MAJOR ARTICLE

Diagnosis of Enterovirus Infection in the First 2 Months of Life by Real-Time Polymerase Chain Reaction

M. A. Verboon-Maciolek,[1] M. Nijhuis,[2] A. M. van Loon,[2] N. van Maarssenveen,[2] H. van Wieringen,[3] M. A. Pekelharing-Berghuis,[4] T. G. Krediet,[1] L. J. Gerards,[1] A. Fleer,[2] R. J. A. Diepersloot,[5] and S. F. T. Thijsen[5]

Departments of [1]Neonatology and [2]Virology, Eijkman Winkler Institute for Microbiology, Infectious Diseases, and Inflammation, University Medical Center; [3]Department of Pediatrics, Medical Center Mesos, and Departments of [4]Pediatrics and [5]Medical Microbiology, Diakonessenhuis, Utrecht, The Netherlands

During summer and fall, enterovirus infections are responsible for a considerable proportion of hospitalizations of young infants. We prospectively studied the incidence of enterovirus infections via real-time polymerase chain reaction (PCR) in blood, feces, and cerebrospinal fluid samples from infants ≤60 days old who had received a clinical diagnosis of sepsis. Forty-five patients were included: 19 were admitted to the pediatric wards of 2 general hospitals, and 26 had been hospitalized since birth in the neonatal intensive care unit (NICU) of a tertiary care hospital. None of the NICU patients developed enteroviral disease. In contrast, an enterovirus was detected in 11 (58%) of the patients admitted to the 2 general hospitals, 10 of whom (53%) showed evidence of systemic infection. Enterovirus infections are an important cause of sepsis in infants admitted to the hospital. Real-time PCR in serum was a rapid and sensitive method for diagnosis of enterovirus infection.

Human enteroviruses are well-known agents of infection in young infants, especially in the summer and fall months [1–3]. These viruses are usually transmitted horizontally, through fecal-oral and possibly oral-oral (respiratory) routes, but they may occasionally be transmitted vertically (perinatal infection). Clinical presentations can vary from a benign febrile illness to severe disease, such as meningitis or meningoencephalitis, severe hepatitis, and myocarditis [1, 2, 4–6]. Because of its possible bacterial origin, fever in young infants necessitates a complete assessment, including blood and urine culture, lumbar puncture, and the initiation of broad-spectrum antibiotic therapy. This all might be unnecessary if a rapid diagnosis of enterovirus infection could be made.

The incidence of enterovirus infection among febrile infants admitted to the hospital with systemic infection or infants with suspected sepsis has been reported by different authors to range from 3% to 50% [2, 7–10]. Many of the studies were performed using viral cultures from rectal swabs and throat swabs. Cell culture has limited sensitivity and does not detect all enteroviruses. Recently, it has been shown that the sensitivity of PCR is superior to that of culture [11–13]. Nevertheless, classical PCR for enteroviruses is still cumbersome and labor intensive and therefore is less well suited for routine application. It also has a potential for false-positive reactions. In real-time PCR, amplification and detection take place at the same time, thus reducing hands-on time, turnaround time, and contamination risk [14–18]. Rapid turnaround times will enhance the clinical impact of PCR for enteroviruses [19–21]. In addition, real-time PCR can easily produce (semi)-quantitative data, enabling assessment of virus load in comparison

Received 1 October 2002; accepted 7 February 2003; electronically published 20 June 2003.

Reprints or correspondence: Dr. M. A. Verboon-Maciolek, University Medical Center, Dept. of Neonatology, Lundlaan 6, 3584 EA Utrecht, The Netherlands (M.Verboon-Maciolek@wkz.azu.nl).

Clinical Infectious Diseases 2003; 37:1–6

1058-4838/2003/3701-0001$15.00

to clinical presentation, as well as of the efficacy of antiviral therapy [15, 22].

We studied the contribution of enterovirus infection in infants who received a clinical diagnosis of sepsis. We used real-time PCR and viral culture to investigate 2 groups of patients in whom sepsis had been clinically diagnosed: 19 infants admitted to 2 general hospitals and 26 infants from a neonatal intensive care unit (NICU). Twenty infants admitted to the NICU without any signs of infection at the time of the study were enrolled in the study as control subjects.

