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Human Parechovirus 1, 3 and 4 Neutralizing Antibodies in Dutch Mothers and Infants and Their Role in Protection Against Disease

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Background: Human parechoviruses (HPeVs) are common pathogens in young children, and in the Netherlands, HPeV1, HPeV3 and HPeV4 are the most frequently detected genotypes. HPeV3 in particular has been associated with severe disease in young infants below 3 months of age while the other genotypes more often infect older children and elicit mild symptoms. We investigated if maternal neutralizing antibodies (nAbs) against HPeV1, HPeV3 and HPeV4 protect young Dutch infants from severe disease related to HPeV infection.

Methods: We conducted a prospective case–control study of Dutch mother–infant pairs. Thirty-eight HPeV-infected infants and their mothers were included as cases, and 65 HPeV-negative children and their mothers as controls.

Results: In control infants, we observed nAb seropositivity rates of 41.4%, 33.3% and 27.6%, with median nAb titers of 1:16, 1:12 and 1:8, against HPeV1, HPeV3 and HPeV4, respectively. In control mothers, nAb seropositivity rates were 84.6%, 55.4% and 60.0% with median nAb titers of 1:128, 1:32 and 1:45 against HPeV1, HPeV3 and HPeV4, respectively. The HPeV3 nAb seroprevalence was significantly lower in HPeV3-infected infants and their mothers (0.0% with $P < 0.05$ and 10.0% with $P < 0.001$, respectively). In contrast, no differences in nAb seroprevalence against HPeV1 or HPeV4 could be detected between case and control infants or mothers.

Conclusions: Our results suggest that young Dutch infants are protected against severe disease related to HPeV1 and HPeV4 by maternal nAbs, but less so against HPeV3 explaining the distinct age distributions and disease severity profiles of children infected with these HPeV genotypes.

Key Words: human parechovirus, maternal antibodies, case–control study

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Human parechoviruses (HPeVs), belonging to the Picornaviridae family, are associated with mild respiratory and gastrointestinal disease, meningitis, encephalitis, sepsis-like illness (SLI) and myocarditis.[1] Based on the viral capsid protein (VP1) nucleotide sequence diversity, HPeVs are classified into 17 genotypes of which HPeV1, 3 and 4 are the most frequently detected types in the Netherlands.[1,2] HPeV1 and HPeV4 elicit mild disease in children 1–5 years of age, while HPeV3 causes severe disease in infants younger than 3 months of age.[3,4]

Humoral immune responses are essential in protection against and clearance of enteroviruses (EVs), a picornavirus genus structurally and clinically related to HPeVs.[5,6] For neonates, lack of transplacental maternal antibodies is a risk factor for developing severe EV infection.[5] Previously, we detected a seroprevalence of 99%–92% for HPeV1 and 60%–62% for HPeV4, but only 10%–13% for HPeV3, in Finnish and Dutch adults. In contrast, HPeV1 neutralizing antibody (nAb) seropositivity rates of 80%–100% and 60%–80% for HPeV3 have been reported in Japan.[7–9] A recent report showed that at disease onset HPeV3-infected Japanese infants were seronegative for HPeV3 nAbs suggesting that maternal antibodies protect neonates against disease related to HPeV3.[10]

Given the distinct age distributions of children infected with different HPeV genotypes and the contrasting HPeV3 nAb seroprevalence levels in Europe and Japan, we investigated the role of HPeV-specific maternal antibodies in a Dutch patient cohort. Our aim was to determine if maternal nAbs can protect young infants against severe disease related to HPeV1, HPeV3 and HPeV4 infection.

MATERIALS AND METHODS

Study Design

This was a prospective multicenter case–control study of mother–child pairs in the Netherlands. We adhered to the tenets of the Declaration of Helsinki, and informed consent was obtained from all parents. The study was approved by the investigational review board at the Academic Medical Center in Amsterdam.

Infants, defined as children younger than 1 year of age, with a clinical suspicion of a viral infection in whom viral diagnostic tests were performed, were included in the study together with their biologic mothers. Mother–child pairs were included as cases if the child was polymerase chain reaction (PCR)-positive for HPeV in one or more of the following clinical samples: feces, blood (serum or ethylenediaminetetraacetic acid (EDTA)/heparin plasma), cerebrospinal fluid (CSF), nasopharyngeal aspirate or throat swab. Control mother–child pairs were children tested PCR-negative for HPeV in any clinical sample. Patients were included between July 2008 and November 2012 in the following hospitals: Academic Medical Center Amsterdam (n = 62), Amstelland Hospital Amstelveen (n = 12), Zuwe Hofpoort Hospital Woerden (n = 20), Meander Medical Center Amersfoort (n = 7), University Medical Center Utrecht (n = 1) and Free University Medical Center Amsterdam (n = 1).

