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Cytomegalovirus in Urine: Detection of Viral DNA by Sandwich Hybridization

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A cytomegalovirus (CMV)-specific sandwich hybridization test was constructed by using two adjacent *Bam*HI DNA fragments of CMV DNA as reagents. The fragments were cloned into two different vectors. One of the recombinants was attached to the filter, and the other was the labeled probe. When present in the sample, CMV DNA mediated labeling of the filter by hybridizing to both the filter-bound DNA and the probe. The sandwich hybridization test was applied for the detection of CMV DNA from urine. DNA was released from virus by 2% Sarkosyl, concentrated by 2-butanol extraction and isopropanol precipitation, denatured, and finally subjected to the sandwich hybridization test. As a result, 70 to 90% of the original viral DNA could be recovered and demonstrated by the quantitative hybridization reaction. Urine could be stored at room temperature in Sarkosyl for at least 2 days without affecting the detectability of CMV. The clinical applicability of the test was evaluated by studying urine samples from four infants excreting CMV. Sandwich hybridization demonstrated the presence of CMV DNA in all of the specimens. These contained originally 10^5 to 10^8 CMV DNA molecules per ml.

Human cytomegalovirus (CMV) is common in the general population. Thus, between 30 and 100% of adults show serological evidence of prior CMV infection (6, 31). Although most CMV infections are mild or symptomless, there are two major groups of patients who may manifest serious CMV infections. One group includes congenitally infected newborn infants, and the other group is patients receiving immunosuppressive therapy. Between 0.3 and 2% of newborn infants excrete CMV in their urine (18, 26), and up to 100% of allograft recipients show evidence of a CMV infection (5). Because of recent developments in chemotherapy, treatment of CMV disease is becoming possible (14). The need for a rapid CMV diagnosis is increasing accordingly.

The present methods for diagnosing CMV infections include serological analysis and virus isolation. Both methods are usually time consuming. Furthermore, virus isolation requires human embryonic fibroblast cells that may be difficult to obtain. Alternative diagnostic approaches include the use of monoclonal antibodies to demonstrate the presence of viral antigens (7, 11, 29). The presence of viral DNA has been demonstrated in concentrated urine by spot hybridization (1; H. Gadler, Ph. D. thesis, National Bacteriological Laboratory, Stockholm, Sweden, 1983), and viral RNA has been detected in various tissue specimens by in situ hybridization (3).

Using recombinant DNA techniques, we created a pair of sharply specific CMV DNA reagents to develop a sandwich hybridization assay. Sandwich hybridization has been previously demonstrated to be a suitable method for testing crude samples for the presence of viruses (20, 21, 28) and bacteria (16, 17). Here, we show that CMV DNA can be rapidly concentrated 300-fold from urine and subsequently detected by sandwich hybridization. In the study was included some clinical specimens to which the method was successfully applied.

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MATERIALS AND METHODS

Cells and virus. MRC-5 cells or human embryo skin fibroblasts, obtained from the Department of Virology, University of Helsinki, were grown in Eagle minimal essential medium supplemented with 10% fetal bovine serum. The cells were infected with human CMV (AD169) and grown in the presence of Eagle minimal essential medium with 2% fetal bovine serum, and the extracellular virus was collected over a period of several days after the appearance of the complete cytopathic effect (CPE) as described previously (15). For DNA labeling, [^{3}H]thymidine (1 mCi/175-cm^2 bottle, 104 Ci/mmol; Amersham Corp., Arlington Heights, Ill.) was added to some cultures 7 days after inoculation just before the appearance of the complete CPE.

Virus purification comprised the following steps. After low-speed centrifugation, the culture medium was concentrated by ultrafiltration (CH4 concentrator with H1P100 hollow fiber cartridge; Amicon Corp., Danvers, Mass.), and the virus was pelleted by centrifugation through a 30% (wt/wt) sucrose cushion in an SW 27 rotor (Beckman Instruments, Inc., Fullerton, Calif.) at 25,000 rpm for 2 h at 4°C. The pellets were suspended in 50 mM Tris-hydrochloride (pH 7.5)–100 mM NaCl (TN), layered over a 20 to 55% (wt/wt) sucrose gradient in TN, and centrifuged in an SW 27 rotor at 25,000 rpm for 17 h at 4°C. The virus band was collected, concentrated by pelleting in the ultracentrifuge, and finally stored at −70°C. The quantity of virus after purification was estimated by the DNA content which was measured after phenol extraction and ethanol precipitation by UV spectrophotometry (Uvikon 810; Kontron), assuming a molecular weight of 150 × 10^6 for the CMV DNA. The [^{3}H]thymidine label, which was confirmed to be entirely in

the DNA, had a specific activity of 460 cpm/10^7 virus particles.

