (fewer than 30) in each group, information that can only come from patient participation in well-designed clinical trials is needed to improve the management of PTB. Second, we did not check the level of Treg cells in peripheral blood and therefore could not compare the relationship between the fecal microbiota of the mother and the level of Treg cells. Further investigation is needed to address whether the difference in gut microbiota is related to the expression of immunoregulatory Treg cells or inflammation-induced Th17 cells that are induced by gut *Clostridium* strains and segmented filamentous bacteria, respectively. Third, it is possible that the unidentified confounders may have an effect on changes in the microbial communities. An alternative interpretation is that, as Aagaard et al. [12] reported, the vaginal microbial 16S rRNA gene catalogue may uniquely differ in pregnancy, with variation of taxa across vaginal subsites and depending on gestational age. Further study is required to determine the relationship between the difference in average OTU for some phylotypes and PTB. Fourth, owing to the cross-sectional nature of the study, our finding about the causal relationships between fecal microbiota and PTB should be interpreted with caution. Fifth, we didn't measure the concentrations of organic acids, indole, and ammonia. And we also didn't measure pH and moisture and didn't compare these values with those from normal pregnancy. Sixth, the selection of optimal T-RFLP probes is a subject of considerable ongoing discussion in the field of microbiome research. T-RFLP analysis only identify differences and similarities in microbial community and the resulting OTUs aren't assigned to a specific species. Unfortunately, there is no perfect set of T-RFLP probes for fecal and vaginal microbiome. Yet, the T-RFLP data can readily be analyzed using various statistical algorithms to quantitatively ascertain similarities and differences among communities and to infer plausible community of intestine and vagina. Seventh, we are unable to grasp the importance of the results that patients had preterm babies had a higher average OTU for Lactobacillares phylotypes than those who delivered term babies. Yet, the identification of significant differences in average OTUs provides evidence that the study of the intestinal microbiota during pregnancy can yield important insights into the relationship between the fluctuation of microbial communities and adverse pregnancy outcome like PTB. Further studies are required to confirm this finding and elucidate the role of intestinal microbiota in PTB. Eighth, the principal component analysis is not a prefect technique to analyze the T-RFLP data. Therefore, the performance of principal component analysis should be interpreted with caution since there were no validation samples to test.

Many studies have been performed to identify the differences in microbial diversity between healthy individuals and patients with rheumatoid arthritis (RA) [32], inflammatory bowel disease (IBD) [33], and type 1 diabetes [34]. However, the results about the fecal level of *Clostridium* in these patients are conflicting (decrease [32],

no change [33], and increase [34]). When abnormality in fecal microbiota in the PTL group is found, the question arises of whether microbiotic alteration, dysbiosis, is a cause or a result of preterm delivery. We speculate that the changes in the immune system through fecal dysbiosis may change the uterine activity. In addition, the underlying mechanisms resulting in alteration of the microbiota remain to be clarified. Recently, intake of probiotic food was found to be associated with a reduced risk of spontaneous preterm delivery and preeclampsia [35,36]. Prospective studies are needed to clarify whether intestinal dysbiosis before pregnancy might cause uterine inflammation and induce uterine contraction or cervical ripening during pregnancy.

Conclusion

Disturbance of the intestinal flora, dysbiosis, during pregnancy was first observed in the PTB group in this study. This may cause inflammatory reactions in the uterus leading to PTB. Further study is needed to clarify the relationship between PTB and dysbiosis of intestinal bacterial flora.

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Author Contributions

Conceived and designed the experiments: AS SY NY RY TM GS SS. Performed the experiments: AS SY NY RY TM GS SS. Analyzed the data: AS TM GS SS. Contributed reagents/materials/analysis tools: AS SY NY RY TM GS SS. Contributed to the writing of the manuscript: AS TM GS SS.

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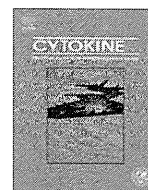

Extensive serum biomarker analysis in patients with enterohemorrhagic *Escherichia coli* O111-induced hemolytic-uremic syndrome

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ABSTRACT

Proinflammatory cytokines are related to the pathogenesis of enterohemorrhagic *Escherichia coli* infection and hemolytic-uremic syndrome (HUS). We employed an antibody array that simultaneously detects 174 serum cytokines. We identified five serum biomarkers, namely insulin growth factor-binding protein-2, angiopoietin-2, soluble interleukin-6 receptor, soluble tumor necrosis factor receptor type II, and matrix metalloprotease protein-3 whose levels increased with the development of HUS. Furthermore, the levels of these cytokines were significantly increased in severe HUS compared with mild HUS. These cytokines might play an important role in the pathogenesis of HUS and may also be used to predict the severity of HUS.