PATIENTS AND METHODS

Patients. From April 2000 through January 2001, 45 infants who received a clinical diagnosis of sepsis were enrolled in our study. Twenty-six patients treated at the NICU of a tertiary care hospital, Wilhelmina Children's Hospital, University Medical Center (Utrecht, The Netherlands), were included in the study at the time of onset of sepsis, and 19 patients who had received a clinical diagnosis of sepsis were included in the study at the time of admission to 2 general hospitals (Diakonessenhuis and Medical Center Mesos, Utrecht, The Netherlands). Inclusion criteria were age $\leqslant$60 days and $\geqslant$2 of the following symptoms: fever, irritability, lethargy, apnea, tachycardia, hypotension, abdominal discomfort, and diarrhea. The main difference between the groups was that all infants from the NICU had been hospitalized since birth, whereas all infants from the general hospitals had been at home before admission to the hospital. Standard hygienic procedures were used before entering the neonatal ward: all jewelry was removed, and hands were washed carefully with disinfectant before contact with the patients. Visitors with symptoms of infection were not allowed to enter the unit or were asked to use mouth caps. Infants could be visited by a limited number of people only.

Twenty infants without any symptoms of infection who were admitted to the NICU were enrolled as control subjects. Blood and stool samples were obtained for analysis from patients and control subjects, and, in some cases, CSF was obtained from septic patients. We analyzed the following parameters: gestational age, birth weight, sex, day on which symptoms first appeared, number of siblings, whether the child was home before symptoms first appeared, body temperature, and results of laboratory tests done at admission, such as C-reactive protein level and WBC count. Informed consent was obtained from the parents of patients entering the study. The ethics committee of the University Medical Center, Utrecht, approved the study (project 00/249).

Virological examinations. Stool and CSF samples were inoculated on different cell cultures, including tertiary monkey kidney cells, human embryonic diploid fibroblasts, and RD cells (derived from human rhabdomyosarcoma), by conventional techniques. Cultures showing a cytopathologic effect were typed by neutralization tests that used pools of antisera and specific antisera, according to standard techniques [23]. Real-time PCR was carried out as described elsewhere [18].

Molecular typing. All enterovirus strains isolated by culture were typed by means of sequence analysis of the *VP1* gene, as described by Oberste et al. [24]. Two different PCRs (PCR A and B) were performed. PCR A was directed at the 5′ part of the *VP1* gene, and PCR B was directed at the 3′ part of the *VP1* gene. RNA was isolated from 100 μL of culture supernatant by the RNeasy kit (Qiagen). An aliquot of 1 μL of isolated viral RNA was reverse transcribed and amplified with 5 μL of 10$\times$ reverse transcriptase (RT) buffer (1 mol/L Tris-HCl [pH 8.8], 1 mol/L ammonium sulfate, and 0.5 mol/L EDTA [pH 8.0]), 1.5 mmol/L $MgCl_2$, 200 μmol/L each of the deoxynucleotide triphosphates, 11 U of RNasin Ribonuclease Inhibitor (Promega), 3.6 U of avian myeloblastosis virus RT (Finnzymes), 2.7 U of *Taq* DNA polymerase (MBI Fermentas), and 2 μmol/L each PCR A forward primer plus 4 μmol/L reverse primer or 4 μmol/L each of the PCR B primers (DNA Technology). Reactions were performed with a PTC 100 Programmable Thermal Controller (MJ Research). The mixture was heated at 50°C for 30 min for cDNA synthesis, followed by 3 min at 94°C for inactivation of the RT enzyme. Subsequently, 35 cycles were run under the following conditions: 30 s at 94°C, 30 s at 42°C, and 30 s at 72°C. PCR products with visible bands after gel electrophoresis were purified with the QIAquick PCR purification kit (Qiagen). PCR products were sequenced with the ABI Prism BigDye Terminator cycle sequencing ready reaction kit (Applied Biosciences) in combination with either the reverse or forward primers of PCR A and B. Cycle sequence PCR was performed with a RoboCycler Gradient 96 Temperature cycler (Stratagene), and PCR products were purified by ethanol–sodium acetate precipitation and analyzed on an ABI Prism 310 DNA sequencer. Sequence results were analyzed with the BLAST 2.1 search engine of GenBank (National Center for Biotechnology Information, Bethesda, MD).

