

Infant Deaths Associated with Human Parechovirus Infection in Wisconsin

Gerald Sedmak,¹ W. Allan Nix,⁴ Jeffrey Jentzen,^{2,a} Thomas E. Haupt,³ Jeffrey P. Davis,³ Sanjib Bhattacharyya,¹ Mark A. Pallansch,⁴ and M. Steven Oberste⁴

¹Virology and Molecular Science Laboratory, City of Milwaukee Health Department, ²Milwaukee County Medical Examiner's Office, Milwaukee, ³Bureau of Communicable Diseases, Wisconsin Division of Public Health, Madison, Wisconsin; and ⁴Division of Viral Diseases, Centers for Disease Control and Prevention, Atlanta, Georgia

(See the editorial commentary by Landry, on pages 362–3.)

Background. From December 1987 through August 2004, lung tissue, nasopharyngeal swabs, and colon swab specimens obtained during 1263 autopsies of infants and young children were examined to assess the role of viruses in deaths of children aged <2 years.

Methods. Multiple cell cultures were used to isolate viruses. With 4 exceptions, virus isolates were identified by neutralization, immunofluorescence assay, or enzyme immunoassay. RNA extracted from these 4 isolates and associated autopsy specimens was tested using parechovirus-specific real-time polymerase chain reaction (RT-PCR) and sequencing assays.

Results. Specimens from 426 (34%) autopsies were positive for at least 1 virus; enteroviruses and adenoviruses were the most commonly identified. Human parechoviruses (HPeVs) were identified antigenically in isolates from 18 decedents (all HPeV type 1) and by RT-PCR in isolates and multiple autopsy specimens from 4 decedents with untypeable virus isolates. Sequencing of the VP1 region identified these 4 HPeVs as HPeV type 3 ($n = 3$) and HPeV type 6 ($n = 1$). Despite the detection of HPeV, the deaths of decedents 3 and 4 were determined to have been from noninfectious causes.

Conclusions. These are the first confirmed HPeV type 3 and HPeV type 6 detections in the United States. This is also the initial report of fatal cases with associated HPeV type 3 infection. These results support prior findings associating HPeVs with serious disease in young children. Clinical testing for HPeVs and routine HPeV surveillance by public health laboratories will help determine the burden of disease caused by HPeVs.

Young infants are susceptible to a wide range of serious, potentially life-threatening infections because of the immaturity of their immune systems and their constant exposure to pathogens. In particular, neonates and other children aged <2 years are at increased risk of lower respiratory tract infection and sepsis [1, 2], 2 major causes of infant mortality worldwide [3]. Sudden unexplained infant death includes sudden infant death

syndrome, accidental suffocation or strangulation, and neglect, abandonment, and other maltreatment syndromes [4]. The annual reported incidence of sudden unexplained infant death in the United States during 1999–2001 was 100 deaths per 100,000 live births [4].

To determine the role of viruses in the deaths of infants and young children, the Milwaukee County Medical Examiner's Office and the City of Milwaukee Health Department Laboratory conducted a long-term investigation involving virus identification in autopsy specimens from deceased children aged <24 months. This report briefly summarizes the results of routine testing of infant decedents for viruses during 1987–2004 and describes in detail data from a subset of 4 infants from whom human parechoviruses (HPeVs) were isolated. These data provide additional evidence that parechoviruses may cause severe, life-threatening illness in infants.

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^a Present affiliation: Department of Pathology, University of Michigan, Ann Arbor.

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Reprints or correspondence: M. Steven Oberste, 1600 Clifton Rd NE, Mailstop G-17, Atlanta, GA, 30333 (soberste@cdc.gov).