Herpes simplex virus type 1 (ATCC VR-733) and herpes simplex virus type 2 (ATCC VR-734) were propagated in human embryo fibroblasts. The cells were disrupted and collected in a 2% sodium dodecyl sulfate (SDS) solution after appearance of the complete CPE.

Preparation of DNA from clinical CMV isolates. Human embryo fibroblast cell cultures inoculated with clinical specimens were scraped into the growth medium after showing a CPE typical of CMV. The presence of CMV was confirmed by immune fluorescence on a portion of the culture with human convalescent serum. Fresh cell cultures were inoculated with a mixture of infected cells and medium and incubated until the CPE appeared. Further passages as described above were carried out (a total of three to five) until 50 to 75% of the cells were infected, as judged by the typical CPE. The cells were finally collected in 2% SDS, homogenized by aspirating through a thin hypodermic needle, treated with proteinase K (1 mg/ml, 37°C, 1 h; E. Merck AG, Darmstadt, Federal Republic of Germany), and phenol extracted, and the DNA was recovered after ethanol precipitation.

The CMV strains (Fig. 1) were isolated from two children, two adult patients that had received kidney transplants, and one adult patient that had received a bone marrow transplant.

Cloning procedures, vectors, and hosts. The *Hin*dIII L-fragment of AD169 CMV DNA cloned in the plasmid vector pAT153 (15) was digested with *Bam*HI restriction enzyme (Bethesda Research Laboratories, Inc., Bethesda, Md.). Two adjacent CMV-derived fragments were separated by electrophoresis in a 0.6% agarose gel, isolated by electroelution (model 1750 Electrophoretic Concentrator; Isco, Lincoln, Nebr.), phenol extracted, ethanol precipitated, and finally subcloned into pBR322 or M13mp7.

The bacterial strains *Escherichia coli* K-12 HB101 and *E. coli* K-12 JM103 and their growth conditions were as described previously (20). The construction of recombinant plasmids with the plasmid vector pBR322 and their transformation into the host and subsequent analysis were as published previously (20). Plasmid preparation on a preparative scale was done by the method of Clewell and Helinski (2). Cloning into the phage vector M13mp7 (13) and the subsequent phage growth on a preparative scale and DNA isolation have also been described previously (20).

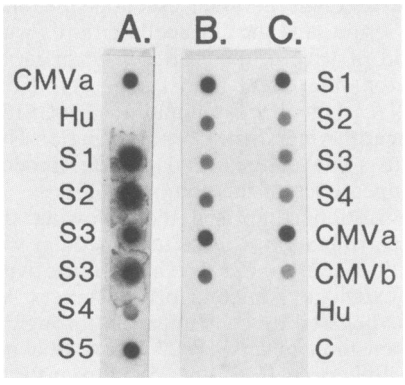

FIG. 1. Specificity of the CMV sandwich hybridization reagents as analyzed by spot hybridization. DNA isolated from 10^6 human embryo fibroblasts infected with different clinical isolates (S1 through S5), from 5×10^5 or 5×10^4 cells infected with CMV AD169 (CMVa and b, respectively), and from 5×10^5 uninfected cells (Hu) were spotted onto each parallel filter. C denotes spots to which 3 μg of calf thymus DNA was applied. Hybridizations were run for 17 h with 2×10^6 cpm of the recombinant phage reagent (A) and for 14 h with 2×10^5 cpm of CMV DNA (B) and 2×10^5 cpm of the recombinant plasmid reagent (C). The specific activities of the probes were 1.2×10^8, 1.1×10^8, and 1.9×10^8 cpm per μg of DNA, respectively. Autoradiographs are presented.