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1. Introduction

Hemolytic-uremic syndrome (HUS) is a multisystem disease characterized by the triad of microangiopathic hemolytic anemia, thrombocytopenia, and renal failure. HUS occurs after a prodrome of hemorrhagic colitis (HC) caused by Shiga toxin-producing *Escherichia coli*, also known as enterohemorrhagic *E. coli* (EHEC) [1,2]. The most prevalent serotype is EHEC O157 however EHEC O111 can also cause these complications [3,4]. An outbreak of EHEC O111 occurred in Toyama and other prefectures in Japan between late April and early May 2011. The courses in some patients were extremely aggressive and some cases were fatal [3].

Some laboratory parameters including increased number of white blood cells, decreased serum sodium and total protein levels, and increased serum alanine aminotransferase levels have been used for predicting the severity of HUS [5,6]. Recently, however, roles of several cytokines and chemokines in EHEC-induced HUS have been identified [7–15].

Enzyme-linked immunosorbent assay (ELISA)-based methods are considered to be the most robust platforms for biomarker discovery and are known for their high degree of sensitivity [16]. Recent advancement in protein array technology has created a high-throughput platform for biomarker screening by ELISA. In this study, we employed the Raybiotech C-Series 2000 antibody array system, an antibody array that simultaneously detects 174 cytokines, to identify potential predictive markers for EHEC-induced HUS.

We report the identification of five serum biomarkers, namely insulin growth factor-binding protein (IGFBP)-2, angiopoietin-2 (Ang-2), soluble interleukin-6 receptor (sIL-6R), soluble tumor necrosis factor receptor type II (sTNFRII), and matrix metalloprotease protein-3 (MMP-3), that were significantly elevated in patients with developing and severe HUS.

2. Materials and methods

2.1. Patients and samples

Serum samples were obtained from nine patients during the outbreak of EHEC O111 HUS that occurred in Toyama, Japan

Abbreviations: EHEC, enterohemorrhagic *Escherichia coli*; HUS, hemolytic-uremic syndrome; HC, hemorrhagic colitis; ELISA, enzyme-linked immunosorbent assay; IGFBP, insulin growth factor-binding protein; IL, interleukin; TNF, tumor necrosis factor; sTNF-RII, soluble tumor necrosis factor receptor type II; MMP, matrix metalloprotease protein.

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Table 1
Clinical characteristics of patients with EHEC O111/HUS.

Case	Age (years)	Sex	STx1	STx2	ARF	Encephalopathy	Severity	Outcome
1	7	M	–	+	+	+	Severe	Died
2	14	M	–	–	+	+	Severe	Died
3	7	F	+	+	+	+	Severe	Alive
4	7	F	–	+	+	+	Severe	Alive
5	26	F	+	+	+	+	Severe	Alive
6	13	M	+	+	–	+	Severe	Alive
7	6	F	–	+	–	–	Mild	Alive
8	8	F	–	+	–	–	Mild	Alive
9	16	M	+	+	–	–	Mild	Alive

between late April and early May 2011. Samples from six patients with EHEC O111 infection were serially obtained from the HC phase to the HUS phase. The clinical characteristics of patients with HUS are shown in Table 1. EHEC O111 infection was diagnosed when one or both of the following criteria were fulfilled: bloody diarrhea, vomiting, or bowel cramps with microbiological identification of EHEC O111 infection. The presence of HUS was defined by thrombocytopenia (platelet count of <150,000/mm^3), hemolytic anemia, and acute renal dysfunction. Acute renal dysfunction was defined as renal injury evidenced by hematuria, proteinuria, or elevated creatinine levels (⩾1.0 mg/dl in children aged <13 years, ⩾1.5 mg/dl in patients ⩾13 years old, ⩾50% increase over baseline) [17]. The severity of HUS was classified according to Gianantonio's criteria as follows: (i) mild, no anuria; (ii) moderate, <7 days of anuria; or (iii) severe, ⩾7 days of anuria [18]. One patient (case 2) did not have anuria but died of acute encephalopathy with diffuse brain edema 2 days after the diagnosis of HUS. This patient was classified as having severe HUS. Another patient (case 6) also did not have anuria but suffered from acute encephalopathy, acute lung injury, and acute pancreatitis. This patient was also classified as having severe HUS.

Three patients were classified as having mild HUS and six were classified as having severe HUS. No patient was classified as having moderate HUS. Serum samples from the patients were separated, divided into aliquots, frozen, and stored at −80 °C until analysis. This study was approved by the Institutional Review Board of Kanazawa University, and all specimens were used after the receipt of informed patient consent.