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MATERIALS AND METHODS

Medical examiner methods. Postmortem nasopharyngeal swabs, colon swabs, and lung tissue specimens were obtained aseptically from each of the deceased children aged <24 months included in this study; additional specimens were also obtained from some individuals. All specimens were processed in a laminar-flow biosafety cabinet with use of normal aseptic technique and precautions to prevent cross-contamination between specimens. Swab specimens were expressed in 5 mL of minimum essential medium containing antibiotics. Portions of autopsy tissues were placed in minimum essential medium with antibiotics, homogenized with a sterile tissue grinder, and centrifuged to remove debris. All unused original autopsy specimens were stored at -70°C .

Virus isolation. Clinical specimens (0.5 mL of swab supernatant or clarified tissue homogenate) were inoculated onto RMK (primary rhesus monkey kidney), HEp-2 (human epithelium), HFS (human foreskin), HEL (human embryonic lung), BGM (Buffalo green monkey kidney), Vero (monkey kidney), Caco-2 (human colonic adenocarcinoma), and MDCK (Madin-Darby canine kidney) cells in 24-well cell culture plates and observed for up to 14 days for the appearance of viral cytopathic effect; BGM, Caco-2, and Vero cells were used only for samples collected from 1994 through 2004. Viruses producing an enterovirus-like cytopathic effect were identified by neutralization of cell culture infectivity by serotype-specific polyclonal antibodies or by indirect immunofluorescence with monoclonal antibodies, as described elsewhere [5]. Isolates that could not be typed were submitted to the Wisconsin State Laboratory of Hygiene virus laboratory for enterovirus polymerase chain reaction (PCR) testing and, if the tests were still negative, the virus isolates were forwarded to the Centers for Disease Control and Prevention (CDC) Picornavirus Laboratory for HPeV-specific PCR and sequence analysis. Virus isolates were passaged once on Vero cells at CDC prior to molecular testing.

Serology. Serum specimens were obtained from household contacts of decedents 1 and 2 several months after the children's deaths. The serum samples were tested for neutralizing antibody against HPeV type 3 (HPeV3) by microneutralization assay [6] and by an indirect immunofluorescence assay (IFA). The nasopharyngeal isolate from decedent 1 was used as the standard virus for both assays. Each specimen was tested in triplicate, with the final titer estimated using the Spearman-Kärber method [7]. For the IFA, HPeV3-specific antibody was detected using infected Vero cells and 2-fold serial dilutions of serum (1:20 to 1:160). Human IgG was detected using FITC-conjugated goat anti-human immunoglobulin G (Bion Enterprises).

Molecular detection of parechovirus RNA genome. RNA extraction and pan-parechovirus real-time reverse-transcription PCR (RT-PCR) were performed as described elsewhere

[8], using the QuantiTect Probe RT-PCR Kit (Qiagen) with 5 μL of RNA for the autopsy specimens, according to the manufacturer's instructions. Parechovirus VP1 reverse-transcription semi-nested PCR, sequencing, and sequence analysis were conducted (W.A.N., K. Maher, M.A.P., and M.S.O., unpublished data). The nucleotide sequences described here were deposited in GenBank under accession numbers FJ652380–FJ652384.

Epidemiologic investigation. The Wisconsin Division of Public Health conducted follow-up investigation of the deaths of the children from whom HPeV3 was isolated. Parents of 2 decedents were interviewed regarding recent travel of family members, past and current illnesses, and contacts of each of the children during the 30 days prior to their deaths. In addition, police reports from the original death investigations and notes from the decedents' last health care visits were requested.

RESULTS

From December 1987 through August 2004, autopsy specimens from 1263 infants and young children were examined for viruses. Over 90% of the deceased children were aged ≤ 12 months (median age, 3 months). Specimens from 426 autopsies (34%) had positive results for at least 1 virus. From these 426 decedents, there were 472 positive specimens; there were 46 decedents with 2 positive specimens. Two viruses were detected in 19 cases, for a total of 445 unique viruses. Enteroviruses, adenoviruses, and rotavirus were the most commonly identified viruses, together representing 81% of the confirmed infections (data not shown). Cytomegalovirus and parechoviruses each represented roughly 5% of virus infections. Other viruses included herpes simplex virus, rhinovirus, influenza A and B viruses, human parainfluenza viruses 1 and 3, respiratory syncytial virus, and reovirus (<2% each).