Sandwich hybridization. Recombinant plasmid DNA (the filter reagent) was denatured and attached to nitrocellulose filters (400 ng/10-mm filter; BA85; Schleicher & Schull Co., Keene, N.H.) as described previously (20). Each hybridization included one filter with the CMV-specific DNA and one empty filter as a control. The 400-μl reaction mixture contained the denatured sample (250 μl), the labeled probe (150,000 to 200,000 cpm) in 4× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate)–0.02% Ficoll (Pharmacia Fine Chemicals [Div. of Pharmacia, Inc.], Piscataway, N.J.)–0.02% polyvinylpyrrolidone (Fluka)–200 μg of denatured herring sperm DNA (Boehringer-Mannheim Corp., New York, N.Y.) per ml–0.25% SDS. The filters were preincubated under the same conditions, without the sample and probe, before the reactions were started. The sample DNA was boiled in 7 mM NaOH–0.3% SDS for 5 min, and then it was quickly cooled, neutralized, and subjected to testing. Virus particles were disrupted by 2% Sarkosyl and proteinase K treatment (1 mg/ml, 37°C, 1 h), and the DNA was denatured as described above. Hybridizations were carried out for 19 to 24 h at 65°C. The filters were then carefully washed in 0.1× SSC–0.2% SDS at 37°C for 2 h with one buffer change. Finally, they were counted in a 1282 Compugamma counter (Wallac, Finland).

Spot hybridization. DNA was applied onto nitrocellulose filters largely as described by Kafatos et al. (10). The DNA was denatured in 0.3 N NaOH at 100°C for 5 min, cooled to 0°C, mixed with an equal volume of 2 M ammonium acetate, and applied to the filter as 3-mm diameter spots by using the Hybri-Dot System (Bethesda Research Laboratories). The preincubation and the final hybridization conditions, as well as the washing conditions after hybridization, were as described above for the sandwich hybridization. The filters were dried and subjected to autoradiography with Kodak X-Omat film.

Labeling of DNA. The single-stranded recombinant phage DNA (probe reagent) was chemically iodinated with ^{125}I (13 to 16 mCi/μg; Amersham), as described previously (16, 20), to specific activities of about 10^7 to 10^8 cpm/μg of DNA. Double-stranded DNA was labeled by nick translation with [^{125}I]dCTP (>1,500 Ci/mmol; Amersham) as substrate (22).

Concentration of DNA from urine. Pretreatment of urine included the addition of 2% Sarkosyl–5 mM EDTA–gentamicin (40 μg/ml)–herring sperm DNA as carrier (2 to 4 μg/ml) (pretreatment mixture). Thereafter, the urine was treated with proteinase K (0.1 mg/ml, 37°C, 1.5 h) if not otherwise stated. For achievement of volume reduction, the urine was then extracted with an equal volume of 2-butanol (Merck), the phases were separated by centrifugation at 1,000 × *g* for 5 min at room temperature, and the butanol was removed (25). The extraction could be repeated up to six times as desired. The final aqueous phase was extracted once with diethyl ether, and the DNA was precipitated by the addition of an equal volume of isopropanol at room temperature (12). After 10 min the DNA was collected by centrifugation at 27,000 × *g* for 30 min at 4°C. The pellet was washed once with 70% ethanol, briefly dried, and dissolved in 10 mM Tris-hydrochloride (pH 7.5)–1 mM EDTA (TE). For volumes of

TABLE 1. Sensitivity and specificity of the CMV sandwich hybridization test

Sample[a]	cpm hybridized[b]
CMV DNA	
10 ng	626
5 ng	314
1 ng	121
HSV-1-infected cells (3×10^5)	46
HSV-2-infected cells (3×10^5)	60
Uninfected cells (3×10^5)	44
No DNA	60

[a] Infected cells were lysed in 2% SDS, homogenized by aspirating through a hypodermic needle, treated with proteinase K (1 mg/ml, 37°C, 1 h), and finally denatured by boiling in 7 mM NaOH for 5 min. Purified CMV DNA was similarly denatured. HSV-1, Herpes simplex virus type 1; HSV-2, herpes simplex virus type 2.

[b] Hybridization was for 19 h with 160,000 cpm of probe (specific activity, 6×10^7 cpm per μg of DNA)

urine up to 18 ml, the DNA was directly precipitated with isopropanol after the addition of the pretreatment mixture without incubating with proteinase K.

Collection of urine specimens. Pooled batches of urine from healthy laboratory personnel were used in the reconstitution experiments. No sterility precautions were taken while the urine was collected.

