Comparison of Rapid Methods of Detection of Cytomegalovirus in Saliva with Virus Isolation in Tissue Culture

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Two rapid methods for the detection of cytomegalovirus (CMV) in saliva from congenitally and perinatally infected children were assessed by comparison with traditional virus isolation in tissue culture (TC). The polymerase chain reaction (PCR) was used to amplify a 300-bp segment of the CMV gB gene which was detected in ethidium bromide-stained agarose gels. A centrifugation-enhanced microtiter culture method with a monoclonal antibody for the detection of early-antigen fluorescent foci (DEAFF) was also used. Saliva specimens were collected with mouth swabs from children who were between the ages of 1 month and 14 years and who had either prenatal or perinatal CMV infection. One hundred sixty samples were tested by PCR and TC; 65 (40.6%) were found positive by TC, and 58 (36.8%) were found positive by PCR. Although four samples were found positive by PCR and negative by TC, saliva from seronegative and seropositive TC-negative adults were never found positive by PCR. One hundred fifty-two samples were tested by DEAFF and TC; 64 (42.1%) were found positive by TC, and 58 (38.2%) were found positive by DEAFF. With TC results as a standard, the sensitivity and specificity of DEAFF were, respectively, 90.6 and 97.7%. Because of the greater ease of collecting saliva than urine from newborns, both of these rapid methods merit evaluation in screening for congenital infection.

The diagnosis of congenital cytomegalovirus (CMV) infection is made by the detection of the virus in body fluids (usually urine) of a newborn (8). The "gold standard" for the detection of CMV has been the isolation of virus in human fibroblast tissue culture (TC), a procedure which requires culturing inoculated TC for 4 or more weeks, with regular observation and changing of media, before a negative result can be declared (7). A more rapid approach that uses shell vials for TC with centrifugation enhancement and monoclonal antibodies to early antigens provides results in about 24 to 48 h, with a sensitivity and a specificity similar to those of the more traditional TC (3, 5). However, the shell vial approach also requires the maintenance of TC and multistep processing of specimens. Neither of these methods appears to be suitable for the screening of newborns for congenital CMV infection. In addition to the disadvantages of these laboratory methods, collecting a satisfactory urine specimen from a newborn can often prove problematic. Since over 90% of newborns with congenital CMV infection have no clinical evidence of disease at birth (6), the vast majority will go undetected unless routine screening is used.

Early detection of congenital CMV infection will allow the anticipation of sequelae such as hearing loss, developmental delay, or motor abnormalities and planning for appropriate intervention. If new developments in antiviral therapy provide a treatment which can be used to prevent or ameliorate some of the central nervous system sequelae associated with congenital CMV infection, then the screening of newborns will become a top priority. Newborn screening will require methods that are inexpensive, rapid, accurate, and amenable to automation. We investigated the performance of two rapid methods for the detection of CMV in saliva, the polymerase chain reaction (PCR) and a microtiter TC procedure based on the detection of early-antigen fluorescent foci (DEAFF).

MATERIALS AND METHODS

Clinical samples. Saliva specimens were collected from 153 CMV-infected infants and children who ranged in age from 1 month to 14 years and who were being monitored in a special clinic. One hundred forty had congenital CMV infection proven by isolation of virus from urine collected within the first 3 weeks of life. Thirteen subjects acquired CMV perinatally; they were not viruric at birth but began to shed CMV at between 1 and 3 months of age. Control samples were collected from one seropositive and two seronegative laboratory workers.

Saliva was collected by swabbing the inside of the subject's mouth with a cotton-tipped applicator (Baxter S/P Sterile Swab; GIBCO Laboratories, Grand Island, N.Y.) until the applicator was soaked; it was then placed in a tube containing 1.5 ml of medium 199 (GIBCO) with the antibiotics nystatin (100,000 U/100 ml) and gentamicin (50 mg/100 ml). In the laboratory, medium was expressed from the applicator, which was then discarded; the tube containing saliva in medium was centrifuged at $1,000 \times g$ for 10 min. The supernatant was tested for CMV by TC inoculation, PCR, and DEAFF.

Virus isolation. Duplicate tubes of locally prepared human foreskin fibroblast tissue were inoculated with 0.2 ml of the supernatant material from the saliva specimen. CMV was identified by the detection of its characteristic slowly developing focal cytopathic effect by light microscopy as previously described (7, 8).