Within 1 week after diagnostic testing of the children, feces for HPeV PCR testing and blood for nAb detection were collected from the mothers. Exclusion criteria were prematurity before a gestational age of 34 weeks and maternal use of intravenous immunoglobulins (IVIGs) during pregnancy. Demographics, data on clinical signs and symptoms, use of antibiotics, the presence and site of isolation of other microorganisms and diagnosis at discharge were collected from the patient's files and discharge letters. Clinical symptoms were defined as gastrointestinal symptoms (diarrhoea, nausea and/or vomiting), respiratory symptoms (rhinorrhoea, cough, otitis, tonsillitis, dyspnoea, tachypnoea, wheezing, inspiratory stridor and/or abnormalities on chest radiography), central nervous system symptoms and signs (irritability, seizures and/or paralysis) and/or meningitis [defined as >19 leukocytes/μL in CSF for children 0–28 days of age and >9 leukocytes/μL in CSF for children 29 days of age or older[11] with or without elevated protein level (0.35 g/L) and/or decreased glucose level (<2.8 mM)] and/or encephalitis, skin changes (rash and/or mottled skin) and SLI. SLI was defined as signs of respiratory or circulatory dysfunction [tachycardia or bradycardia, low blood pressure and/or decreased saturation rate (<95%)]. Fever was defined as a body temperature above 38.5°C, and severe disease as having SLI, meningitis and/or encephalitis or myocarditis and/or pericarditis.

HPeV Detection and Genotyping

RNA from all clinical specimens was isolated by automatic extraction using the MagnaPure LC instrument (Roche Diagnostics GmbH, Almere, Netherlands). RNA was reverse-transcribed and HPeV was detected by real-time PCR targeting the 5′ untranslated region of the viral genome.[12] For HPeV genotyping, sample RNA was reverse-transcribed and PCR-amplified using HPeV VP1-specific primers.[13] The amplicons were sequenced using the BigDye Terminator Cycle Sequencing Ready Reaction Kit and automated sequencer (Applied Biosystems, Foster City, CA).

HPeV Strains and Cell Lines

HPeV1 Harris strain was obtained from the National Institute of Public Health and the Environment (RIVM, Bilthoven, The Netherlands). HPeV4-K251176 strain was isolated from a Dutch clinical specimen in 2002.[14] HPeV1 and HPeV4 were cultured in HT29 cells (human colorectal adenocarcinoma; ATCC, Manassas, VA). HPeV3-251825 strain, isolated from a Dutch clinical specimen in 2010, was cultured in LLCMK2 cells (rhesus monkey kidney cell line, kindly provided by the Municipal Health Services, Rotterdam, the Netherlands). HT29 and LLCMK2 cells were maintained in Eagle's minimum essential medium (Lonza, Basel, Switzerland) supplemented with 8% heat-inactivated fetal bovine serum (Sigma-Aldrich, St. Louis, MO), streptomycin (100 μg/mL, Bio Whittaker; Lonza, Basel, Switzerland), penicillin (100 U/mL, BioWhittaker; Lonza, Basel, Switzerland), nonessential amino acids (ScienCell Research Laboratories, Carlsbad, CA) and L-glutamine (200 nM; Lonza, Basel, Switzerland). We recently observed that chloroform treatment renders HPeV3 more susceptible for in vitro neutralization.[13] HPeV3 virus stock was therefore chloroform-treated as follows: 10% (v/v) chloroform (Sigma-Aldrich, St. Louis, MO) was added to each virus culture, vortexed vigorously for 5 minutes and removed by centrifugation for 10 minutes at 3000 rpm. The 50% cell culture infective dose of virus stocks was determined and calculated by using the formula by Reed and Muench.[15]

In Vitro Neutralization Assay

Two-fold serial dilutions of the serum or plasma samples were incubated with an equal volume of hundred 50% cell culture infective dose virus at 37°C, 5% CO_2, for 1 hour. HT29 (HPeV1 and HPeV4) or LLCMK2 (HPeV3) in Eagle's minimum essential medium supplemented with 8% fetal bovine serum were subsequently added and plates were incubated at 37°C, 5% CO_2 for 7 days. The nAb titer was calculated on the basis of the number of wells showing cytopathogenic effect using the method by Reed and Muench[15] and reported as the reciprocal titers of serum dilutions that exhibited 50% neutralization. A nAb titer of ≥1:32 was set as a cut-off level for seropositivity as this has been correlated with protection against HPeV-related illness and used in recently published reports.[8,10] Undetectable nAb titers were assigned as 1:2 for median titer calculations. If children received IVIG, only samples taken before IVIG treatment were evaluated.

