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DETECTION OF CYTOMEGALOVIRUS IN URINE OF HIV-INFECTED PATIENTS BY DNA-DNA HYBRIDIZATION COMPARISON WITH VIRUS ISOLATION, IMMUNOFLUORESCENCE AND IMMUNOPEROXIDASE

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Immunofluorescence and immunoperoxidase test directed against early viral antigens, and DNA-DNA hybridization were compared with viral isolation for their abilities to detect Cytomegalovirus (CMV) in the urine of 89 HIV infected patients. From the 100 urine samples collected, 70 were found positive by at least one method. Considering viral isolation as the "gold standard" technique, immunofluorescence and immunoperoxidase had a sensitivity of 92.3% and 88% respectively, with a specificity in both cases of 95%. DNA-DNA hybridization showed a sensitivity of 90% but with lower (60%) specificity. All of the three assays were effective in detecting CMV from urine and the technical advantage of each is discussed.

Key words: cytomegalovirus – rapid diagnosis – monoclonal antybodies – hybridization

Because of recent development in viral chemotherapy, treatment of human cytomegalovirus (HCMV) is becoming avilable and the need for rapid CMV diagnosis is increasing (Snydman, 1988). The available methods for HCMV diagnosis include serology and viral isolation. The interpretation of serological finding is difficult, particularly in immunosupresed patients, while in viral isolation attempts, the cytopathical effect (CPE) of this virus often does not develop until several weeks after inoculation (Tyms et al., 1989). Moreover, virus isolation may be dificult because of microbial contaminants in specimens.

The imediate detection of eraly nuclear antigen in cell culture by immunofluorescence using monoclonal antibodies plus centrifugal enhancement of infectivity provides a sensitive and specific method for the rapid detection of HCMV in urine (Mazeron et al., 1984). This method is less susceptible to cellular toxicity or microbiological contamination than virus isolation, but still it requires cell culture. On the other hand, molecular biology techniques like DNA-DNA or RNA-DNA hybridization, as well as the recently developed polymerase chain reaction (PCR) (Shuster et al., 1986; Demmler et al., 1988) represent a more powerful approach for the rapid detection of HCMV in urine.

We report here our experience using two techniques based on monoclonal antibody technology: immunofluorescence and immunoperoxidase detection of early antigens of CMV in cell culture, and DNA-DNA hybridization, a potentially useful method for rapid viral diagnosis.

MATERIALS AND METHODS

Viruses and cells – The strain AD 169 CMV was kindly supplied by Profcssor F. Bricout of the Trousseau Hospital, Paris, France. Clinical isolates of HCMV, HSV1 and HSV2 were obtained from the Laboratory of Clinical Virology of the Institute of Tropical Medicine "Pedro Kouri". Epstein Barr virus transformed cell lines (P3HR1 and RAJI) were also obtained from the Trousseau Hospital.

Low passage Human Embryonic Lung (LH) fibroblast in approximately their 15th passage were grown in Minimum Essential Medium (MEM) containing 10% of Fetal Calf Serum, 1% of glutamine, 100 Iu/ml penicillin and 100 µg/ml streptomycin sulfate.

Processing of clinical specimens – 100 urine samples were obtained from 89 patients at

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different stages of HIV infection suspected CMV infection. Each sample (10 to 30 ml) was collected in a sterile container, clarified by centrifugation and treated with penicillin and streptomycin as preservatives. About 2 ml of each sample were immediately used for viral isolation, immunofluorescence or immunoperoxidase assays; the rest was stored at – 70 °C until further use for hybridization.

Viral isolation and identification was conducted according to previously reported procedures (Reynolds et al., 1979). If no evidence of HCMV infection was found after 30 days of incubation, the samples were discarded as negative.

Immunofluorescence (IF) and immunoperoxidase (IP) techniques were performed as previously described as previously described (Mazeron et al., 1984; Swenson & Kaplan, 1985). E13 monoclonal antibody (Biosoft, France) against the early antigens of CMV was used for both tests. Affinity-purified, goat-antimouse IgG serum conjugated with fluorescein isothiocyanate (BIOCEN, Cuba) or peroxidase (IPK, Cuba) were used to detected specifically bound IgG.