The patients were selected on the basis of positive CMV isolation from urine. A second specimen was collected 3 to 28 days later. Because all patients were infants, collection was into a collection bag, and the volume varied. The pretreatment mixture was added to the specimens immediately after collection. Thereafter, they were stored at room temperature until they were tested.

RESULTS

Sandwich hybridization reagents. The *Hin*dIII L-fragment which maps in the long unique region of CMV DNA (map position 0.3 to 0.35) (15, 23) was chosen as the starting material for creating the sandwich hybridization reagents. From a clone containing this fragment, two adjacent *Bam*HI fragments of 4.2 and 2.5 kilobases were subcloned into pBR322 and the M13mp7 phage vector, respectively. The recombinant plasmid was fixed onto nitrocellulose filters, and the single-stranded recombinant phage DNA was chemically iodinated (16, 20) to function as the labeled probe.

The specificity of the cloned DNA fragments is shown in Fig. 1 and Table 1. Genetic variation, reflected as antigenic heterogeneity (30), exists among naturally occurring CMV strains, the overall DNA homology being about 80% (9). We therefore decided to ensure that the genomic region from which the DNA reagents were selected was not subject to extensive variation. The radioactively labeled recombinant DNA reagents were hybridized with DNA derived from five different clinical CMV isolates. Human DNA was included in the assay to control repeated sequences common to human DNA and the genomes of herpesviruses (19) so that they did not occur in the cloned hybridization reagents. In addition, specific human genes, or parts of them, may be incorporated into the viral genomes as demonstrated for the *myc* sequences (4, 24). The DNA fragments did not show any cross-hybridization with human or calf DNA (Fig. 1). They also did not hybridize with herpes simplex virus types 1 or 2 DNA in a sandwich hybridization assay to which a lysate of infected cells was added (Table 1). It has been previously shown that viral DNA from infected cells, lysed as in this experiment, can be efficiently detected by the sandwich hybridization method (20). The reagents are thus specific for CMV, recognizing all clinical isolates and the prototype strain with equal efficiency.

The sensitivity of the sandwich hybridization assay in detecting purified CMV DNA was 4×10^6 DNA molecules (Table 1).

Concentration of CMV DNA from urine. It was evident that the sensitivity of the sandwich hybridization test would not allow the detection of CMV from unconcentrated urine of patients suspected of excreting virus. Preliminary experiments with radiolabeled virus indicated that CMV is fragile in urine and cannot be recovered quantitatively by pelleting in an ultracentrifuge. We therefore designed a process to concentrate the viral DNA instead. Two concentration procedures depending on the initial volume of urine were explored.

The results of reconstitution experiments, in which [^{3}H]thymidine-labeled CMV was added to urine and the recovery of the labeled DNA was measured, are shown in Table 2. After pretreatment (see above), DNA was simply precipitated with an equal volume of isopropanol (experiment 1). Recovery of the label in the dissolved precipitate was 82 to 87%. When larger volumes were to be treated, decreasing the urine volume was desirable before DNA precipitation was carried out. This was achieved by extraction with 2-butanol, which effectively reduced the water volume by taking up water to 40% of its original volume (25). By carrying out four successive extractions (experiment 2), the initial 100-ml volume was concentrated 10-fold. The recovery of DNA in the latter process (experiment 2) was 53 to 72%, depending on the proteinase K treatment. Proteinase K treatment of urine decreased the loss of DNA to the butanol phase by a factor of two (from 29 to 15% loss). Sarkosyl concentrations between 1 and 2% did not affect the results.

Detection of CMV by sandwich hybridization from urine after concentration. Increasing amounts of purified CMV were added to constant volumes of urine, viral DNA was released from the virions, and the DNA was concentrated by the two processes described above. The DNA recovered

TABLE 2. Recovery of labeled CMV DNA from urine after concentration

Expt no.[a]	Urine sample		Proteinase K treatment	DNA recovered (cpm) (% mean)[b]
	Vol (ml)	CMV (cpm)		
1	18	3,192	+	2,800 (82) 2,432
			−	2,806 (87) 2,737
2	100	10,904	+	8,170 (72) 7,539
			−	5,678 (53) 5,791

[a] In experiment 1, DNA precipitation was done directly with isopropanol; in experiment 2, 2-butanol extractions repeated four times were included. Two independent measurements are shown.

[b] DNA precipitate was dissolved in 400 μl of TE and counted in Insta-Gel (Packard Instrument Co., Inc., Rockville, Md.).