Microbiological examination. Blood cultures were performed by the BacT/ALERT automated system (Organon Teknica) with Pediabact pediatric blood culture bottles. Bottles with positive results were subcultured on blood agar plates. Isolates were identified by standard bacteriological methods.

Statistical analysis. The differences between C-reactive protein levels, WBC counts at onset of sepsis, and clinical data for infants with and infants without enterovirus infection were analyzed by Student's t test. Differences were considered to be statistically significant at $P < .05$.

Table 1. Characteristics of 65 infants ≤60 days old in whom sepsis was clinically diagnosed.

Characteristic	Group 1 (n = 19)	Group 2 (n = 26)	Group 3 (n = 20)
Gestational age, median weeks (range)	40 (38–41)	30 (26–41)	29 (26–31)
Birth weight, median g (range)	3600 (2760–4480)	1400 (690–3770)	1300 (690–1920)
Age at onset/admission, median days (range)	22 (1–55)	12 (5–35)	14 (7–24)
Sex, no. of male/no. of female patients (ratio)	13/6 (2.2)	14/12 (1.2)	12/8 (1.5)
No. of siblings, median (range)	1.0 (0–3)	0.5 (0–3)	0.3 (0–3)
Positive results of PCR for enterovirus, no. of patients	11	0	0
Positive results of bacterial blood culture, no. of patients	0	17	ND

NOTE. Group 1 included patients admitted to the general hospitals who received a clinical diagnosis of sepsis. Group 2 included neonatal intensive care unit patients with symptoms of sepsis. Group 3 included neonatal intensive care unit patients without any symptoms of infection. ND, not done; PCR, polymerase chain reaction.

RESULTS

The 65 infants included in the study were divided into 3 groups. Group 1 included patients admitted to the general hospitals in whom sepsis had been clinically diagnosed. Group 2 included NICU patients with symptoms of sepsis. Group 3 included NICU patients without any symptoms of infection (table 1).

Group 1. Nineteen patients were included in group 1. Their characteristics are presented in table 2. All patients had been at home before admission to the hospital. In 11 patients (58%), an enterovirus infection was demonstrated. Two (18%) of the 11 patients with an enterovirus infection became ill within the first week of life (at 5 and 6 days of age); the remaining patients (9 [82%] of 11) were admitted after ⩾10 days of life. Analysis of fluid drawn by lumbar puncture, which was performed in 8 patients (all with enterovirus disease), showed increased cell counts in 50% of the patients (range, 488–4800 leukocytes/mm^3) and normal protein and glucose levels. The clinical symptoms of the patients with enterovirus infection were as follows: temperature >38°C (11 of 11 patients), irritability (7 [64%] of 11), and diarrhea (4 [36%] of 11). The mean temperature in patients with enterovirus infection was significantly higher than that in patients without enterovirus infection ($P < .01$).

The results of culture for enterovirus, real-time PCR, and molecular typing of enteroviruses are presented in table 3. Average virus load values were higher in feces (threshold cycle [Ct] value, 30.2) than in serum (Ct value, 34.5) ($P = .06$), corresponding with a virus load in feces that was ~200-fold higher than that in serum. Thirteen (68%) of 19 patients received broad-spectrum antibiotics. All patients with enterovirus infection had an uncomplicated recovery within 3 days.

Group 2. The 26 patients (4 term and 22 preterm infants) in group 2 had been hospitalized since birth in the NICU of the tertiary care hospital because of prematurity, respiratory

Table 2. Characteristics of 19 infants ≤60 days old who were admitted to 2 general hospitals with a clinical diagnosis of sepsis.