Cosmid DNA isolation and probe preparation – The cosmid PcM 1015 (Fleckenstein et al., 1982), containing Hind III and EcoR1 fragments of HCMV, was kindly donated by J. C. Nicolas (Trousseau Hospital, Paris). The bacteria were grown and the cosmid DNA was extracted by alkaline lysis (Manniatis et al., 1982) and digested by EcoR1 accordingly to manufacture's recommendations (Enzibiot, Cuba). The digests were subjected to overnight electrophoresis at 40 V in 0.8% low gelling agarose gels (Pharmacia). The 9.9 Kb K EcoR1 and the 8.6 Kb G EcoR1 fragments were excised from the gels and purified by glass powder elution (Davis et al., 1986). The KG EcoR1 probe represents approximately the 50% of the original cosmid and 10% of the total HCMV genome.

Labeling of DNA probes – DNA probes (100ng) were labeled by random priming (Enzibiot, Cuba) and used without furthers purification steps.

Dot blot hybridization – For DNA-DNA hybridization, urine samples were processed according to the method previously described (Spector et al., 1984). DNA of HCMV, HSV1, HSV2, Epstein Barr virus cell lines (P3HR1 and RAJI) and uninoculated human lung fibroblast were prepared in the same manner and used to evaluate the hybridization test. DNA was denatured heating samples and controls at 100 °C for 5 min, and chilled it on ice for 5 min, then applied to a nitrocellulose filter using a filter manifold (Schleicher & Schuel Inc.). Immobilized samples were fixed to the solid support by baking at 80 °C for 2 h.

Nitrocellulose filters were pre-hybridized and hybridized by conventional procedures (Chou & Merigan, 1983) using a radiolabeled probe at 10^6 counts per min/ml. Following hybridization, the filters were washed repeatedly in SSC (SSC is 0.15 M NaCl and 0.015 M Sodium Citrate) and in Sodium Dodecyl Sulphate (SDS) at different temperatures. The bound ^{32}p dATP labeled probe was detected autoradiographically by exposure for 24 h to X-ray film (Fuji) at -70 °C using an intensifying screen.

RESULTS

The results obtained in the analysis of samples are shown in Table I. Viral isolation was positive in 52 samples (x = 10.2 days for CPE detection); 50 samples were also positive by IF and 48 were positive by IP. Using dot blot hybridization, 63 urine samples were recorded as positive. The four test were in complete agreement in 70 samples, among which 41 were positive (40.5%) and 29 were negative (28.7%). The remaining 30 samples were discordant for the four tests employed. Fifteen dot blot-positive samples were negative in the other assays. There was no evidence for non-specific hybridization, as none of the samples hybridized when the labeled vector was used as probe.

As shown in Table II, all of the three assays gave a similar sensitivity (about 90%) when compared to virus isolation. The specificity of either IF or IP was high (95.8% and

TABLE I

Detection of HCMV in urine specimens

Techniques	Samples	Positives
Viral isolation	100	52
Immunofluorescence	100	50
Immunoperoxidase	100	48
Dot Blot	100	63

TABLE II

Comparison between obtained results using Viral Isolation, Immunofluorescence, Immunoperoxidase and Dot-Blot Hybridization for the detection of HCMV in clinical samples

		Viral isolation							
		+	–		+	–		+	–
IF	+	48	2	IP	46	2	DB	46	17
	–	4	46		6	46		6	31
		Sensitivity: 92.3%			Sensitivity: 88.4%			Sensitivity: 88.4%	
		Specificity: 95.8%			Specificity: 95.8%			Specificity: 64.5%	
		Coincidence: 94 %			Coincidence: 92 %			Coincidence: 77 %	
		PPV: 92 %			PPV: 95.8%			PPV: 49.2%	
		NPV: 92 %			NPV: 88.4%			NPV: 83.7%	

IF = immunofluorescence
IP = immunoperoxidase
DB = dot blot hybridization
PPV = positive predictive value
NPV = negative predictive value

95.8%, respectively), the monoclonal antibodies based tests showed more than 92% agreement with cell culture, while dot blot showed only 77% agreement with a specificity of 64.5%.

DISCUSSION

The aim of the present work was to compare the use of different techniques for the detection of HCMV in urine samples from HIV infected patients.

