Detection of Echovirus 18 in Human Breast Milk

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We detected enteroviral RNA and cultured infectious virus from a series of banked breast milk samples from the mother of an infant with neonatal sepsis; sequencing of the enterovirus isolate identified it as echovirus type 18. In this case, it is possible that enterovirus transmission occurred through the breast milk.

CASE REPORT

A female infant was the 3,470-g product of a 36-week-gestation pregnancy, born to a 29-year-old G3P2012 mother via elective repeat Caesarian section. The infant’s Apgar score was 9 at 1 min and at 5 min, and she required only routine resuscitation. The pregnancy course and maternal prenatal laboratory tests were unremarkable. The infant had a normal physical examination at birth and was admitted to the well-baby nursery but was transferred to the neonatal intensive care unit (NICU) at 24 h, when it was noted that she was in respiratory distress. As part of her sepsis evaluation in the NICU, a complete blood count (CBC) and serum electrolytes were normal, and a C-reactive protein (CRP) concentration was low, at 1.3 mg/dl (normal is <1.0 mg/dl). The cerebrospinal fluid (CSF) profile was normal, and an enteroviral PCR from the CSF was negative. Her respiratory symptoms resolved rapidly, and she was quickly weaned from nasal cannula oxygen. She had elevated unconjugated bilirubin to a maximum value of 15.9 mg/dl (the normal range is 0.2 to 1.0 mg/dl) and required phototherapy until day 4 of life.

On day 4 of life, the patient’s temperature rose to 100.9°F (38.2°C), and she became difficult to arouse. Her suck diminished in strength, but she continued to tolerate oral bottle feedings of freshly expressed breast milk. She again required oxygen by nasal cannula. Her physical examination was significant for a diffusely erythematous macular blanching rash. A sepsis evaluation was repeated, and she was treated with ampicillin, gentamicin, and acyclovir. Her CBC was within normal limits, and her CRP concentration was found to be elevated to 5.3 mg/dl and continued to rise over the next 24 h to a peak of 18.3 mg/dl (Fig. 1). A repeated analysis of the CSF was significant for monocytosis, with a glucose concentration of 44 mg/dl (normal is 45 to 75 mg/dl), a protein concentration of 94 mg/dl (normal is 15 to 45 mg/dl), and a white blood cell count of 40/μl, with 80% monocytes, 3% lymphocytes, and 17% segmented neutrophils, and no red blood cells. Blood and CSF samples were submitted for bacterial culture, and CSF and serum samples were sent for herpes simplex virus (HSV) and enterovirus analysis by PCR. No bacteria were seen by Gram’s stain of the CSF, and all cultures were negative. HSV PCR of the CSF was negative, but the quantity of CSF was insufficient to test for enteroviruses by PCR. However, an enterovirus PCR of her serum was positive, and all antimicrobials were discontinued. Except for a minimal transaminitis, the remainder of her laboratory evaluation was normal. There were no other signs of liver dysfunction.

Over the next 3 days, the patient continued to have a low-grade fever, a rash, and an oxygen requirement. By day 7 of life, the patient defervesced and was appropriately alert. Her CRP concentration had fallen to 5.4 mg/dl. Her oral intake of bottle-fed breast milk began to improve, and her rash continued to fade. The patient was weaned from a minimal oxygen requirement over 3 days and was discharged home on day 11 of life. At the time of this patient’s presentation, there were no other patients in the NICU with enteroviral sepsis, and there was no reported enteroviral outbreak of infection in the NICU.

At the time of the infant’s birth, all three of her family members had recently had manifestations of enteroviral infection (Fig. 1). Three days prior to delivery, the mother developed a periumbilical maculopapular blanching rash but was afebrile and otherwise asymptomatic. This rash persisted until 2 days after delivery and was initially attributed to pruritic urticarial papules and plaques of pregnancy. In retrospect, the mother’s rash was most likely due to enteroviral infection. The mother had no respiratory, gastrointestinal, or other constitutional symptoms but did have contact with the infant from birth throughout the hospitalization. Twelve hours before the patient’s birth, the patient’s father presented to the emergency department of the same hospital complaining of fever, head-
ach, photophobia, and a macular rash on his trunk. This clinical presentation and his CSF profile were consistent with viral meningitis. His CSF was positive for an enterovirus by PCR, although this information was not known until day 2 of the infant’s life. The father was admitted for empirical antibiotic treatment and pain control and did not have physical contact with the patient until day 6 of the patient’s life, after she had clinical symptoms. Nine days before the patient’s birth, her 3-year-old brother had a mild illness characterized by low-grade fever and vomiting for 4 days, followed by a maculopapular rash with wide distribution that lasted for an additional 3 days. The patient had no exposure to her brother until she was discharged. Her brother did attend preschool, where his teacher, at least two classmates, and a parent had manifestations of enteroviral disease. It is noteworthy that the teacher and the parent had also been admitted to a local hospital with viral meningitis, and the children had gastrointestinal symptoms within 2 weeks of the patient’s birth, all in the month of July.

