**Association Between Neutralizing Antibody Titers against Parechovirus A3 in Maternal and Cord Blood Pairs and Perinatal Factors**

**（パレコウイルス A3 型に対する母体血、臍帯血の中和抗体価と周産期因子との関連）**


## ABSTRACT

***Background.*** Parechovirus A3(PeV-A3) is a pathogen that causes severe infectious diseases such as sepsis and meningoencephalitis in neonates and young infants. This study aimed to measure neutralizing antibody titers (NAT) against PeV-A3 in paired maternal blood and cord blood samples and to clarify the serum epidemiology of PeV-A3 as well as the association among NAT and perinatal factors.

***Methods.*** NAT against PeV-A3 were measured in 1033 mothers (maternal blood and cord blood pairs; total 2066 samples) who delivered in Fukushima Prefecture between December 2013 and June 2014. RD-18S cells were used to measure NAT against PeV-A3. The association among NAT against PeV-A3 in maternal blood and cord blood and perinatal factors was determined using multivariate logistic regression analysis.

***Results.*** The median gestational age of the infants was 39 weeks 4 days (interquartile range: 38 weeks 4 days–40 weeks 3 days). NAT against PeV-A3 in maternal blood and cord blood were almost the same. The rate of low titer group (NAT ≤ 1:16) was approximately 70% and that of high titer group tended to increase with gestational age. The rate of high titer group and geometric mean titer decreased with increased maternal age.

***Conclusions.*** Neonates born to shorter gestational age and older mothers have low NAT against PeV-A3 in cord blood. Thus, more attention should be paid to the onset of severe PeV-A3 disease in such neonates and young infants.

**Keywords:** parechovirus A3; The Japan Environment and Children's Study; neutralizing antibody; severe infectious disease; perinatal factors

**Abbreviation:**

PeV-A3: parechovirus A3

NAT: Neutralizing Antibody Titers

JECS: the Japan Environment and Children's Study

GA: gestational age

MEM: Eagle's minimum essential medium

FBS: fetal bovine serum

MM: maintenance medium

CPE: cytopathic effect

$TCID_{50}$: the 50% tissue culture infective dose

HTG: high titer group

LTG: ligh titer group

GMT: geometric mean titer

IQR: interquartile range

aOR: adjusted odds ratio

## INTRODUCTION

Parechoviruses (PeVs) are non-enveloped RNA viruses within the Parechovirus genus of the Picornaviridae family and mainly cause gastroenteritis and respiratory infections in children [1]. To date, 17 genotypes of PeVs have been identified; the most common genotypes isolated from patients in Japan are Parechovirus A1(PeV-A1), A3(PeV-A3), and A6 [2]. Ito et al. first reported PeV-A3 in Japan in 2004 [3]. PeV-A3 has recently attracted attention due to causing severe infectious diseases with sepsis and meningoencephalitis in neonates and young infants [4–9]. The onset of severe infectious disease is thought to be associated with a lack of or low levels of maternal antibody [10–12]. To date, there have been no reports on neutralizing antibody titers (NAT) against PeV-A3 in maternal blood and cord blood pairs. This study aimed to clarify the serum epidemiology of NAT against PeV-A3 and to determine the association among the NAT and perinatal factors in mothers and their neonates. Our results may help predict the onset of infection and prevent severe PeV-A3 disease in neonates and young infants.

## MATERIALS AND METHODS

### Participants

This study was conducted by Fukushima Regional Center as an adjunct study of the Japan Environment and Children's Study (JECS). Paired maternal blood and cord blood samples of 1033 mothers (total 2066 samples) who delivered in Fukushima Prefecture between December 2013 and June 2014 were analyzed. Information about this study was provided on our web site and via newsletters mailed to all participants in the JECS. Mothers did not consent to participate in this study were excluded from the study (opt-out system). This study was approved by the Ministry of the Environment Government of Japan and the ethics committee of the Fukushima Medical University (number #2124).