Characteristic	Enterovirus positive (n = 11)	Enterovirus negative (n = 8)
Gestational age, median weeks (range)	40 (38–41)	40 (38–41)
Birth weight, median g (range)	3700 (3300–4480)	3400 (2760–3860)
Age at admission, median days (range)	28 (5–55)	2 (1–55)
Sex, no. of male/no. of female patients (ratio)	8/3 (2.7)	5/3 (1.7)
Duration of hospital stay, median days (range)	5 (4–6)	4 (2–7)
No. of siblings, median (range)	1.0 (0–3)	1.0 (0–3)
Duration of antibiotic therapy, median days (range)	4 (0–10)	1.5 (0–10)
C-reactive protein level at admission, median mg/L (range)	14 (5–63)	8 (3–98)
WBC count at admission, median cells $\times 10^9$/L (range)	9.7 (6.7–16.2)	10.5 (3.9–25.4)
Temperature at admission, median °C (range)	39.2 (38.2–39.9)[a]	38.2 (35.8–39)

[a] $P < .01$.

Table 3. Results of culture for enterovirus, real-time PCR, and molecular typing in feces, CSF, and serum of enterovirus-positive infants ≤60 days old in whom sepsis had been clinically diagnosed.

Patient	Results of testing of feces			Results of testing of CSF			Results of PCR testing of serum, Ct value
	Culture	Molecular typing	PCR, Ct value	Culture	Molecular typing	PCR, Ct value	
1	–	NA	37.4	NA	NA	NA	39.9
9	Echo-6	Echo-6	29.8	–	NA	38.7	29.3
10	Echo-16	Echo-16	29.3	–	NA	–	26.8
11	NA	NA	NA	–	NA	32.4	38.5
12	Echo-16	Echo-16	25.7	NA	NA	NA	35.5
13	Echo-16	Echo-16	25.0	–	NA	–	28.9
14	Echo-13	Echo-13	29.0	Echo-13	Echo-13	27.1	33.9
15	Echo-13	Echo-13	28.4	Echo-13	Echo-13	32.4	40.9
17	–	NA	36.8	–	NA	–	–
18	NA	Echo-16	30.9	Echo-16	Echo-16	NA	NA
19	Echo-6	Echo-6	30.1	Echo-6	Echo-6	38.3	36.7
Mean			30.2[a]			33.8	34.5

NOTE. Ct values ≥45 were considered to be negative. Ct, threshold cycle; echo, echovirus; NA, not analyzed; –, negative.

[a] $P = .06$, compared with serum value.

problems, infection, or asphyxia. Their characteristics are presented in table 1. In 9 patients (35%), intubation was necessary at the onset of sepsis because of respiratory insufficiency. Fourteen (53%) developed fever or temperature instability; 16 (62%), circulatory problems; 19 (73%), respiratory problems, including apnea; and 12 (46%), abdominal discomfort. All patients received broad-spectrum antibiotics at the onset of symptoms. Enterovirus was detected in none of these patients, but parechovirus 1 was isolated from the feces of 1 patient. In 17 patients (65%), the bacterial blood culture showed coagulase-negative staphylococci within 24 h of culture.

Group 3. The control group consisted of 20 patients who had been hospitalized since birth in the NICU and who were without signs of infection at the moment of viral evaluation. The characteristics of these patients are presented in table 1. None of these patients had positive results of culture or PCR for enterovirus.

DISCUSSION

Sepsis in neonates and young infants is an important clinical entity that leads to large numbers of hospital admissions each year. Review of the literature shows that 0.6%–3% of infants are readmitted to the hospital for suspected sepsis in the first month of life, and the majority of the patients are infected with enterovirus [2, 7]. These findings are supported by recent studies that used PCR to detect enterovirus. The incidence of enterovirus infection in young, febrile infants admitted to the hospital with systemic infection, especially in the summer, reaches 50% [8, 10].

In a previous retrospective Dutch study that used viral cultures only, the incidence of laboratory-diagnosed enteroviral disease during a 3-year period in infants aged <30 days was 26 cases/100,000 live births [3]. This number is an underestimation of the true incidence, because Dutch pediatricians often do not submit specimens for viral diagnosis from infants with suspected sepsis, and data were obtained on the basis of viral cultures only.

In the present prospective study, we used viral culture and real-time PCR to identify enterovirus infection. Fifty-seven percent of the infants admitted to a general hospital who received a clinical diagnosis of sepsis during April through January were found to have an enterovirus infection. The results of real-time PCR in plasma were positive in 9 of 10 infants with enterovirus infection. All 5 patients with positive results of PCR in CSF also had positive results of PCR in serum. Average Ct values for serum and CSF were 34.5 and 33.8, respectively.