2.2. Quantification of serum cytokines

RayBio human cytokine antibody array (C-Series 2000 antibody array, RayBiotech, Norcross, GA) was used for detection of 174 cytokines in frozen stock sera of the patients with EHEC O111 induced HUS, according to the manufacturer's protocol. Relative levels were determined by the ratio of intensity of each sample to that of the internal positive control. The intensity of each sample and control was measured using the software image processing and analysis in Java (Image J).

Serum levels of IGFBP2 and sTNFRII were evaluated by ELISA according to the manufacturer's instructions (IGFBP2: RayBiotech, Inc., Norcross, GA, sTNF-RII: R&D Systems, Minneapolis, MN, USA).

2.3. Statistical analysis

Within-group comparisons were analyzed using the Mann–Whitney *U*-test or paired *t* test. A *p*-value of <0.05 was considered statistically significant.

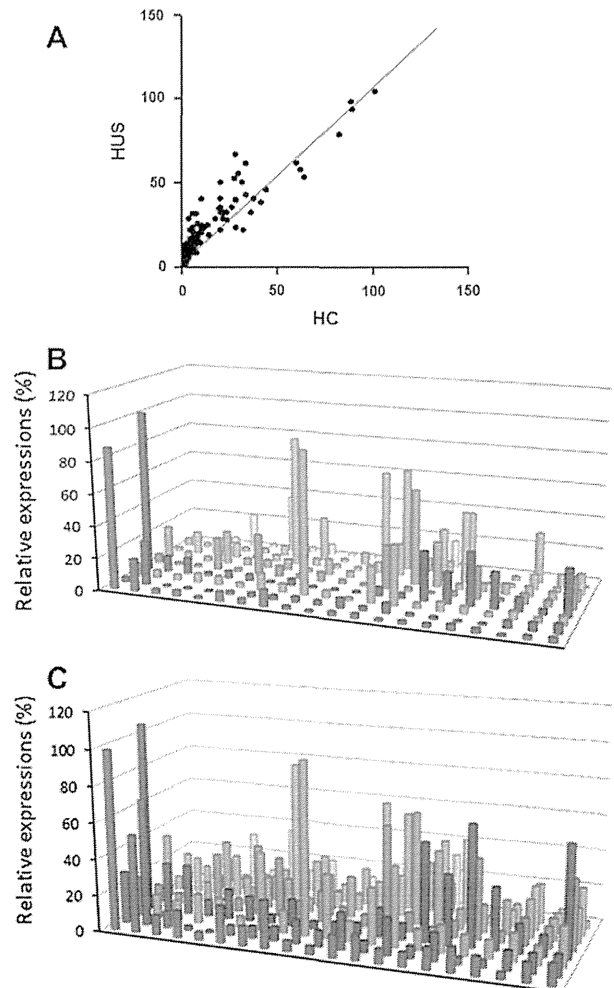

Fig. 1. Changes in the relative levels of 174 cytokines with the development of hemolytic-uremic syndrome (HUS). The relative levels were determined by the ratio of intensity of each sample to that of internal positive control. (A) Plot of the mean relative density values of the 174 cytokines during the hemorrhagic colitis (HC) phase and the HUS phase. (B) Mean relative density values of 174 cytokines of six patients during the HC phase. (C) Mean relative density values of 174 cytokines of nine patients during the HUS phase. The mapping of each bar in Fig. 1B and C are shown in Supplementary Table 1.

3. Results

3.1. The levels of 126 out of 174 cytokines increased with the development of HUS

The levels of 174 cytokines were analyzed during the HC phase ($n = 6$) and the HUS phase ($n = 9$) and the mean relative density

values for each group were shown as plot files (Fig. 1A). The levels of 126 out of the 174 cytokines significantly increased during the HUS phase (Fig. 1A–C) compared with the HC phase (Fig. 1A and B). These 126 cytokines were as follows: BDNF, BLC, BMP-4, CKβ8-1, eotaxin-3, FGF-6, FGF-7, Flt-3 ligand, fractalkine, GCP-2, GDNF, I-309, IFN-γ, IGFBP-1, IGFBP-2, IGFBP-4, IL-15, IL-16, IL-1α, IL-1β, IL-1ra, IL-3, IL-4, IL-5, IL-6, IL-7, Leptin, MCP-2, MCP-3, MCP-4, M-CSF, MDC, MIG, MIP-1δ, MIP-3α, NT-3, PARC, SCF, SDF-1, TGF-β1, TGF-β3, TNF-α, TNF-β, AgRP, Ang-2, amphiregulin, Axi, bFGF, bNGF, BTC, CTACK, Dtk, EGF-R, ENA78, Fas/TNFRSF6, FGF-4, FGF-9, GITR, GRO, HCC-4, ICAM3, IGFBP-6, IGF-I SR, IL-1R4/ST2, IL-1R1, IL-11, IL-12p40, IL-12p70, IL-17, IL-2Rα, IL-6R, IL-8, MIF, MIP-1α, MIP-1β, MIP-3β, MSP-α, NT-4, osteoprogerin, oncostatin M, PIGF, sgp130, sTNFRII, sTNFRI, thrombopoietin, TRAIL R3, TRAIL R4, uPAR, VEGF, VEGF D, ActivinA, CD80, BMP-5, BMP-7, cardiotrophin-1, CXCL-16, endoglin, ErbB3, Fas ligand, IL-1RII, IL-10Rβ, IL-13Rα2, IL-18BPα, IL-18Rβ, MMP-3, IL-2Rβ, IL-2Rγ, IL-21R, IL-5Rα, IP-10, LeptinR, LIF, MMP-1, MPIF-1, PDGFAA, PDGFRα, PECAM-1, Prolactin, Siglec-5, TGF-α, TGF-β2, Tie-1, Tie-2, TIMP-4, VE-cadherin, VEGF R2, and VEGF R3.