### Perinatal background

The perinatal background was obtained from JECS dataset. Maternal background included maternal age, gestational age (GA), maternal smoking (defined as smoking when pregnancy had become apparent and/or during pregnancy), maternal obesity (defined as body mass index ≥ 25 kg/m$^2$ prior to pregnancy), the number of deliveries, mode of delivery, and obstetric complications. Neonatal information included birth weight, sex, the number of siblings, Apgar score, umbilical arterial blood pH, and morbidity at 6 months (Subjects diagnosed with non-PeV-A3 infections such as respiratory syncytial virus, rotavirus, and adenovirus infections were excluded). In addition, serum IgG levels in the maternal blood and cord blood were measured by a commercial company (SRL, Inc. Tokyo, Japan) using enzyme-linked immunosorbent assay.

**Viruses and cells**

*Virus Strains*

PeV-A3 1356-Yamagata-2008 strain was used for analysis and was cultured in RD-18S cells. These viruses were stored at −80°C until completion of the neutralization test.

*Cell Culture*

RD-18S cells were used for replicating PeV-A3 strain. Eagle's minimum essential medium (MEM) (Thermo Fisher Scientific, Waltham, MA USA) comprising 10% heat-inactivated fetal bovine serum (FBS), glutamine (600 μg/mL), penicillin (100 U/mL), gentamicin (50 μg/mL), and amphotericin B (1 μg/mL) was used as a growth medium for RD-18S cells, which were cultured at 37°C in 5% $CO_2$ for 5 days.

*Virus Titration*

Virus was serially diluted using maintenance medium (MM), which was MEM with 5% FBS. The diluted virus solution was inoculated on RD-18S cells in a 96-well microplate and cultured for 5 days in MM. The cytopathic effect (CPE) was observed by microscope. The virus titer was determined according to the 50% tissue culture infective dose ($TCID_{50}$).

*Neutralization Test*

Serum samples were inactivated at 56°C for 30 min and serially mixed in two-fold dilutions from 1:4 to 1:1024 using MM. 200 $TCID_{50}$/0.05 mL virus was added per well and incubated at 37°C in 5% $CO_2$ for 2 h. Confluent monolayers of the RD-18S cells were prepared in a 96-well microplate, and the incubated virus–serum mixture was added to each well. Subsequently, these plates were cultured at 34°C in 5% $CO_2$ for 5 days. The CPE was assessed by microscope. NAT were determined as the reciprocal of the highest serum dilution that inhibited the CPE by 50%. The titer for preventing the onset of severe PeV-A3 disease was set at 1:32, as previously reported [11]. Serum samples were classified according to NAT into the high titer group (HTG) (NAT ≥ 1:32) or the low titer group (LTG) (NAT ≤ 1:16).

**Statistical analysis**

Statistical analysis was performed using Stata/SE 14 (Stata Corp. LLC, Texas, USA). The association between NAT against PeV-A3 in maternal blood and cord blood was examined using Kappa statistics. The rate of HTG was compared using the chi-square test and geometric mean titer (GMT) was compared using the Kruskal–Wallis test and Mann–Whitney test. Categorical variables and continuous variables between LTG and HTG were compared using the chi-square test and the Mann–Whitney test. The association among NAT against PeV-A3 and perinatal factors was evaluated by multivariate logistic regression analysis. $P < 0.05$ was considered statistically significant.

**RESULTS**

**Clinical Characteristics of mothers and neonates**

NAT were measured in 1033 maternal blood and cord blood pairs. The median maternal age was 30 years [interquartile range (IQR): 27–34 years)]; median GA was 39 weeks 4 days (IQR: 38 weeks 4 days–40 weeks 3 days); and median birth weight was 3064 g (IQR: 2790–3305 g). In total, 612 mothers (59.2%) had previously given birth, and 175 mothers

(16.9%) delivered by cesarean section. (Table 1).