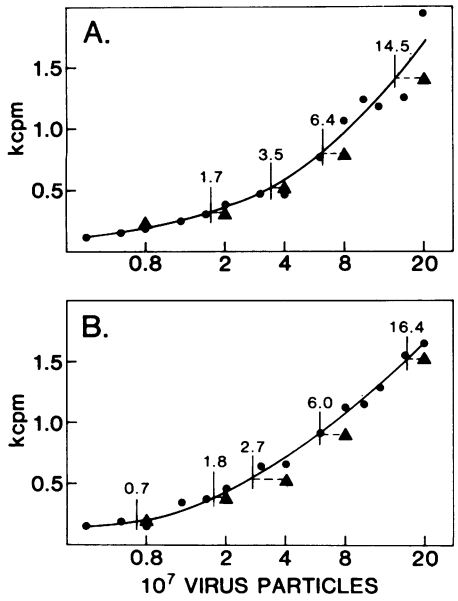

FIG. 2. Detection of CMV DNA by sandwich hybridization from urine after concentration. Concentration was done with 2-butanol followed by precipitation with isopropanol from an initial volume of 100 ml (A), or by direct precipitation with isopropanol from an initial volume of 18 ml (B). The number of CMV particles added to each urine sample is depicted (▲). The respective yield, estimated according to the standard curve (●), is given as the number of DNA molecules. Duplicate samples were measured. Hybridizations were for 20 h (A) and 23 h (B) with 155,000 cpm of probe (specific activity, 2×10^7 cpm per μg of DNA). The amount hybridized to the CMV filters in the absence of CMV DNA was 65 cpm ($\bar{x}_4$) (A) and 53 cpm ($\bar{x}_4$) (B).

was dissolved in 250 μl of TE, rendered to the single-stranded form, and subjected to sandwich hybridization. Assays with standard amounts of CMV DNA were carried out simultaneously (Fig. 2). Because sandwich hybridization is a quantitative assay (20), the number of CMV DNA molecules recovered after concentration can be estimated by a standard curve. The number of CMV particles added to each urine sample are shown (Fig. 2), and the number recovered is shown after extrapolation from the standard curve. Identical urine samples without CMV but with the carrier DNA were concentrated in parallel, and the concentrates were added to sandwich hybridizations with various amounts of CMV DNA. The standards measured in the presence of the mock concentrates were within 80 to 106% of the control values, and the background was unaffected. About 80% of the CMV present in 100 ml of urine could be detected by a sandwich hybridization test, indicating a 300-fold concentration (Fig. 2A). The results are in accordance with those shown in Table 2. Thus, components interfering with the hybridization were not concentrated by either process (Fig. 2A and B).

The detectability of CMV was not affected by the presence of albumin (200 mg/100 ml) or *E. coli* (10^9/100 ml) in urine. It was also not affected by storing for up to 48 h at room temperature, provided that the pretreatment mixture was added. These measurements were done as described in the legend to Fig. 2A (unpublished data).

Demonstration of CMV in clinical urine specimens. Patients were selected for this study on the basis of positive viruria, which was confirmed by virus isolation. All of the patients that were still hospitalized by the time the virus isolation results were available were children less than 1 year of age. A separate urine specimen to be tested by sandwich hybridization was collected. The specimen size varied, because we tested all of the urine that was voided into the collection bag. The pretreatment mixture was added immediately upon collection, and the urine was concentrated within 48 h. All of the isolation-positive urine specimens were also positive by the sandwich hybridization test (Table 3). Standards were run in each experiment, giving the limit of positivity (twice

TABLE 3. Detection of CMV by nucleic acid sandwich hybridization of urine specimens from children

Patient no.	Age	Diagnosis	Sample vol (ml)[a]	Amt of CMV DNA in control test (ng)	cpm hybridized[b]	Concn of CMV DNA molecules (× 10^6 per ml)[c]
1	1 mo	Congenital CMV infection	0.27		527	87
			10		2,224	
			90		4,027	
				0	153	
				1	268	
				5	349	
2	13 d	Microcephaly	30		3,040	18
				0	130	
				5	418	
3	7 mo	Infantile spasm	8		297	1.5
			25		615	
				0	79	
				1	161	
				5	433	
4	8 mo	Neurological disorder	50		261	0.2
				0	49	
				1	175	
				5	526	

[a] The volumes are aliquots from urine voided into a collection bag. The concentration procedure was as described in Table 2, experiment 2, except for 0.27 ml of urine from patient 1, which was tested unconcentrated, and 8 ml of urine from patient 3, which was precipitated with isopropanol without prior volume reduction. The urine from patient 1 was concentrated in one batch (100 ml), but the final concentrate, dissolved in 200 μl, was divided into 20 and 180 μl to be tested separately.