Statistical Analysis

Data were analyzed using SPSS for windows, version 23 (IBM Corp. Armonk, NY). Categorical variables were compared by χ^2 or Fisher exact test. Normally distributed continuous variables were compared using student *t* test and one-way analysis of variance. For unequally distributed continuous variables, the Kruskal–Wallis and Mann–Whitney *U* test were used. Ninety-five percent confidence intervals of the seroprevalence rates were calculated according to the E.B. Wilson method, using the VassarStats website for Statistical Computation.[16] Linear regression analysis was performed to test correlations. Two-tailed *P* values <0.05 were considered to be significant.

RESULTS

Study Population Demographics

A total of 103 mother–infant pairs were included in this study, of which 38 HPeV-infected infants (including 1 twin) and their mothers were defined as cases, and 65 HPeV-negative children and their mothers as controls. Feces, CSF, blood, nasopharyngeal aspirate or throat swab was tested for HPeV presence (number of positive/tested samples) in 102/103 (99%), 40/103 (39%), 17/103 (17%) and 24/103 (23%) of the children, respectively. HPeV and EV double infections in stool samples were included in the HPeV group (6 children), unless EV was detected as the only pathogen in CSF or blood (1 child). Feces samples of 24 case mothers were available for HPeV PCR detection. In the case group, most children were infected with HPeV3 (n = 22, 58%), while HPeV1 was found in 8 (21%), and HPeV4 in 6 (16%) of the children. One child was infected with HPeV6 and 1 with

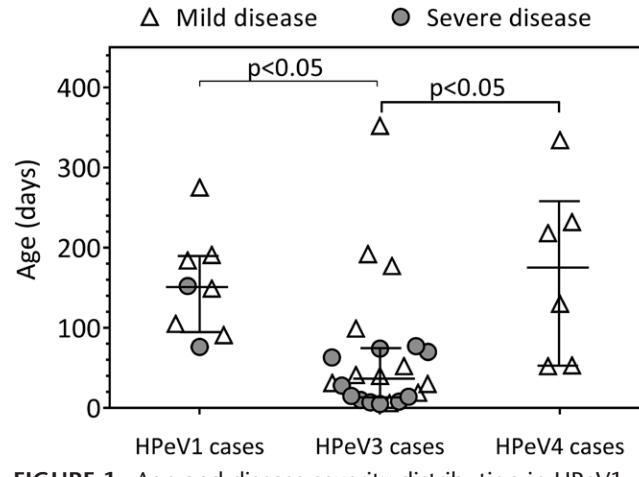

FIGURE 1. Age and disease severity distribution in HPeV1-, HPeV3- and HPeV4-infected children with median age and interquartile range.

HPeV7. These latter 2 cases were excluded from the analysis of nAbs against HPeV1, HPeV3 and HPeV4.

There were no significant differences in epidemiologic or clinical characteristics between cases and controls (Table, Supplemental Digital Content 1, http://links.lww.com/INF/D47). HPeV3-infected children were significantly younger (median age, 1.2 months) than HPeV1- and HPeV4-infected children (median age, 5.0 and 5.8 months, respectively) (Fig 1). HPeV-infected children with severe disease were significantly younger (median age, 0.9 months) than HPeV-infected children with mild disease (median age, 3.5 months). Severe disease was diagnosed in 50% (11/22) of HPeV3-infected infants, compared with 14.3% (2/14) in HPeV1 and HPeV4-infected children ($P = 0.039$). Seven of the mothers were positive for the same HPeV genotype as detected in their children (6 HPeV3, 1 HPeV1), and no HPeV was detected in the remaining 17 mothers tested. One HPeV3-infected mother had clinical symptoms (of diarrhoea), the other HPeV-positive mothers were asymptomatic.