Due to the paucity of maternal symptoms and the mode of delivery, we sought to investigate other potential sources of infection for our patient. Since the infant was continuously fed freshly expressed colostrum and then breast milk throughout her hospitalization, we hypothesized that the infant might have acquired the enterovirus through her mother’s milk. Laboratory analyses of stored breast milk samples were performed with the mother’s consent. The work was deemed not to be human subject research and was declared to be exempt by the institutional review boards at Children’s Hospital of Philadelphia and the Hospital of the University of Pennsylvania.

A series of 11 banked milk samples from days 6 to 11 of the infant’s life had been stored at -20°C for approximately 10 weeks prior to testing. Samples of colostrum or breast milk from before day 6 of life were not available for testing. Conventional tube cultures and real-time PCR were performed for all breast milk samples.

For viral isolation, a total of 0.2 ml of each specimen was inoculated onto one tube each of primary rhesus monkey kidney cells (PRMK), human embryonic lung fibroblasts (MRC-5), and human carcinoma cells of the lung (A549) and larynx (HEp-2). The culture tubes were maintained in slanted stationary racks at 37°C and 5% CO₂ and observed daily for cytopathic effect for a period of 14 days. Twice weekly, the monolayers were refed with fresh modified Eagle’s minimum essential medium (BioWhittaker, Walkersville, MD) containing 2% fetal bovine serum, gentamicin (5 μg/ml), vancomycin (10 μg/ml), and amphotericin B (10 μg/ml). Growth of suspected enterovirus isolates was confirmed by real-time PCR. Attempts were made to further characterize the isolates by immunofluorescent staining of acetone-fixed infected cells, using murine monoclonal antibodies directed against coxsackievirus serotypes A9 and B1-6; echovirus types 4, 6, 9, 11, and 13; poliovirus types 1 to 3; and enterovirus types 70 and 71 (Chemicon International, Temecula, CA). Isolates were also submitted to an outside reference laboratory for identification by neutralization with Lim Benyesh-Melnick equine antiserum typing pools A to H.

For PCR, enteroviral RNA was extracted from 200 μl of each breast milk sample, using an automated MagNA Pure LC instrument and a total nucleic acid isolation kit from Roche Diagnostics, Indianapolis, IN. Real-time TaqMan PCR was performed in 50-μl volumes using a one-step reverse transcription (RT)-PCR system (Applied Biosystems, Foster City, CA), 900 nM of forward and reverse primers, 200 nM of fluorogenic probe, Multiscribe reverse transcriptase/ RNase inhibitor mixture (Applied Biosystems), nuclease-free water with yeast tRNA (60 ng/ml; Roche Molecular Biochemicals, Indianapolis, IN), and 15 μl of isolated total nucleic acid. The primers and probe are from a portion of the enterovirus genome that carries the highly conserved 5’ nontranslated region and includes the forward primer 5’-CGG CCC CTG ATT GCG GCT AA-3’, the reverse primer 5’-GAA ACA CGG ACA CCC AAA GTA-3’, and the probe 5’(FAM reporter)-TCY GYR GCG GAA CCG ACT A(TAMRA quencher)-3’. This allows for the broad recognition of all enteroviruses without differentiation of individual serotypes. The amplification and detection were completed using a 7500 real-time PCR system (Applied Biosystems). The thermal cycling parameters consisted of 1 cycle for 30 min at 48°C, 1 cycle for 10 min at 95°C, and 45 two-step cycles of 15 s at 95°C and 60 s at 60°C. Positive and negative controls consisting of PRMK cells in-
fected with echovirus 11 and uninfected A549 human lung carcinoma cells were processed with each batch of clinical samples from the extraction of nucleic acids through the detection of the amplified product. Specimens and controls were tested singly and were considered positive when the fluorescence signal generated at the threshold cycle value exceeded a defined threshold limit. Appropriate no-template controls were included in each reaction plate. A human albumin gene was amplified as an internal positive control for human nucleic acid, to ensure that negative results were not due to poor nucleic acid extraction or inhibition of the PCR assay. The quantity of enteroviral RNA in each specimen or control was determined from a standard curve generated using a set of five nucleic acid standards ranging from $10^8$ to $10^4$ copies/ml.

Multiple samples of breast milk obtained over time were found to be positive for an enterovirus by real-time PCR. The highest viral loads were found in the earliest samples, with measurements ranging from $7.8 \times 10^4$ to $1.0 \times 10^5$ copies/ml on day 6 of the infant's life and from $7.2 \times 10^4$ to $2.5 \times 10^4$ copies/ml on day 7 of life. The viral load then continued to fall, and the last positive sample on day 9 of life contained 513 copies/ml (Fig. 1). An enterovirus was also readily cultured from these same breast milk samples. Although the isolates were confirmed to be an enterovirus by our pan-specific real-time PCR, they could not be further typed by the immunofluorescence or neutralization assays described above. Subsequently, a reverse-transcriptase seminested PCR was used to amplify RNA from the enteroviral isolate, and sequencing of the amplicon identified the virus as an echovirus type 18 (23).