3.2. Five cytokines were associated with the severity of EHEC O111-induced HUS

To identify potential predictive markers for the severity of EHEC-induced HUS, we evaluated the differences of the levels of these 126 cytokines between the three mild and the six severe cases during the HUS phase. The levels of 11 cytokines including IGFBP-1, IGFBP2, IL-5, IL-6, NT-3, Ang-2, IGFBP-6, sIL-6R, sTNFRII, and MMP-3 significantly increased during the HUS phase in patients with severe HUS compared with those with mild HUS (Fig. 2). We then evaluated how the levels of these 11 cytokines changed from the HC phase to the HUS phase in four patients with severe HUS. The levels of IGFBP2, Ang-2, sIL-6R, sTNFRII, and MMP-3 increased significantly with the development of severe HUS (Fig. 3). To verify these results, we measured serum levels of IGFBP2 and sTNFRII by ELISA. Serum levels of IGFBP2 and sTNFRII significantly increased during the HUS phase in patients with severe HUS compared with those with mild HUS (Supplemental Fig. 1).

4. Discussion

Previous reports have demonstrated that proinflammatory cytokines play an important role in the pathogenesis of EHEC infection and development of severe complications such as HUS [7–15]. In this study, we employed the Raybiotech C-Series 2000 antibody array system and identified five serum cytokines (IGFBP-2, Ang-2, sIL-6R, sTNFRII, and MMP-3) that were significantly elevated in patients with severe HUS. The levels of these five cytokines also increased with the development of HUS. Our findings indicate that these particular cytokines play an important role in the pathogenesis of EHEC infection and the development of HUS. Furthermore, these cytokines might be useful biomarkers to predict the severity of EHEC-induced HUS.

The IGF system plays a key role in the regulation of cellular proliferation, differentiation, and apoptosis [19]. The IGF system comprises the ligands IGF-I and IGFII, their receptors, and a family of six high-affinity IGF-binding proteins (IGFBPs) [19,20]. Most of