DEAFF. For DEAFF, 0.1-ml aliquots of the saliva-medium supernatant were inoculated into triplicate wells of a 96-well TC plate (Linbro; Flow Laboratories, Inc., McLean, Va.) containing locally derived human foreskin fibroblasts. The plate was centrifuged at $300 \times g$ for 1 h. The inoculum was aspirated and replaced with medium 199 containing 2% calf serum. After 24 h of incubation at 37°C in an atmosphere of 5% CO₂, the medium was aspirated and replaced with a locally prepared murine monoclonal antibody to CMV im-

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TABLE 1. Comparison of PCR and DEAFF with virus isolation in TC for the detection of CMV in saliva

TC result	No. samples that were			
	PCR		DEAFF	
	Positive	Negative	Positive	Negative
Positive	58	7	58	6
Negative	4	91	2	86

mediate-early antigen (1). After 1 h of incubation at 37°C, the antibody was replaced with fluorescein isothiocyanate-labeled goat anti-mouse immunoglobulin (Tago, Inc., Burlingame, Calif.). After an additional hour of incubation, the conjugate was removed and the plate was washed with phosphate-buffered saline. Foci were identified by examination of the plate with an inverted fluorescence microscope (Leitz Diavert).

PCR. A 0.1-ml aliquot of the saliva-medium supernatant was added to 0.1 ml of lysis buffer–20 µl of proteinase K (Sigma Biochemicals, St. Louis, Mo.), and the mixture was incubated overnight at 56°C. After deactivation of the proteinase K by heating to 95°C for 10 min, 10 µl was used as a source of template DNA. PCR was performed with reagents supplied in the GENEAMP kit (Perkin Elmer Cetus, Norwalk, Conn.) in accordance with the manufacturer's instructions. Samples were amplified for 30 cycles on an Ericomp (San Diego, Calif.) thermal cycler with denaturation at 95°C for 1 min, annealing at 55°C for 2 min, and extension at 72°C for 3 min. In the 31st cycle, the extension time was lengthened to 7 min. A pair of 20-bp primers bracketing a 300-bp segment between bases 1850 and 2150 in a conserved region of the gB envelope gene was used (4). Primer sequences were 5'-ACGTGAAGGAATCGCCAGGA-3' and 5'-AGTTCCAGTACCCTGAACTC-3'. Each PCR run included a sample with no template (distilled water), DNA extracted from CMV Ad169 as a positive control, and saliva samples from a seronegative adult and a seropositive, normal adult not shedding CMV. PCR products were electrophoresed in 1.8% agarose for 15 h at 25 V. Bands were visualized under UV light. A sample was considered positive for CMV when it yielded the characteristic band corresponding to 300 bp.

RESULTS

One hundred sixty saliva samples were tested for CMV by TC and by PCR; a comparison of the results is shown in Table 1. Sixty-five of the 160 (40.6%) were found positive by virus isolation, and 58 of the 160 (36.3%) were found positive by PCR. An example of the results obtained with PCR is shown in Fig. 1. Positive samples yielded characteristic bands readily visible in agarose gels. With TC results as a reference, the sensitivity of PCR was 58 of 65 (89.2%). Four samples that were found positive by PCR were found negative by TC; the specificity of the PCR compared with the latter method was 95.8%. No positive PCR result was obtained for any of the no-template controls or for any of the saliva samples from seronegative or seropositive adults. The initial 100 PCR results were run twice to check for reproducibility; there were no discrepancies between runs. One hundred fifty-two saliva samples were tested in parallel by TC and by DEAFF; the results are shown in Table 1. Sixty-four of the 152 (42.1%) were found positive by TC, and 58 of the 152 (38.2%) were found positive by DEAFF; the

FIG. 1. Typical result for the amplification of a 300-bp segment of the CMV gB gene from saliva. Lanes: 1, saliva from a seronegative adult with CMV Ad169 added; 2, saliva from a seropositive, TC-negative adult; 3, saliva from a seronegative adult; 4, 8 to 13, 16, and 17, negative result for saliva from children with congenital or perinatal CMV infection; 5 and 6, no-template controls; 7, control template from the GENEAMP kit; 14 and 15, positive result for saliva from children with congenital CMV infection; 18, DNA ladder.

sensitivity and specificity of DEAFF compared with TC were, respectively, 90.6 and 97.7%.

Samples with discordant PCR and TC results are compared in Table 2. There were four apparent false-positives in PCR; two of these samples were also positive in DEAFF. Young infants and patients with symptomatic congenital CMV infections shed larger quantities of virus; only one of four PCR-positive, TC-negative samples was from an asymptomatic child over 6 months of age. Three of seven PCR-negative, TC-positive samples were also DEAFF positive. Five of these samples discordant for PCR and TC were from asymptomatic children, and four were from children over 6 months of age.

PCR, DEAFF, and TC results are shown according to age in Table 3. The proportion of positive samples declined with advancing age for all three methods, and there were no statistically significant differences between methods in the proportion of positive results at any age. With TC as the standard, the sensitivity and specificity of PCR for children under 6 months of age were 92.5 and 86.7%, respectively. The sensitivity and specificity of DEAFF compared with TC for samples from children under 6 months of age were 94.6 and 83.3%, respectively.