Neutralizing Antibodies in Mothers and Infants

Seroprevalence and median titers of nAbs against HPeV1, HPeV3 and HPeV4 were compared between HPeV-infected and control infants and between their mothers. There was sufficient material available from 13 HPeV3-, 4 HPeV1-, 1 HPeV4-infected infants and from 29 control infants for comparisons. Seroprevalence of HPeV1, HPeV3 and HPeV4 nAbs in control children was 41.4%, 33.3% and 27.6% (Fig 2A), with median nAb titers of 1:16, 1:12 and 1:8 (Fig 2B), respectively. None of the HPeV3-infected children were seropositive for HPeV3 nAbs (n = 13) and their median nAb titer (1:4) was significantly lower than that of control children. HPeV1-infected children were seronegative for HPeV1 nAbs, and the median nAb titer was significantly lower (1:4) when compared with the control children (1:16). The one HPeV4-infected infant tested had a HPeV4 nAb titer of 1:32 (Fig 2B).

nAb seropositivity rates in the control mothers were 84.6%, 55.4% and 60% against HPeV1, HPeV3 and HPeV4, respectively (Fig 2C). The seropositivity rate of HPeV3 nAbs was significantly

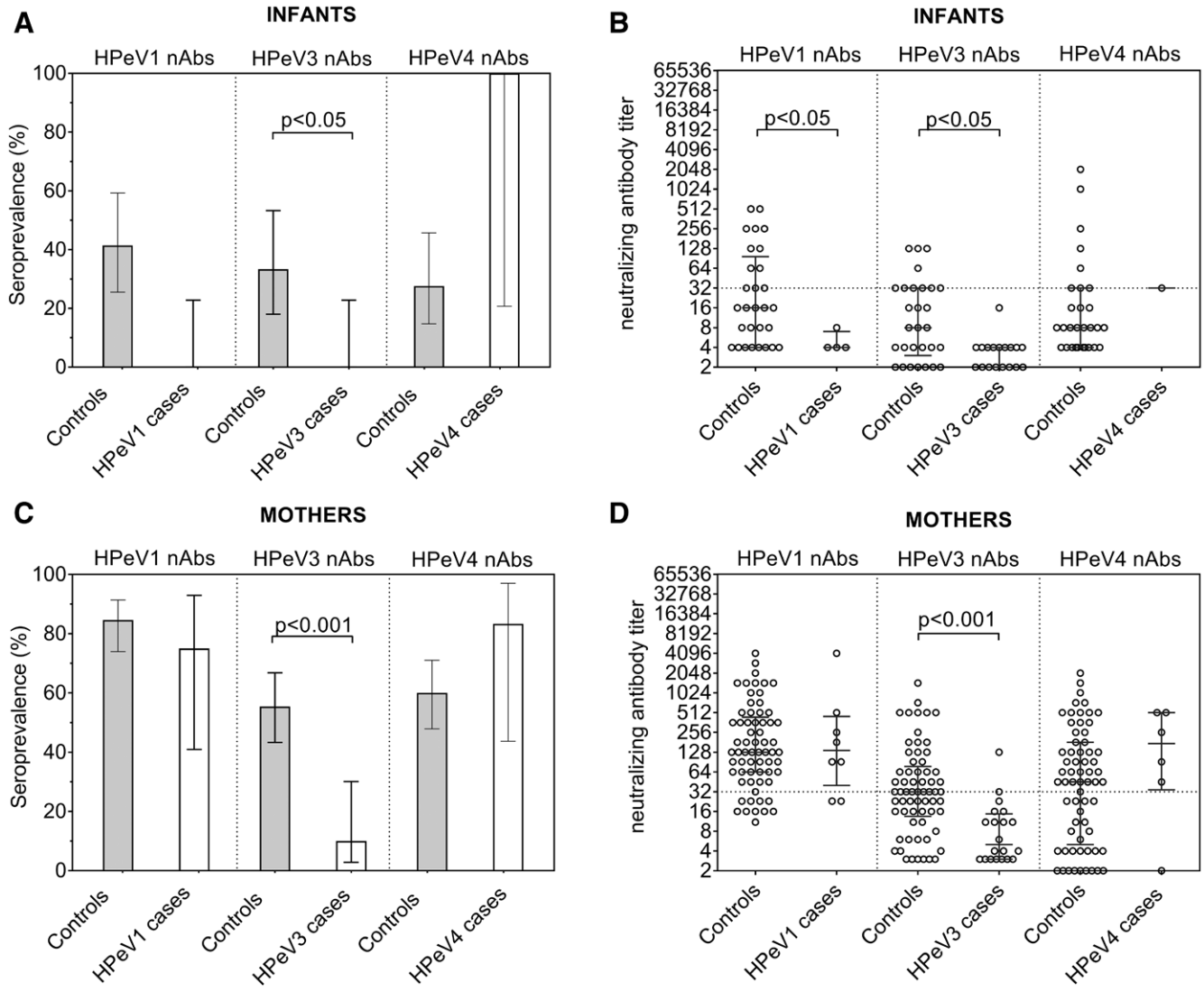

FIGURE 2. Neutralizing antibodies (nAbs) in mothers and infants. nAb seroprevalence with 95% CI (A) and distribution of nAb titers (B) against HPeV1, HPeV3 and HPeV4 in control and HPeV-infected infants. nAb seroprevalence with 95% CI (C) and distribution of nAb titers (D) against HPeV1, HPeV3 and HPeV4 in mothers of control and HPeV-infected infants. The nAb seroprevalence cut-off value of titer ≤1:32 is presented as a horizontal line in graphs B and D. CI indicates confidence interval.

TABLE 1. nAb Seroprevalence and Median nAb Titers Against HPeV1, HPeV3 and HPeV4 in Mothers Below and Above 32 Years of Age

Mothers	HPeV1	HPeV3	HPeV4
≤32 yr			
Seropositivity % (95% CI)	79.6 (66.4–88.5)	63.3 (49.3–75.3)	65.3 (51.3–77.1)
Median nAb titer (IQR)	1:128 (1:45–1:512)	1:45 (1:12–1:128)	1:91 (1:10–1:256)
≥32 yr			
Seropositivity % (95% CI)	92.3 (81.8–97.0)	27.0 (16.8–40.3)*	53.8 (40.5–66.7)
Median nAb titer (IQR)	1:155 (1:91–1:512)	1:16 (1:6–1:32)*	1:45 (1:4–1:237)

*$P \leq 0.001$.
CI indicates confidence interval; IQR, interquartile range.

lower (10%) in mothers of HPeV3-infected children compared with the control mothers, whereas the seropositivity rates of HPeV1 (75%) and HPeV4 (83.6%) nAbs in mothers of HPeV1- and HPeV4-infected infants did not significantly differ from control mothers (Fig 2C). In control mothers, median nAb titers against HPeV1, HPeV3 and HPeV4 were 1:128, 1:32 and 1:45, respectively (Fig 2D). Median nAb titer against