HPeV type 1 was identified in 20 specimens from 18 (4%) of the 426 autopsies by neutralization with standard antisera for echovirus type 22 (previous name for HPeV type 1). Isolates obtained from 4 decedents could not be identified by standard virologic methods. For all 4, enterovirus-like cytopathic effect was detected in 4 days on Vero cells, and some cytopathic effect was noted on BGM cells for decedent 2. All isolates were hemadsorption-negative and could not be identified by standard methods for enterovirus typing (data not shown). Enterovirus-specific RT-PCR was negative in the Wisconsin State Laboratory of Hygiene virology laboratory (data not shown).

Description of cases. Decedent 1 was a female infant aged 4 weeks who had experienced a cold-like illness, including a fever (temperature, 39.5°C) lasting 48 h, and 2 episodes of vomiting during the week prior to death. During a clinic visit 4 days prior to death and after the fever had resolved, her lungs were clear and she had normal chest radiographic findings. She received a diagnosis of a cold and was sent home. Other family members reported having had mild upper respiratory tract ill-

ness at the time of the child's death. The infant was described as fussy when put to bed at 10 PM on the night of her death and was found unresponsive, in a prone position, at 1:30 AM. The autopsy did not reveal signs of injury, trauma, or other suspicious circumstances. The physical examination was unremarkable, and toxicology screening tests were negative. Microscopic examination of the lungs demonstrated diffuse intra-alveolar edema and scattered multinuclear cells but no significant inflammation. The cause of death was recorded as sudden unexplained infant death associated with viral syndrome. There was moderate growth of *Haemophilus influenzae* and light growth of alpha Streptococci from lung tissue. Colon swab, nasopharyngeal swab, lung swab, and lung tissue specimens all yielded viral cytopathic effect in Vero cells (Table 1). The nasopharyngeal swab isolate submitted to CDC was identified as HPeV3 by VP1 PCR and sequencing.

Decedent 2 was a female infant aged 4 months who was healthy during a physician examination 2 weeks prior to death but had signs of a mild upper respiratory tract infection without fever at the time of death. The evening of the infant's death, she was put to bed and found unresponsive, in a prone position, the following morning. The autopsy did not reveal signs of injury, trauma, or other suspicious circumstances. The general examination was unremarkable, and toxicology screening test results were negative. Microscopic examination of the lungs showed diffuse intra-alveolar edema with associated multinucleated giant cells and small areas of light chronic inflammatory cell infiltrates. The cause of death was recorded as sudden unexplained infant death associated with mild respiratory changes. Colon swab, nasopharyngeal swab, and lung tissue specimens all yielded viral cytopathic effect in Vero cells, but heart tissue was culture negative (Table 1). The nasopharyngeal swab isolate submitted to CDC was identified as HPeV3 by VP1 PCR and sequencing.

Decedent 3 was a female child aged 16 months who was born at 30 weeks gestation. She had respiratory distress at birth and chronic lung disease up to the time of death. Soon after birth, she had open-heart surgery. At the age of 7 months, she had a tracheostomy placed; she had vocal cord paralysis and a history of tracheostomy tube self-removal. Terminally, she removed the tracheostomy tube in the presence of a baby sitter who could not replace it. The child was pronounced dead in an emergency department of a local hospital. The autopsy did not reveal signs of injury, trauma, or other suspicious circumstances. The general examination was unremarkable, but the neck had a midline trachea with a well established tracheostomy opening. The lungs were partially collapsed, but there were no obvious masses or inflammatory infiltrates. The heart was enlarged with dilated pulmonary trunk. Microscopically, lung sections exhibited congestion and edema. The cause of death was recorded as asphyxia caused by self-removal of the tracheos-

Table 1. Analysis of Lung and Spleen Tissues, Colon Swab, and Nasopharyngeal (NP) Swab Specimens by Real-Time Reverse-Transcription Polymerase Chain Reaction (RT-PCR) and VP1 Semi-nested RT-PCR