**NAT in maternal blood and cord blood**

The rate of HTG and GMT in maternal blood and cord blood were 27.3% and 9.1 and 28.7% and 8.3, respectively. There was no significant difference in the rate of HTG and GMT between the maternal blood and cord blood. The NAT was ≤1:16 in approximately 70% of mothers and neonates. In addition, NAT in maternal blood and cord blood samples were almost the same using the Kappa statistic (kappa coefficient 0.95, $p < 0.01$).

**NAT by maternal age and GA**

When the data were stratified by maternal age, the rate of HTG and GMT decreased with increased maternal age, these were significant in both maternal blood and cord blood. There was no difference in the rate of HTG and GMT between maternal blood and cord blood, which was excepted for the groups of ≥35 years of age in GMT (Table 2.1). When the data were stratified by GA and analyzed with multiple comparisons, the rate of HTG in cord blood in the group of ≥40 weeks was higher than that in the group of 37–39 weeks. There was no difference in the rate of HTG and GMT between maternal blood and cord blood, which was excepted for the group of 37–39 weeks in GMT (Table 2.2).

**Differences in Perinatal Factors between LTG and HTG**

The number of maternal blood samples in the LTG and HTG were 751 (72.7%) and 282 (27.3%), respectively. The median maternal age in the LTG and HTG was 31 years (IQR: 28–35 years) and 28 years (IQR: 25–31 years), respectively. The median GA in the LTG and HTG was 39 weeks 4 days (IQR: 38 weeks 4 days–40 weeks 3 days) and 39 weeks 6 days (IQR: 39 weeks 0 days–40 weeks 3 days), respectively. Pregnancy-induced hypertension was observed in 4.0% and 1.1% of subjects in LTG and HTG, respectively. There were no significant differences between the LTG and HTG with serum IgG levels, maternal smoking, maternal obesity, sex, and the number of siblings.

The number of cord blood samples in the LTG and HTG were 737 (71.3%) and 296 (28.7%), respectively. The median maternal age in the LTG and HTG was 31 years (IQR: 28–35 years) and 28 years (IQR: 25–31, years), respectively. The median GA in the LTG and HTG was 39 weeks 3 days (IQR: 38 weeks 4 days–40 weeks 2 days) and 39 weeks 6 days (IQR: 39 weeks 0 days–40 weeks 4 days), respectively. The median serum IgG level in the LTG and HTG was 1165 mg/dL (IQR: 1005–1365 mg/dL) and 1268 mg/dL (IQR: 1077–1440 mg/dL), respectively. There were no significant differences between the LTG and HTG with maternal smoking, maternal obesity, pregnancy-induced hypertension, sex, and the number of siblings (Table 3).

**Association between NAT and Perinatal Factors by Multivariate Logistic Regression Analysis**

Multivariate logistic regression analysis revealed significant differences between the LTG and HTG in maternal blood with maternal age [adjusted odds ratio (aOR): 0.89, 95% confidence interval (CI): 0.86–0.92], serum IgG levels (aOR: 1.00, 95% CI: 1.00–1.01) and number of siblings (aOR: 1.22, 95% CI: 1.02–1.45). There were significant differences between the LTG and HTG in cord blood with maternal age (aOR: 0.90, 95% CI: 0.87–0.93), GA (aOR: 1.16, 95% CI: 1.01–1.33), and serum IgG levels (aOR: 1.00, 95% CI: 1.00–1.01) (Table 4).

**DISCUSSION**

To the best of our knowledge, this is the first study to evaluate NAT against PeV-A3 in maternal blood and cord blood pairs. This study revealed the serum epidemiology of NAT against PeV-A3 in neonates and the association among NAT and perinatal factors because many perinatal factors were simultaneously investigated with large sample size. In conclusion, the factors associated with NAT against PeV-A3 in cord blood are maternal age, GA, and serum IgG level.