[b] Each patient represents a separate experiment. The mean of three parallel positive and negative controls is shown. Hybridizations were run for 19 h with 196,000 cpm (patient 1), 172,000 cpm (patient 2), and 155,000 cpm (patients 3 and 4) of probe (specific activity, 6×10^7 to 8×10^7 cpm per μg of DNA).

[c] Concentration in the original specimen, assuming 80% recovery during concentration.

the number of background counts per minute). The amount of CMV DNA molecules in the unconcentrated urine was calculated. The quantity of CMV secreted was highest in the two youngest patients, who were probably suffering from congenital CMV infection. The two additional patients were 7 and 8 months old. They secreted 2×10^5 to 15×10^5 virus particles per ml of urine. Whether they suffered from congenital or neonatal CMV infection was not known. The amount of infective virus in all of the urine samples was high, as judged by the rapidity of virus isolation (positive results were obtained within 3 to 8 days of inoculation).

DISCUSSION

CMV is a difficult virus to study. It has an extremely large genome of 230 kb, potentially coding for some 250 proteins. Because of the high structural complexity and the fact that the virus grows poorly, no modern diagnostic methods had been developed until recently. The cloning of the entire genome (15, 23, 27) and hybridoma technology have provided means for this development.

When nucleic acid hybridization is used to identify the virus, it is not necessary to know the gene content of the selected probes, as long as these DNA fragments fulfill the specificity criteria of the diagnostic test. The larger viral genomes are, the more there are repeated sequences in them that are common to all eucaryotic DNA (8, 19). These must be avoided when choosing a probe. Therefore, careful selection of a probe with proper hybridization controls is important. Several genomic fragments have proved to be suitable for diagnostic purposes (1; Gadler, Ph.D. thesis). Chou and Merigan (1) chose to use as a probe a sequence derived from a region repeated twice in the genome, thus increasing the sensitivity of the test. Because there are reports suggesting that the sequence variation among CMV strains might be concentrated in the repeat areas (32), we decided to select the reagents from the long unique region. The two *Bam*HI fragments, representing only 3% of the genome, proved to be CMV specific and were sufficiently homologous to the DNA of a variety of independent clinical CMV strains to recognize them. The gene content of this fragment is not known.

The sensitivity of the sandwich hybridization test is lower than that of a direct spot hybridization in which the sample DNA is fixed to the nitrocellulose. We are, however, using it because of the following advantages. The method is suitable for studying crude samples containing bacteria, cell lysates, or secretions such as nasopharyngeal mucus (16, 20, 21, 28), probably because the specimen is kept in solution. Urine, from which CMV most often is recovered, also is a crude sample of undefined nature. Futhermore, urine may contain enterobacteria which, in turn, may harbor plasmids or phages related to the cloning vectors. These would give specific background reactivity in direct spot hybridization when a recombinant DNA probe is used. Thus, Chou and Merigan (1) had to remove the vector part of their probe when studying CMV in urine by spot hybridization. In sandwich hybridization, however, this problem is circumvented.

The sample pretreatment developed here involving precipitation of viral DNA from crude urine by isopropanol is a reproducible method that also can be carried out routinely in a laboratory. Combined with extractions with 2-butanol, it can be applied to urine volumes of 500 ml. Furthermore, the method allows transportation and storage of the specimens at room temperature, which increases its applicability.

Even if the number of clinical samples used in this study was small, it demonstrated that the test and the sample pretreatment developed are applicable to diagnostic use. The results of the study suggest that at least congenital, and perhaps other primary CMV infections, could be diagnosed by hybridization. This would permit more extensive screening of patients than has been possible so far. Hybridization is applicable to any group of patients. More sensitive hybrid detection methods must be developed for general application. The hybridization test can also detect virus in tissue specimens. Our preliminary data indicate that sandwich hybridization can be applied to studying lung biopsies for the presence of CMV.

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