Decedent, specimen	Virus isolation	Real-time PCR	VP1 RT-PCR ^a
1			
Lung	Positive	NA	NA
Colon swab	Positive	NA	NA
NP swab	Positive	NA	HPeV3
2			
Lung	Positive	NA	NA
Heart	Negative	NA	NA
NP swab	Positive	NA	HPeV3
Colon swab	Positive	NA	NA
3			
Lung	Negative	Negative	HPeV3
Spleen	Negative	Positive	HPeV3
NP swab	Negative	Positive	HPeV3
Colon swab	Positive	Negative	HPeV3
4			
Lung	Negative	Negative	NA
Spleen	Negative	Negative	HPeV6
NP swab	Negative	Positive	HPeV6
Colon swab	Positive	Positive	HPeV6

NOTE. HPeV, human parechovirus; NA, specimen not available for molecular testing.

^a Virus type determined by RT-PCR and sequencing of the VP1 gene, from the Vero cell isolate.

tomy tube. The family reported a sibling had vomiting and diarrhea at time of the infant's death. Culture of colon swab material yielded viral cytopathic effect in Vero cells, but lung and spleen tissues and nasopharyngeal swab material were culture negative (Table 1). The colon swab isolate was identified as harboring HPeV3 by VP1 PCR and sequencing. The HPeV3 genome was detected in the spleen tissue and nasopharyngeal swab specimens by real-time RT-PCR and in nasopharyngeal swab, colon swab, lung, and spleen specimens by VP1 PCR and sequencing (Table 1).

Decedent 4 was a male infant aged 3 months. He was found unresponsive, in a seated position close to the bed. At autopsy, the decedent had multiple blunt force injuries to the head, both recent and old abrasions of the face, and multiple scars of the body; the abdomen was markedly protuberant. Neuropathologic diagnoses were congestion of the posterior cerebellum and epidural hematoma. Microscopic examination of the lungs and heart were unremarkable. An isolate was obtained in Vero cells from the colon swab specimen, but the lung tissue, spleen tissue, and nasopharyngeal swab samples were negative (Table 1). The isolate was identified as harboring HPeV type 6 by VP1 PCR and sequencing. The HPeV type 6 genome was detected in the nasopharyngeal and colon swabs by real-time PCR. VP1 PCR and sequencing detected HPeV type 6 genome in the

spleen tissue, nasopharyngeal swab, and colon swab samples. Lung tissue was negative by real-time PCR; there was insufficient material to test the lung tissue with VP1 PCR (Table 1).

Serology. The father of decedent 1 had some neutralizing antibody to HPeV3 at a 1:20 dilution, but no antibody was detected by IFA. Her mother had negative results for HPeV3 antibody (neutralization titer, <1:10; negative by IFA), but her sister, aged 3 years, was HPeV3-positive by neutralization (titer, 1:40) and strongly positive by IFA (titer, >1:160), indicating a history of HPeV3 infection. Her grandmother's serum specimen was negative in the neutralization test but antibody-positive by IFA. Both parents and the sister reportedly had nasal congestion but no other signs of acute illness at the time of the child's death; the grandmother had no recent history of respiratory illness or diarrhea.

The father of decedent 2 was negative for neutralizing antibody (titer, <1:10) but antibody-positive by IFA (titer, >1:160). Her mother and 3 siblings were all HPeV3-positive in both assays, indicating a history of HPeV3 infection. The mother and a sister aged 9 years each had neutralizing titers of 1:40 and a 4-year-old sister and 2-year-old brother each had neutralizing titers of 1:20. None of the family members reported a recent history of respiratory illness or diarrhea.

No serum samples were collected from family contacts of decedents 3 or 4. As a crude gauge of HPeV3 seroprevalence in the community, serum samples previously submitted for human immunodeficiency virus testing of 59 Milwaukee-area adults (age range, 16–60 years; 34 male, 25 female) were blinded and tested for HPeV3 antibody by IFA and the neutralization test; all sera were HPeV3-negative in both tests.