Severe enterovirus infections in neonates are associated with low NAT [12] and a lack of

maternal antibodies is a risk factor for severe infectious disease [13]. Maternal antibody plays an important role in preventing severe PeV-A3 disease [11]. To date, the prevalence of PeV-A3 antibody has been reported in Japan (Aichi and Niigata Prefectures), Finland, and the Netherlands [3,14,15], and the seroprevalence was found to be 74%, 61%, 13%, and 10%, respectively. In this study, the rates of HTG and GMT to PeV-A3 decreased with increased maternal age in both maternal blood and cord blood, which was consistent with previous studies. PeV-A1 has also been reported to cause gastroenteritis and respiratory infection in children; however, there are few reports of severe infectious disease in neonates and young infants [16, 17]. An epidemiological study in Finland revealed that 95% of neonates had neutralizing antibody against PeV-A1 [18]. Watanabe et al. reported the NAT against both PeV-A1 and PeV-A3. The rate of HTG against PeV-A1 peaked at 90% by 3 years of age and remained to be high with no decrease after 35 years of age. However, in the PeV-A3 began to decrease at 35 years of age [14]. Globally, PeV-A3 is epidemic every 2–3 years and PeV-A1 is epidemic every year [19]; therefore, NAT against PeV-A3 may decrease because of reduced reinfection or boosting. In addition, because the viral protein region (the antigenic determinant of PeV-A3) is always changing, the latest strains of PeV-A3 cannot be neutralized by serum from older age [20]. These reasons were considered to be the decrease in NAT against PeV-A3 with age.

Generally, the maternal antibody transfer is believed to begin at around the GA of 16 weeks and shows a linear increase; thereafter, the antibody levels in term infants are similar to or higher than those in mothers. Multivariate analysis showed a positive correlation between the serum IgG level and NAT and suggested that there is a certain amount of PeV-A3-antibody in the maternal antibody. In addition, there was also a positive correlation between NAT and GA in cord blood which suggested that transplacental transfer including PeV-A3-antibody from mother to the fetus increases as GA prolongs (Table 4). We hypothesized that NAT against

PeV-A3 would show significant differences between maternal blood and cord blood in premature infants. However, there were no differences between maternal blood and cord blood samples in NAT in premature infants (Table 2.2). The reason for no significant difference was believed to be that almost all premature infants (median: 36 weeks 0 day, IQR: 33 weeks 4 days–36 weeks 5 days) were late-preterm infants; therefore, further research is required comprising cases with shorter GA.

Some studies have found that PeV-A3 infection occurs predominantly in males [21–23], but there was no difference with sex between the HTG and LTG in this study.

PeV-A3 tends to infect young children, and going to nursery school and kindergarten is believed to be risk factors for infection. Many children who developed severe PeV-A3 disease had sick contacts within the family [24]. Recently, an asymptomatic PeV-A3-infected child reportedly transmitted the virus to family members [25], and the adults with low NAT are infected with PeV-A3 and they may also be the source of infection. In our study, the number of siblings was examined as neonatal information and there was association between them and NAT in maternal blood in multivariate analysis. Because mothers have an increased risk of reinfection in PeV-A3 from children with increase in the number of their children, NAT may therefore increase.

The strengths of this study are the large sample size, the use of maternal and cord blood paired sample, and data on many perinatal variables. Low NAT in cord blood could be distinguished into impaired placental transmission or inadequate production of antibody in mother, owing to the use of paired samples.

However, this study has some limitations. First, we could not track whether infants with low NAT in cord blood actually had PeV-A3 infection. Second, because the proportion of late-preterm infants in premature birth was high, it could not completely clarify the association between premature birth and NAT in cord blood.

We conclude that NAT against PeV-A3 in maternal blood and cord blood are almost the same in full-term infants. Infants born to shorter GA and older mothers may have low NAT against PeV-A3. Therefore, more attention should be paid to the onset of severe PeV-A3 diseases.