Using real-time PCR, we tried to correlate virus load with severity of enterovirus disease. Two patients (patients 1 and 17; table 3) had very high Ct values in feces (37.4 and 36.8, respectively) that corresponded to a very low virus load. Viral culture of these fecal samples yielded negative results. The serum sample from patient 17 remained negative on PCR, indicating that this patient was shedding virus at low copy numbers in the feces only and was probably already recovering from the enterovirus infection. Patient 1 had a positive result of

serum PCR; the number of enterovirus copies in this patient's serum, however, was >40 times lower than the average number in PCR-positive serum samples from other patients. Bacterial urinary tract infections were finally diagnosed in both patients.

Our results suggest that serum real-time PCR is as least as good as or better than real-time PCR of CSF as a diagnostic tool for systemic enterovirus infection, although more patients should be analyzed to confirm this observation. A rapid diagnosis of enterovirus infection in young infants is important to prevent unnecessary exposure to broad-spectrum antibiotics, to reduce the anxiety of parents about prognosis, and possibly to indicate that antiviral treatment should be initiated, in severe cases. Rapid diagnosis of enterovirus disease has been demonstrated to significantly reduce hospital stay and costs [19–21, 25]. All of our enterovirus-positive patients recovered within 3 days, and if the patients could have received the diagnosis of enterovirus infection sooner, it is likely that earlier discharge from the hospital would have been possible.

Interestingly, an enterovirus infection was not detected in any of the NICU patients. All these patients had been hospitalized since birth, and visitors and medical staff had undergone a regimen of mandatory hygienic measures before contact with the infants. Nor did any of the patients hospitalized since birth in the study of Jenista et al. [7] have cultures positive for enterovirus. Enteroviruses are known to be transmitted horizontally, via the fecal-oral route; however, transmission may also occur perinatally. Because symptoms of perinatally acquired enteroviral disease are more severe and occur within the first days of life, it is probable that most of our patients were infected horizontally [1, 4–6]. Even for the 2 patients who were admitted to the hospital within the first week after birth (at 5 and 6 days of age), horizontal transmission cannot be excluded. We assume that the lack of adequate hygienic measures at home, compared with the hospital, is probably responsible for the increased risk of enteroviral disease.

In 1 of the patients hospitalized in the NICU, parechovirus 1 (previously "echovirus 22") was found in the feces. The clinical spectrum of neonatal infection caused by this virus is not much different from that caused by enteroviruses [1]. Therefore, parechovirus primers and probes need to be included in real-time PCR assays [16, 26].

In conclusion, febrile infants who are admitted to a hospital from home are very likely to be infected with an enterovirus. The NICU population seems not to be at a high risk of horizontal transmission of enteroviruses. Real-time PCR offers a tool for rapid diagnosis, but future prospective studies are needed to determine whether there is a relationship between virus load and clinical presentation.

Acknowledgments

We thank Tapani Hovi and Pia Lukkari of the National Public Health Institute (Helsinki, Finland) for their generous help with molecular typing.