HPeV3 (1:5) was significantly lower in mothers of HPeV3-infected children compared with the control mothers, whereas the median nAb titers against HPeV1 (1:136) and HPeV4 (1:174) of the control mothers did not significantly differ from those of mothers of HPeV1- and HPeV4-infected children (Fig 2D). In mothers older than the median age of 32 years, the seropositivity rate and median nAb HPeV3 titer were significantly lower (23.3% and 1:16, respectively) compared with the younger group (63.3% and 1:45, respectively) (Table 1).

Linear regression analysis indicated significant positive correlations between the nAb titers of infants below 3 months of age and their mothers (Fig 3). No significant correlations were found between nAb titers of infants above 3 months of age and their mothers (data not shown).

DISCUSSION

In this prospective case–control study, we assessed if maternal nAbs could protect young Dutch infants against disease related to HPeV1, HPeV3 and HPeV4. We showed that nAb seroprevalence against HPeV3 in the control mothers was low, and that none of the HPeV3-infected children nor the majority of their mothers had protecting levels of nAbs against HPeV3. No significant difference in the nAb seroprevalence against HPeV1 or HPeV4 was observed between the cases and controls.

In agreement with previous reports, the 3 most detected HPeV genotypes in this patient cohort were HPeV1, HPeV3 and HPeV4.[2,17,18] Also the age and disease severity profiles of these infants were in line with previous data.[1,19,20] HPeV3 nAbs in the control mothers were less prevalent than HPeV1 or HPeV4 nAbs potentially reflecting different exposure patterns to these viruses. Importantly, we observed low levels of nAbs against HPeV3 in HPeV3-infected children and in their mothers suggesting that the lack of maternal nAbs puts young infants at risk of disease related to HPeV3 infection. Furthermore, in line with the seroepidemiologic reports from Japan, we found evidence of declining protection as the HPeV3 nAb seroprevalence drastically decreased in mothers above 32 years of age.[8,9] HPeV3 is not often detected in adults; however, cases of severe myalgia have been reported in 30- to 40-year-old adults during an HPeV3 outbreak in infants in Japan.[21,22] Six of the 7 HPeV PCR-positive mothers in this study were HPeV3-infected but none had symptoms resembling myositis. In addition, in infants under 3 months of age and their mothers, the nAb titers correlated positively whereas, likely due to waning of the maternal antibodies, in older children the correlation was lost.