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Table 1. Subject Characteristics (1033 mothers and neonates)

| Characteristic | Number |
|---|---|
| Maternal age (years), median (IQR) | 30 (27–34) |
| Gestational age (weeks-days), median (IQR) | 39-4 (38-4–40-3) |
| Multipara, n (%) | 612 (59.2) |
| Delivery type: cesarean section, n (%) | 175 (16.9) |
| Gestational diabetes mellitus, n (%) | 13 (1.1) |
| Pregnancy-induced hypertension, n (%) | 33 (3.3) |
| Maternal smoking, n (%) | 193 (18.8) |
| Premature birth, n (%) | 43 (4.2) |
| Birth weight (g), median (IQR) | 3064 (2790–3305) |
| Low birth weight infant, n (%) | 80 (7.7) |
| Male, n (%) | 521 (50.4) |
| Number of siblings in neonates, median (IQR) | 1 (0–1) |
| 5-min Apgar score, median (IQR) | 9 (9–9) |
| Morbidity at 6 months of age, n (%) | 236 (23.4) |

Abbreviations: IQR, interquartile range

Table 2.1. The Rate of High Titer Group and Geometric Mean Titer in Maternal Blood and Cord Blood by Maternal Age

| Maternal age (n) | | < 25years (125) | 25–29 years (347) | 30–34 years (337) | ≥ 35years (224) | p value |
|---|---|---|---|---|---|---|
| The rate of HTG, % (n) | MB | 44.0 (55) | 34.0 (118) | 22.6 (76) | 14.7 (33) | <0.01[a] |
| | CB | 44.8 (56) | 36.9 (128) | 23.1 (78) | 15.2 (34) | <0.01[a] |
| | p value (MB/CB) | 0.90[a] | 0.43[a] | 0.85[a] | 0.90[a] | — |
| GMT (95% CI) | MB | 15.0 (11.2–20.2) | 11.7 (10.0–13.8) | 7.9 (6.8–9.2) | 5.8 (4.8–7.1) | <0.01[b] |
| | CB | 15.4 (11.2–21.2) | 11.5 (9.6–13.8) | 7.0 (5.9–8.3) | 4.6 (3.7–5.7) | <0.01[b] |
| | p value (MB/CB) | 0.75[c] | 0.70[c] | 0.14[c] | 0.03[c] | — |

Abbreviations: HTG, high titer group; MB, maternal blood; CB, cord blood; GMT, geometric mean titer; CI, confidence interval

[a]chi-square test, [b]Kruskal–Wallis test, [c]Mann–Whitney test

Table 2.2. The Rate of High Titer Group and Geometric Mean Titer in Maternal Blood and Cord Blood by Gestational Age

| Gestational age (n) | | A < 37 weeks (43) | B 37–39 weeks (574) | C ≥ 40 weeks (416) | p value (multiple comparisons) | | |
|---|---|---|---|---|---|---|---|
| | | | | | A/B | B/C | A/C |
| Maternal age (years), median (IQR) | | 32 (28–35) | 30 (27–34) | 30 (27–33) | 0.21[a] | 0.21[a] | 0.04[a] |
| The rate of HTG, % (n) | MB | 20.9 (9) | 24.7 (142) | 31.5 (131) | 1.00[b] | 0.07[b] | 0.63[b] |
| | CB | 23.3 (10) | 24.7 (142) | 34.6 (144) | 1.00[b] | <0.01[b] | 0.55[b] |
| | p value (MB/CB) | 0.80[b] | 1.00[b] | 0.34[b] | — | — | — |
| GMT (95% CI) | MB | 8.3 (5.2–13.2) | 8.9 (7.9–10.0) | 9.5 (8.1–11.2) | 0.91[a] | 0.90[a] | 0.86[a] |
| | CB | 6.3 (3.8–10.4) | 7.7 (6.7–8.8) | 9.5 (8.0–11.4) | 0.66[a] | 0.27[a] | 0.36[a] |
| | p value (MB/CB) | 0.36[c] | 0.04[c] | 0.78[c] | — | — | — |