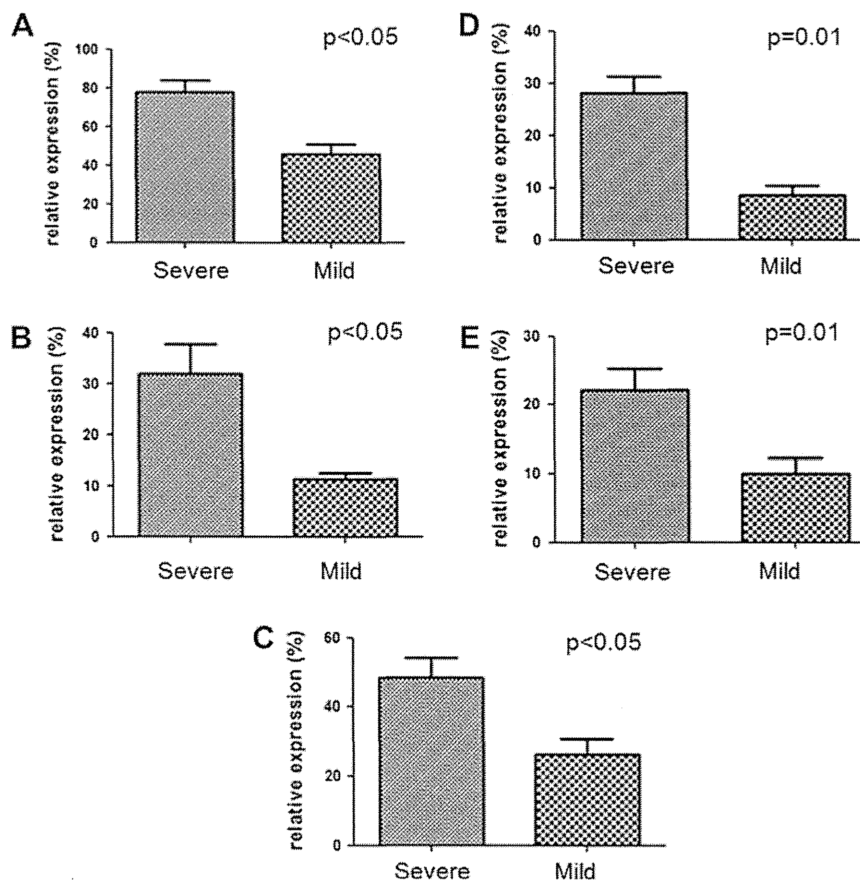

Fig. 2. The levels of five cytokines identified during severe and mild hemolytic-uremic syndrome ((A) IGFBP-2, (B) Angiopoietin-2, (C) sIL-6R, (D) sTNFRII, and (E) MMP-3).

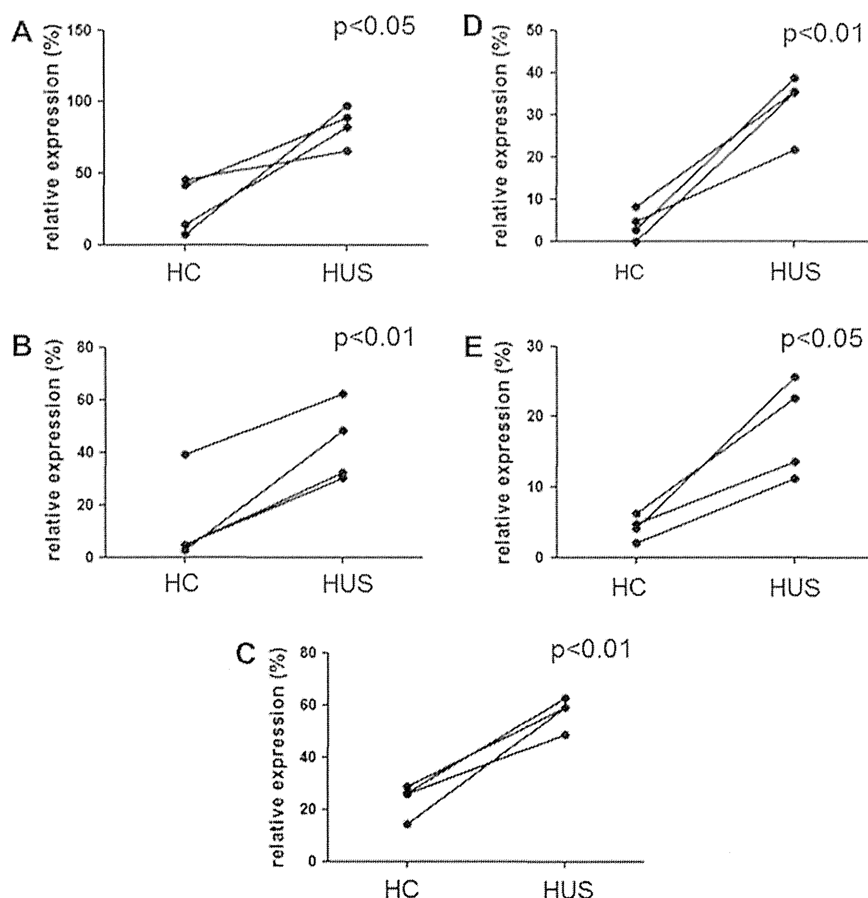

Fig. 3. The changes in levels of five cytokines between hemorrhagic colitis (HC) and hemolytic-uremic syndrome (HUS) in four patients with severe HUS ((A) IGFBP-2, (B) Angiopoietin-2, (C) sIL-6R, (D) sTNFRII, and (E) MMP-3).

the biological actions of the IGF are modulated by the IGFBPs. IGFBP-2 is the second most abundant binding protein in the circulation [20–23]. Although the regulation and physiological roles of IGFBP-2 are less well defined, an association with metabolic homeostasis and insulin sensitivity has been reported [24]. In addition to these roles in metabolism, IGFBP-2 has an important role in cell proliferation/survival, migration, and adhesion, through the interaction with critical genes in tumorigenesis [23]. Interestingly, IGFBP-2 is also associated with angiogenesis and enhances VEGF gene promoter activity [25]. Serum IGFBP-2 levels are elevated under conditions where the total IGF-I plus IGF-II level exceeds the capacity of IGFBP-3 including growth hormone deficiency, with an IGF-II-secreting tumor or following IGF-I administration. A number of disease states in critically ill patients are also characterized by an elevated serum IGFBP-2 level [26]. Although there are certainly nutritional influences on IGFBP-2, it is not clear whether the elevation seen in critical illness is primarily related to nutritional disturbance or to a direct cytokine effect. Mice injected with IL-1α or TNF-α show no change in serum IGFBP-2. However, intravenous injection of *E. coli* endotoxin into healthy humans elicits an increase in IGFBP-2 over several hours [27,28]. Furthermore, IGFBP-2 mRNA shows a dramatic increase by 24–48 h following either CNTF or IL-1 beta injection in a rat model of central nervous system injury [29]. The role of IGFBP-2 in the pathogenesis of HUS is still unknown, but IGFBP-2 might have pleiotropic effects through the interactions with other cytokines.