We hypothesize that Dutch women have not been exposed to HPeV3 as frequently as to HPeV1 and HPeV4 and therefore have lower levels of HPeV3 nAbs. However, the occurrence of HPeV4 infections in the Netherlands is lower than that of HPeV3 and does not explain the seroprevalence rates.[2,17] Most mild and asymptomatic infections are likely excluded in these studies and thus the number of HPeV1 and HPeV4 infections could be underestimated.

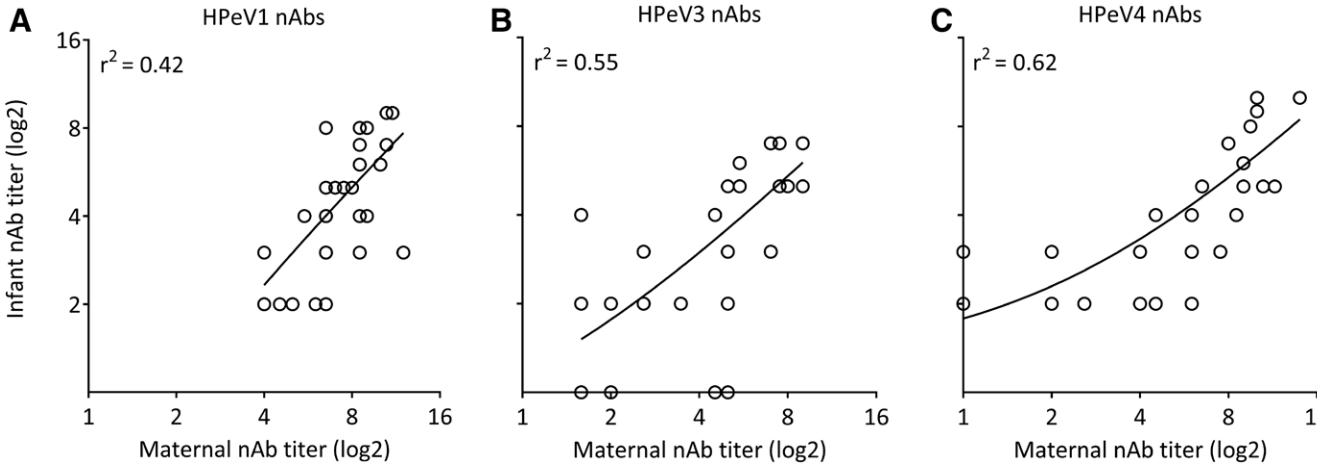

FIGURE 3. XY scatter plot and linear regression analysis of $\log_2$-transformed nAb titers between all children below 3 months of age and their mothers: (A) HPeV1, (B) HPeV3 and (C) HPeV4 nAb titers. r^2 indicates coefficient of determination.

Interestingly, the VP1 capsid protein C-terminus of both HPeV1 and HPeV4, but not of HPeV3, is known to contain an arginine–glycine–aspartic acid (RGD) motif.[23,24] HPeV1 RGD-motif can bind the host cell surface integrin receptors and has shown to be highly immunogenic.[24–26] Therefore, HPeV4 nAb seroprevalence may be the result of cross-neutralizing antibodies raised against the RGD-motif in the highly prevalent HPeV1.[27] Lower nAb seroprevalence in older mothers may also suggest that widespread HPeV3 circulation has occurred fairly recently. Furthermore, we recently observed antigenic variation among HPeV3 strains.[13] This could facilitate virus escape from herd immunity and result in lower nAb seropositivity rates and median nAb titers in mothers not exposed to the circulating HPeV3 strain.

This is the first case–control study to demonstrate the absence of maternal HPeV3 nAbs in HPeV3-infected infants and in their mothers. We acknowledge that the low number of HPeV1- and HPeV4-infected children is a limitation, however an expected one when the study population is children below 1 year of age. Additionally, children in the control group were HPeV PCR-negative, but many were PCR-positive for EVs or other pathogens. Therefore, nAb seroprevalence in the control group may not represent the general Dutch population. Further research on HPeV nAb seropositivity rates is warranted.

In conclusion, our results imply that during first months of life, young Dutch infants are protected by maternal antibodies against disease related to HPeV1 and HPeV4, but not to HPeV3. Lack of maternal nAbs can explain why severe disease related to HPeV3 is predominantly detected in infants under 3 months of age while HPeV1 and HPeV4 infections are associated with mild disease in older children.

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