DISCUSSION

HPeVs, members of the *Parechovirus* genus of *Picornaviridae*, cause a spectrum of diseases similar to that of the human enteroviruses, including aseptic meningitis, gastroenteritis, encephalitis, respiratory diseases, and neonatal sepsis-like disease [9–22]. HPeV3 has been reported in association with gastroenteritis, respiratory illness, meningitis, transient paralysis, and severe neonatal viral sepsis in Japan [17], Canada [23, 24], the Netherlands [14, 25–27], Norway [28], Scotland [29], and the United States (reported here). This is the first report of fatal cases associated with HPeV3 infection.

Two small seroepidemiologic studies, in Japan and Finland, suggest that children are infected with HPeVs early in life, reaching a high cumulative rate of seropositivity by the time they enter primary school [30, 31]. The high rate of infection suggests that, like enteroviruses, many HPeV infections are asymptomatic or subclinical. In addition, like the enteroviruses, the HPeVs can also be detected in the stool samples of healthy children [28]. Very limited serologic testing of serum samples from Milwaukee adults suggests that HPeV3 infections are not

common in southeastern Wisconsin or that waning immunity makes titers difficult to detect in adults.

HPeV3, first detected in Japan in 1999 [17], is common in Japan and has also been detected in neonates in Canada in the fall of 2001 [23, 24, 32]. In our study, HPeV3 was detected in autopsy specimens of 3 infants (decedents 1, 2, and 3) in Wisconsin during a period of 12 months (2003–2004) in communities separated by 100 miles, suggesting widespread circulation in eastern Wisconsin and probably a more-extensive geographic area. It appears that HPeV3 may have contributed to the deaths of decedents 1 and 2. Some relatives of decedents 1 and 2 had detectable antibody to HPeV3, which indicates prior HPeV3 infection. No known common epidemiologic link was identified for any of the infected infants. Despite the detection of HPeV in sterile-site specimens, the deaths of decedents 3 and 4 were determined to have been from noninfectious causes (self-removal of tracheostomy tube and blunt trauma, respectively).

HPeVs were isolated from colon swab specimens from all 4 decedents and from both nasopharyngeal swab and lung tissue specimens from decedents 1 and 2. Virus isolation is generally insensitive for HPeVs [33], suggesting that the virus titer was relatively high in colon sample material from all 4 decedents and in lung tissue from 2 decedents. In decedents 3 and 4, isolates were obtained only from nonsterile specimens (colon swab); however, spleen tissue from both decedents 3 and 4 and lung tissue from decedent 3 were positive by PCR. Vero cells appear to be the optimal cell line for isolation of HPeVs; however, improved sensitivity, rapid turn-around, and ease of use suggest that PCR should be the method of choice for HPeV detection.

It is important to assess the association between HPeV infection and sepsis-like illness and central nervous system involvement in neonates and other infants. The expanding use of the molecular techniques in microbial diagnosis provides emerging opportunities for clinical and public health laboratories to implement PCR testing for HPeVs, particularly in cases of severe disease in young children. Currently, HPeVs are monitored through the National Enterovirus Surveillance System (NESS) because of the historical classification of HPeV type 1 and HPeV type 2 as enteroviruses (echoviruses 22 and 23, respectively). The NESS is a voluntary, passive laboratory-based surveillance system for monitoring temporal and geographic trends and serotypes of enterovirus infections in the United States [34, 35]. Public health and private laboratories and the CDC Picornavirus Laboratory report enterovirus detections to the NESS monthly. Each report includes information on sex, age, state, specimen type and collection date, and enterovirus serotype [35]. Further studies are needed to describe the clinical correlates, disease associations, and disease burden of HPeV infections and to assess the contribution of HPeVs to sudden

unexplained infant death and other severe illness outcomes among infants.

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Potential conflicts of interest. All authors: no conflicts.

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