Ang-1 and Ang-2 are important regulators of endothelial cell function and competitive ligands of the endothelial Tie-2 receptor [30]. Ang-1 is produced constitutively and promotes endothelial cell quiescence. Ang-2 is stored in endothelial cells and leads to endothelial activation upon release by noxious or inflammatory stimuli. In healthy states, circulating Ang-1 levels exceed those of Ang-2, and the Ang-2/Ang-1 ratio is low. Recently, dysregulation of Ang-1 and Ang-2 has been reported in EHEC O157:H7 infection and HUS [31]. In patients with EHEC O157-induced HUS, the Ang-2/Ang-1 ratio increases as a result of decreased Ang-1 levels and increased Ang-2 levels. These findings indicate that endothelial dysfunction, manifested as dysregulation of Ang-1 and Ang-2, could underlie HUS pathophysiology and the Ang-2/Ang-1 ratio might be a useful indicator for the severity of HUS. Although we did not evaluate Ang-1 levels, the increased Ang-2 levels in this study support this finding.

IL-6 performs a prominent role during disease and has been described as both a pro-inflammatory and anti-inflammatory cytokine. IL-6 responses are regulated by sIL-6R which forms a ligand–receptor complex with IL-6 that is capable of stimulating a variety of cellular responses including proliferation, differentiation, and activation of inflammatory processes. Increased sIL-6R levels have been documented in several clinical conditions indicating that its production is coordinated as part of a disease response [32–34]. Thus, sIL-6R has the potential to regulate both local and systemic IL-6-mediated events. Previous reports including have shown that serum IL-6 levels are significantly elevated in patients with severe HUS and increase with the development of HUS, consistent with the findings of the present study [8,14,15]. In this study, IL-6 levels were significantly elevated during the HUS phase

in patients with severe HUS compared with those in patients with mild HUS. IL-6 levels tended to increase with the development of HUS in severe cases, although this was not statistically significant because the number of patients in our study is small. On the other hand, sIL-6R levels were significantly increased during the HUS phase in patients with severe HUS compared with those in patients with mild HUS and significantly increased with the development of HUS in severe cases. These findings indicate that IL-6 might play an important role in the pathogenesis of HUS.

The pro-inflammatory cytokine TNF-α also has a prominent role during HUS. For example, TNF-α increases vascular permeability, injures vascular endothelial cells, and induces necrosis of myelin and oligodendrocytes. sTNFR is a shedding form of the extramembranous domain of the TNF receptor that can interfere with the function of TNF-α. It has been reported that sTNFR is the natural homeostatic regulator of the action of TNF-α and that its level, rather than TNF-α levels, reflects the true biological activity of the cytokine. We previously reported that serum sTNFRII levels are significantly elevated in patients with severe HUS and increase with the development of HUS [15]. We also demonstrated that patients with acute encephalopathy showed elevated TNF-α levels during HUS [15]. In the present study, sTNFRII levels significantly increased during the HUS phase in patients with severe HUS compared with those in patients with mild HUS, and they were significantly increased with the development of HUS in severe cases. These findings indicate that TNF-α might play an important role in the pathogenesis of HUS.

MMPs are a group of proteolytic enzymes that can degrade the principal components of the extracellular matrix. On the basis of these degradation activities, MMPs are widely believed to play a central role in tissue degradation. MMPs influence many basic processes, such as cell proliferation, differentiation, angiogenesis, and apoptosis [35]. Notably, MMP family proteins elicit dual roles in the pathogenesis of inflammation, stimulating protective innate and/or adaptive immune functions as well as tissue destruction [36]. MMP-3 overexpression is associated with tissue destruction in the context of chronic inflammation. For example, MMP-3 is involved in extracellular matrix breakdown and tissue remodeling in affected joints of patients with rheumatoid arthritis (RA) and serum MMP-3 levels are widely used in clinics for RA patients as a disease activity marker [37]. In addition to the role of tissue degradation, MMP-3 has a role as a regulator of inflammation through the interaction with inflammatory cytokines [38,39]. The role of MMP-3 in the pathogenesis of HUS is still obscure, but MMP-3 might have pleiotropic effects on the pathogenesis of HUS.