Abbreviations: MB, maternal blood; HTG, high titer group; GMT, geometric mean titer, CB; cord blood; CI, confidence interval

[a]Kruskal–Wallis test, [b]chi-square test, [c]Mann–Whitney test

Table 3. Comparison of Low Titer Group (NAT ≤ 1:16) with High Titer Group (NAT ≥ 1:32)

| Perinatal factor | Maternal blood | | | Cord blood | | |
|---|---|---|---|---|---|---|
| | LTG | HTG | p value | LTG | HTG | p value |
| Number | 751 | 282 | | 737 | 296 | |
| Maternal age (years), median(IQR) | 31 (28–35) | 28 (25–31) | <0.01 | 31 (28–35) | 28 (25–31) | <0.01 |
| GA(weeks-days), median(IQR) | 39-4 (38-4–40-3) | 39-6 (39-0–40-3) | <0.01 | 39-3 (38-4–40-2) | 39-6 (39-0–40-4) | <0.01 |
| IgG (mg/dl), median (IQR) | 826 (706–942) | 842 (727–958) | 0.11 | 1165 (1005–1365) | 1268 (1077–1440) | <0.01 |
| Maternal smoking, n (%) | 138 (18.5) | 55 (19.6) | 0.67 | 133 (18.1) | 60 (20.6) | 0.40 |
| Maternal obesity, n (%) | 75 (10.3) | 28 (10.2) | 0.96 | 69 (9.6) | 34 (12.2) | 0.32 |
| PIH, n (%) | 30 (4.0) | 3 (1.1) | 0.02 | 28 (3.8) | 5 (1.7) | 0.08 |
| Male, n (%) | 383 (51.0) | 138 (48.9) | 0.56 | 374 (50.8) | 147 (49.7) | 0.75 |
| Number of siblings (No.), median (IQR) | 1 (0–1) | 1 (0–1) | 0.64 | 1 (0–1) | 1 (0–1) | 0.71 |

Abbreviations: LTG, low titer group (NAT ≤ 1:16); HTG, high titer group (NAT ≥ 1:32); IQR, interquartile range; GA, gestational age; PIH, pregnancy-induced hypertension. Note: Categorical variables were compared between the LTG and HTG using chi-square test. Continuous variables were compared using the Mann–Whitney test.

Table 4. Results of Multivariate Logistic Regression Analysis Showing Associations between Perinatal Factors for High Neutralizing Antibody Titers.

| Perinatal factor | Maternal blood | | | Cord blood | | |
|---|---|---|---|---|---|---|
| | aOR | 95% CI | p value | aOR | 95% CI | p value |
| Maternal age | 0.89 | 0.86–0.92 | <0.01 | 0.90 | 0.87–0.93 | <0.01 |
| GA | 1.11 | 0.99–1.24 | 0.06 | 1.16 | 1.01–1.33 | 0.03 |
| IgG | 1.00 | 1.00–1.01 | 0.01 | 1.00 | 1.00–1.01 | 0.02 |
| Maternal smoking | 0.96 | 0.66–1.40 | 0.84 | 1.05 | 0.70–1.57 | 0.81 |
| Maternal obesity | 1.02 | 0.63–1.66 | 0.92 | 1.23 | 0.73–2.05 | 0.44 |
| PIH | 0.31 | 0.09–1.07 | 0.06 | 0.35 | 0.10–1.24 | 0.10 |
| Male | 0.94 | 0.70–1.26 | 0.67 | 1.06 | 0.77–1.47 | 0.71 |
| Number of siblings | 1.22 | 1.02–1.45 | 0.03 | 1.21 | 1.00–1.47 | 0.06 |

Abbreviations: aOR, adjusted odds ratio; CI, confidence interval; GA, gestational age; PIH, pregnancy-induced hypertension.

Note: Low titer group (NAT ≤ 1:16) was set as 0 and high titer group (NAT ≥ 1:32) was set as 1 for the objective variables were examined.