TABLE 2. Samples with discordant PCR and TC results

Result	No. of samples tested	No. of samples that were			
		DEAFF positive	From subjects who were		
			<6 mo old	Symptomatic	Asymptomatic
PCR positive, TC negative	4	2	3	3	1
PCR negative, TC positive	7	3	3	2	5

TABLE 3. Detection of CMV in the saliva of children with congenital or perinatal infection according to age

Age (mo)	No. of samples found positive/total no. tested (%) by:		
	PCR	DEAFF	TC
≤6	41/52 (78.8)	37/51 (72.5)	40/54 (74.1)
7–12	10/15 (66.7)	9/13 (69.2)	8/12 (66.7)
13–24	2/16 (12.5)	3/16 (18.8)	4/14 (28.6)
25–48	1/14 (7.1)	1/10 (10.0)	1/11 (9.1)
≥49	1/40 (2.5)	1/35 (2.9)	1/30 (3.3)

DISCUSSION

Our results suggest that both PCR and DEAFF merit evaluation as screening methods for congenital CMV infection. Both methods provide results in 24 h or less, compared with about 4 weeks for virus isolation. Both methods compare favorably to standard TC isolation of virus in both sensitivity and specificity. Since newborns shed more virus than older infants and children, it is very likely that when these rapid methods are used to screen samples of saliva from newborns, their sensitivity will be even higher. Although we found both PCR and DEAFF to have >95% specificity, it is important to note that all of the children from whom samples were obtained had congenital or perinatal CMV infection. Some of the apparent false-positive samples could well have been samples that contained CMV not detected in TC. The fact that saliva from adult negative controls never gave a positive result in PCR lends support to the conclusion that the true specificity may be even higher than that measured. We were unable to adequately explain differences in results with PCR, DEAFF, and TC. The sensitivity of each method could be diminished by different variables. For example, the presence of mycoplasmas or other microorganisms in saliva could impair the ability to isolate CMV in TC. Enzymes present in saliva could interfere with the action of the polymerase used in PCR; alternatively, nucleases produced by microorganisms could alter the target DNA sequences, preventing amplification.

Demmler et al. evaluated PCR for the detection of CMV in the urine of congenitally infected newborns by using primers directed at both major immediate-early-protein and late-antigen genes (2). For 44 newborns with virus isolated from urine, samples from 41 were found positive by PCR with gel electrophoresis to detect amplified products; CMV DNA was found in all 44 urine samples from infected newborns (in a dot blot assay) when ³²P-labeled oligonucleotide probes were used to detect amplified products. No positive PCR results were found for a group of 27 urine samples that were TC negative. Although we did not achieve this level of sensitivity and specificity with saliva, our samples were collected from infants and children and would be expected to contain much less virus than the urine of a congenitally infected newborn.

Although the diagnosis of congenital CMV infection is usually made by the isolation of virus from the urine of a newborn, we chose to study saliva because it offers some clear advantages over urine. Collection of a urine sample from a newborn requires attaching a plastic, adhesive bag over the perineum. After the infant voids, the bag is removed. This procedure requires a substantial amount of nursing time to place the bag and check frequently for urine. Samples that are contaminated by stool, a frequent occurrence, are usually toxic for TC and therefore yield uninterpretable results. Minor skin irritation is sometimes produced

by the urine bag. With the trend toward shorter newborn nursery stays, many infants leave the hospital without providing an adequate urine specimen. Collection of saliva with a cotton-tipped applicator can be accomplished in seconds without undressing the newborn.

The principal advantage to be gained from screening for congenital CMV infection is identification of the cases that are asymptomatic at birth, over 90% of the total. Early identification of these infected infants will allow audiologic assessment to detect those born with sensorineural hearing loss. About 15% of those with silent congenital CMV infection have a hearing loss at birth or develop it within the first few years of life (6). It is clearly advantageous for hearing loss to be detected before these infants pass the age at which verbal communication skills are normally acquired. A small percentage of these children may have developmental delay, mental retardation, or neuromuscular or visual problems. In addition to the identification of children at risk for handicaps, wide-scale screening could provide a more accurate population-based estimate of the incidence (public health importance) of congenital CMV infection and allow the detection of changes in incidence.

The sensitivity of about 90% observed for each of the rapid methods that we assessed would probably be acceptable in a screening test for congenital CMV infection, especially if the cases with sequelae were among those identified. However, it would be desirable for a screening test for congenital CMV infection to have a specificity of near 100% because the incidence of congenital CMV is relatively low, about 1% of live births (6). With a sensitivity of 89% and a specificity of 96%, the predictive values of positive and negative PCR results would be, respectively, 18 and 99.9%. For DEAFF, with a sensitivity of 91% and a specificity of 98%, the predictive values of positive and negative tests would be, respectively, 31 and 99.9%. As noted above, the true specificity of each test in newborns may be significantly higher than what we measured in infants and children. A more valid assessment of the sensitivity and specificity of rapid methods proposed for screening for congenital CMV infection will require a comparison with virus isolation in a newborn population.

ACKNOWLEDGMENTS

This research was supported in part by grants from The Institute of Child Health and Human Development (HD 10699 and T32 HD07300) and the Division of Research Resources (Clinical Research Center grant RR-32).

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