References

1. Cherry JD. Enteroviruses. In: Remington JS, Klein J, eds. Infectious diseases of the fetus and newborn infant. 5th ed. Philadelphia: Saunders, **2001**:477–508.
2. Dagan R. Non-polio enteroviruses and the febrile young infant: epidemiologic, clinical, and diagnostic aspects. Pediatr Infect Dis J **1996**; 15: 67–77.
3. Verboon-Maciolek MA, Krediet TG, Van Loon AM, et al. Epidemiological survey of neonatal non-polio enterovirus infection in the Netherlands. J Med Virol **2002**; 66:241–5.
4. Modlin JF. Perinatal echovirus infection: insights from a literature review of 61 cases of serious infection and 16 outbreaks in nurseries. Rev Infect Dis **1986**; 8:918–26.
5. Abzug MJ, Levin MJ, Rotbart HA. Profile of enterovirus disease in the first two weeks of life. Pediatr Infect Dis J **1993**; 12:820–4.
6. Verboon-Maciolek MA, Swannink CMA, Krediet TG, et al. Severe neonatal echovirus 20 infection characterized by hepatic failure. Pediatr Infect Dis J **1997**; 16:524–7.
7. Jenista JA, Powell KR, Menegus MA. Epidemiology of neonatal enterovirus infection. J Pediatr **1984**; 104:685–90.
8. Byington CL, Taggart EW, Carroll KC, et al. A polymerase chain reaction–based epidemiologic investigation of the incidence of nonpolio enteroviral infections in febrile and afebrile infants 90 days and younger. Pediatrics **1999**; 103:E27.
9. Rosenlew M, Stenvik M, Roivainen M, et al. A population-based prospective survey of newborn infants with suspected systemic infection: occurrence of sporadic enterovirus and adenovirus infections. J Clin Virol **1999**; 12:211–9.
10. Rotbart HA, McCracken GH Jr, Whitley RJ, et al. Clinical significance of enteroviruses in serious summer febrile illnesses of children. Pediatr Infect Dis J **1999**; 18:869–74.
11. Schlesinger Y, Sawyer MH, Storch GA. Enteroviral meningitis in infancy: potential role of polymerase chain reaction in patient management. Pediatrics **1994**; 94:157–62.
12. Abzug MJ, Loeffelholz M, Rotbart HA. Diagnosis of neonatal enterovirus infection by polymerase chain reaction. J Pediatr **1995**; 126: 447–50.
13. Ahmed A, Brito F, Goto C, et al. Clinical utility of the polymerase chain reaction for diagnosis of enteroviral meningitis in infancy. J Pediatr **1997**; 131:393–7.
14. Read SJ, Mitchell JL, Fink CG, et al. LightCycler multiplex PCR for the laboratory diagnosis of common viral infections of the central nervous system. J Clin Microbiol **2001**; 39:3056–9.
15. Verstrepen WA, Kuhn S, Kockx MM, et al. Rapid detection of enterovirus RNA in cerebrospinal fluid with a novel single-tube real-time reverse transcription–PCR assay. J Clin Microbiol **2001**; 39:4093–6.
16. Corless CE, Guiver M, Borrow R, et al. Development and evaluation of a "real-time" RT-PCR for the detection of enterovirus and parechovirus RNA in CSF and throat swab samples. J Med Virol **2002**; 67: 555–62.
17. Verstrepen WA, Bruynsels P, Mertens AH, et al. Evaluation of a rapid real-time RT-PCR assay for detection of enterovirus RNA in cerebrospinal fluid specimens. J Clin Virol **2002**; 25:S39–43.
18. Nijhuis M, van Maarssenveen N, Schuurman R, et al. Rapid and sensitive routine detection of all members of the genus *Enterovirus* in different clinical specimens by real-time PCR. J Clin Microbiol **2002**; 40:3666–70.

19. Ramers C, Billman G, Hartin M, et al. Impact of a diagnostic cerebrospinal fluid enterovirus polymerase chain reaction test on patient management. JAMA **2000**; 283:2680–5.
20. Robinson CC, Willis M, Meagher A, et al. Impact of rapid polymerase chain reaction results on management of pediatric patients with enteroviral meningitis. Pediatr Infect Dis J **2002**; 21:283–6.
21. Stellrecht KA, Harding I, Woron AM, et al. The impact of an enteroviral RT-PCR assay on the diagnosis of aseptic meningitis and patient management. J Clin Virol **2002**; 25:S19–26.
22. Monpoeho S, Coste-Burel M, Costa-Mattioli M, et al. Application of a real-time polymerase chain reaction with internal positive control for detection and quantification of enterovirus in cerebrospinal fluid. Eur J Clin Microbiol Infect Dis **2002**; 21:532–6.
23. Van Loon AM, Cleator GC, Ras A. External quality assessment of enterovirus detection and typing. Bull World Health Organ **1999**; 77: 217–23.
24. Oberste MS, Maker K, Kilpatrick DR, et al. Typing of human enteroviruses by partial sequencing of VP1. J Clin Microbiol **1999**; 37: 1288–93.
25. Marshall GS, Hauck MA, Buck G, et al. Potential cost saving through rapid diagnosis of enteroviral meningitis. Pediatr Infect Dis J **1997**; 16: 1086–8.
26. Joki-Korpela P, Hyypiä T. Diagnosis and epidemiology of echovirus 22 infections. Clin Infect Dis **1998**; 27:129–36.