A limitation of the present study was the small number of patients with EHEC O111/HUS. A study with a larger sample including other serotypes of EHEC infection with the correction for multiple comparison, may help define the true diagnostic value of these markers. Despite this limitation, our results indicate that IGFBP-2, Ang-2, sIL-6R, sTNFRII and MMP-3 might play important roles in the pathogenesis of HUS. These cytokines may also be useful to predict the severity of HUS.

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Appendix A. Supplementary material

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.cyto.2013.12.005.

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Effects of campaign for postpartum vaccination on seronegative rate against rubella among Japanese women

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Abstract

Background: Japan experienced two rubella outbreaks in the past decade (2004 and 2012 – 2013), resulting in 10 and 20 infants with congenital rubella syndrome (CRS), respectively. This study was performed to determine whether the seronegative rate was lower in multiparous women than in primiparous women in Japan.

Methods: Hemagglutination inhibition (HI) test results during pregnancy were analyzed retrospectively in 11048 primiparous and 9315 multiparous women who gave birth at six hospitals in northern Japan in the 5-year study period (January 2008 through December 2012). Women with HI titer < 1:8 were defined as susceptible to rubella.

Results: The seronegative rate was significantly lower in multiparous than primiparous women aged 30 – 31 years (2.3% [22/967] vs. 4.5% [66/1454], $P = 0.0036$), 36 – 37 years (3.4% [55/1601] vs. 5.7% [79/1389], $P = 0.0030$), and overall women (3.8% [350/9315] aged 34.7 ± 5.2 vs. 5.4% [597/11048] for 33.2 ± 5.9, $P < 0.001$). The susceptible fraction size did not differ largely according to hospital, ranging from 3.5% to 6.3%. Those for each year did not change markedly; 4.5% [150/3369], 5.2% [221/4268], 4.4% [195/4412], 4.6% [186/4056], and 4.6% [195/4258] for 2008, 2009, 2010, 2011, and 2012, respectively. Those for teenagers were consistently high: 22.7% [5/22], 20.7% [6/29], 20.6% [7/34], 13.0% [3/23], and 23.5% [4/17] for 2008, 2009, 2010, 2011, and 2012, respectively.

Conclusions: The seronegative rate was significantly lower in multiparous than primiparous women. However, Japanese rubella vaccination programs were insufficient to eliminate CRS.

Keywords: Congenital rubella syndrome, Outbreak, Vaccine

Background

Public health concern regarding rubella stems from the teratogenic effects that can result from congenital rubella infection, particularly during the first trimester of pregnancy. Japan experienced a rubella outbreak in 2004, in which 10 infants contracted congenital rubella syndrome (CRS) [1]. Supplemental immunization activity targeting adult women and population immunity surveys were strengthened since the outbreak in 2004. Japanese guidelines for obstetric practice recommend determination of immunity status against rubella with hemagglutination inhibition (HI) test during the first trimester and postpartum vaccination in women with low titer of HI test results (≤ 16×) [2].

However, a rubella outbreak occurred again in Japan in 2012 – 2013 [3]. The total number of rubella patients in Japan during the first 9 months of 2013 was 14077 (108 per 1000000 population, 69% of cases were serologically confirmed) [3]. Among 14077 patients in this outbreak, vaccination status was unknown in 8973 patients. Of 5104 patients with known vaccination status, 924 (18.1%) had been vaccinated, while 4180 (81.9%) had not been vaccinated [4]. The majority of rubella cases occurred among adults aged 18 years or older: male and female adults aged 18 years or older accounted for 71.7% and 19.8% of all 14077 cases, respectively [3]. Consequently, 20 infants (1.8 per 100000 live births)

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were diagnosed with CRS during the 12-month period between October 2012 and September 2013 in Japan [3].

The present retrospective and multicenter study was conducted to determine whether the experience of prior birth influenced seronegative rate against rubella among pregnant Japanese women and to assess how many pregnant Japanese women were susceptible to rubella during the rubella outbreak that occurred in Japan in 2012 – 2013.

Methods

This study was conducted after being approved by the Institutional Review Boards of Hokkaido University Hospital, Kitasato University Hospital, National Center for Child Health and Development, Nippon Medical School Tama-Nagayama Hospital, Jichi Medical University Hospital, and Toyama University Hospital.

This retrospective study included 20363 women, all of whom fulfilled the following criteria: rubella immunity was determined in pregnancy by HI test and gave birth during the 5-year period between January 2008 and December 2012 at one of following six hospitals located in northern Japan: Hokkaido University Hospital, Toyama University Hospital, Jichi Medical University Hospital, National Center for Child Health and Development, Nippon Medical School Nagayama Hospital, and Kitasato University Hospital (Table 1). A portion of the results obtained in this study regarding the overall fraction of pregnant women susceptible to rubella was described elsewhere previously [3].