Nonpolio enteroviruses are ubiquitous RNA viruses that can cause protean clinical disease, most commonly in summer and fall in temperate climates like that in the United States. Neonatal enteroviral infections are common (see reference 1 for a review); in one study, 13% of infants were infected with an enterovirus in their first month of life (17). More recently, a reverse-transcriptase seminested PCR was used to amplify RNA from the enteroviral isolate, and sequencing of the amplicon identified the virus as an echovirus type 18 (23).

The mother, a healthcare provider, was quite interested in the potential mode of transmission in this case. After careful examination, the infant's only exposure to an enterovirus was through her mother. Although the infant had other family members who were sick with enteroviral illnesses, she had had no direct or indirect contact with them. Due to her mode of delivery, the infant's exposure to maternal urogenital secretions was less likely. It was possible that the infant acquired the enterovirus through contact with maternal respiratory secretions after birth, though the mother did not have rhinorrhea or cough. Stool is thought to have the largest viral load of enteroviruses, so a fecal-oral transmission was also possible. However, since the infant was in the NICU and the mother knew of her own exposure to an enterovirus, a strict hand washing regimen was observed in an effort to avoid infant exposure. It is also plausible that the infant was infected transplacentally via maternal viremia. However, the presentation of an enteroviral illness on day 4 of life is more suggestive of infection at or after birth rather than in utero. It remains possible that the infant acquired enterovirus either prenatally or postnatally; unfortunately, there were no maternal specimens from the perinatal time period available for testing, though it might have been interesting to test the mother’s urine, blood, stool, and amniotic fluid for the presence of an enterovirus. Thus, we examined the mother’s breast milk as a possible source of the infant’s enteroviral infection. Although we have not excluded other modes of transmission, the high load of live virus in the breast milk presents the possibility that breast milk was the means of postnatal transmission in this case.

Nevertheless, it appears that the presence of enteroviruses in breast milk is relatively uncommon. In a recent study of enteroviral infections in infants, enteroviral RNA could not be found in the breast milk of 234 mothers, although 8 of them had detectable virus in serum samples that were collected simultaneously with the breast milk (26). However, coxsackievirus B3 has recently been detected in the mother’s breast milk of two severely ill infants, although it was unclear that breast milk was the primary mode of transmission that led to disease in these neonates (9). To the best of our knowledge, our case represents only the second report of an enterovirus, in this instance echovirus 18, being detected in the breast milk of a mother whose infant had a documented enteroviral illness. The kinetics of enteroviral infection and shedding in breast milk may play a role in the frequency of detection; alternatively, different types of enteroviruses may have various tropisms for shedding into breast milk. Here, we found that the shedding of echovirus 18 continued in breast milk for 8 days after the mother’s rash had resolved completely. It is notable that the virus remained infectious, with a high titer in the breast milk, despite freezing at $-20^\circ C$ for an extended period of time. This is a reminder that enteroviruses, in general, are more stable under freezing conditions than most viruses and that strategies to eliminate them from breast milk would most likely require pasteurization or thermal processing rather than freezing.

With regard to the particular serotype of enterovirus identified in the breast milk, echovirus 18 has been reported to circulate consistently at relatively low levels within the United States, with periods of increased activity observed between extended intervals of less activity (7). The virus accounts for 2.7% of the reported annual enteroviral infections and ranks 12th among the 15 most common enteroviral serotypes identified. It ranked as high as second in 1987 and 2001. Since 2001, echovirus 18 has emerged as a major cause of aseptic meningitis outbreaks. The virus also has been associated with epidemic diarrhea in infants, neonatal sepsis, exanthema, and
leukoencephalitis (5, 7, 20). Similar to other enteroviruses, echovirus 18 most commonly affects children less than 1 year old. Interestingly, the illnesses in the case of our patient, her family members, and others in the community occurred during a large summertime outbreak of aseptic meningitis.

Although this report identifies echovirus type 18 as another virus that potentially can be transmitted through breast milk, it is important to state that only a few viruses (e.g., cytomegalovirus, human immunodeficiency virus, and human T-cell leukemia virus type 1) frequently cause clinically significant infections through this mode of transmission. Breast milk remains a superior source of nutrition for infants, not least because of its many antiviral and antibacterial properties. The many benefits of breast milk should be carefully considered when making a decision to avoid or stop breast feeding during maternal infection. Also, previous studies have found breast feeding to be protective against symptomatic enteroviral infections due to the presence of neutralizing antibodies (17, 26). Further research is necessary to determine how often breastfed infants present with enteroviral sepsis, whether breast milk is the most likely source of neutralizing antibodies (17, 26). Further research is necessary to determine how often breastfed infants present with enteroviral sepsis, whether breast milk is the most likely source of infection and disease, and whether breast feeding alters the severity of the illness, particularly in the first week of life.

REFERENCES