Titer of rubella antibody determined with HI test was expressed as < 8×, 8×, 16×, 32×, 64×, 128×, 256×, 512×, 1024×, and > 1024×. Women with HI titer < 8× were defined as having no immunity against rubella (susceptible to rubella) in this study. The correlation between HI titer (x, [Log2]) and titer (y, IU/mL [Log2]) determined by enzyme immunoassay (Siemens Healthcare Japan, Tokyo, Japan) is as a follows [5]: $y = 0.736x + 1.6377$.

All data are presented as means ± SD. For statistical analysis of categorical data, Fisher's exact test was applied. The statistical software package StatView 5.0 for Macintosh (SAS Institute Inc. Cary, NC) was used for all data analyses. In all analyses, $P < 0.05$ was taken to indicate statistical significance.

Table 1 Regions (prefectures) and numbers of pregnant women tested

Regions (prefectures)	Direction from Tokyo	No. of women
A (Hokkaido)	830 km N	1450
B (Toyama)	260 km NW	803
C (Tochigi)	100 km N	2467
D (Tokyo 1)	15 km W*	7329
E (Tokyo 2)	40 km W*	3642
F (Kanagawa)	35 km W*	4672

N, north; NW, northwest; W, west; *, from Japan Railroad Tokyo Station.

Results

Regional differences in rubella immunity among pregnant women

Regional differences in size of the fraction susceptible to rubella (HI titer < 8×) were relatively small: 3.5% (86/2467) in Region C (Tochigi), 3.5% (257/7329) in Region D (Tokyo 1), 4.1% (33/803) in Region B (Toyama), 5.4% (78/1450) in Region A (Hokkaido), 5.6% (263/4672) in Region F (Kanagawa), and 6.3% (230/3642) in Region E (Tokyo 2) (Figure 1). Overall, 4.7% (947/20363) of all pregnant women were susceptible to rubella. Overall fractions with HI titer 8×, 16×, 32×, 64×, 128×, 256×, 512×, and ≥ 1024× were 4.5% (908/20363), 11.0% (2250/20363), 21.5% (4381/20363), 25.5% (5201/20363), 18.7% (3800/20363), 10.1% (2063/20363), 3.5% (715/20363), and 0.5% (98/20363), respectively.

Fraction of pregnant women susceptible to rubella according to maternal age and year

The seronegative rates differed greatly between six groups divided according to maternal age (Figure 2). Those for younger women were consistently high: 22.7% [5/22], 20.7%[6/29], 20.6%[7/34], 13.0%[3/23], and 23.5% [4/17] for teenagers, and 11.9%[17/143], 14.3%[28/196], 10.7%[18/168], 13.5%[19/141], and 12.1%[17/140] for women aged 20 – 24 years in 2008, 2009, 2010, 2011, and 2012, respectively. These values decreased with advancing maternal age, and this trend did not vary with time. Overall seronegative rates according to maternal age were as follows [3]: 19.8% [25/126] for women

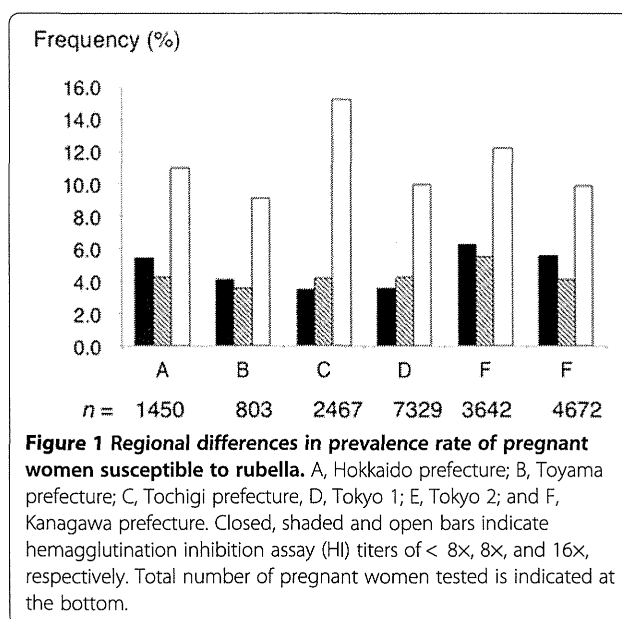

Figure 1 Regional differences in prevalence rate of pregnant women susceptible to rubella. A, Hokkaido prefecture; B, Toyama prefecture; C, Tochigi prefecture, D, Tokyo 1; E, Tokyo 2; and F, Kanagawa prefecture. Closed, shaded and open bars indicate hemagglutination inhibition assay (HI) titers of < 8×, 8×, and 16×, respectively. Total number of pregnant women tested is indicated at the bottom.