Our current work showed that IF and IP procedures are sensitive and specific as diagnostic tests for HCMV detection; both tests, in our hands, demonstrated a good correlation with standard cell culture procedures as has been reported by other authors (Swenson & Kaplan, 1985).

The KG EcoR1 fragments we used do not contain human DNA sequences (Ruger et al., 1984), the specificity of the hybridization assay was confirmed by the negative results obtained with 1 μg of DNA extracted from uninfected LH cells, P3HR1, RAJI, HSV1 or HSV2.

The sensitivity of DNA hybridization with overnight autoradiography for the detection of HCMV in urine was quite similar to earlier reports (Buffone et al., 1988), but CMV DNA could not be detected in six urine samples positive by viral isolation. This might be due to the presence of extremely small amounts of virus below the limits of assay detection (up to 10-20 pcg CMV DNA), which is confirmed by the fact that in these cases the isolation was obtained after some 17 days compared with 10 days for the rest of the samples.

False-positive results in HCMV DNA hybridization assay have been observed by different authors (Shuster et al., 1979); Spector et al., 1984) and we also encountered this problem in 15 samples which were positive by dot blot but negative by the other tests, possibly reflecting the presence of non infectious virus in such specimens.

Our study showed that IF and IP tests for detection of HCMV in urine samples have similar levels of sensitivity and specificity and are feasible for routine diagnosis of CMV infection, considering they are going to provide reliable results in a shorter period of time than the classical isolation procedures. The DNA-DNA hybridization is an important development in microbiological diagnosis, and its sensitivity has been reported previously about of 5 pcg of DNA (Chou & Merigan, 1983). The test doesn't require a tissue culture facility and the constraints derived from handling radioactive material may be overcome in the future with the use of non-radioactive probes. DNA-DNA hybridization showed an unexpected low positive predictive value in our study, due to the large proportion of false positive results.

REFERENCES

BUFFONE, G. J.; DEMMLER, G. J.; SCHIMBOR, C. M. & YOW, M. D., 1988. DNA hybridization assay for congenital cytomegalovirus infection. *J. Clin. Microbiol., 26:* 2184-2186.

CHOU, S. & MERIGAN, T. C., 1983. Rapid detection and quantitation of human Cytomegalovirus in urine through DNA hybridization. *New Engl. J. Med., 308:* 921-925.

DAVIS, G. L.; DIBNER, M. D. & BATTEY, J. F., 1986. *Basic Methods in Molecular Biology,* (p. 123-126), Elsevier Science Publishing Co., Inc, New York.

DEMMLER, G. J.; BUFFONE, G. J.; SCHIMBOR, C. M. & MAY, R. A., 1988. Detection of cytomegalovirus in urine from newborns by using polymerase chain reaction DNA amplification. *J. Infect. Dis., 158:* 1177-1184.

FLECKENSTEIN, B.; MULLER, I. & COLLINS, J., 1982. Cloning the complete human cytomegalovirus genome in cosmids. *Gene, 38:* 39-46.

MANIATIS, T.; FRITSCH, E. F. & SAMBROOK, K. J., 1982. *Molecular Cloning: A laboratory Manual,* p. 178. Cold Spring Harbor Laboratory, Cold Sprin Harbor, New York.

MAZERON, M. C.; COLIMAN, R. C.; ROSETO, A. & PEROL, Y., 1984. Detection of cytomegaloviremia using monoclonal antibodies. *Develop. Biol. Standard., 57:* 287-291.

RUGER, R.; BORNKAMM, G. W. & FLECKENSTEIN, B., 1984. Human cytomegalovirus DNA sequences with homologies to the cellular genome. *J. Gen. Virol., 65:* 1351-1364.

SCHUSTER, V.; MATZ, B.; WREGAND, H.; TRAUB, B.; KAMPA, D.; REYNOLDS, D. W.; STAGNO, S. & ALFORD, CH. A., 1979. Laboratory Diagnosis of Cytomegalovirus infections, p. 399-439. In E. H. Lennette & N. J. Schmidt (eds), *Diagnostic Procedures for Viral, Rickettsial and Chlamydial Infections.* Amer. Pub. Health Assn. Washington, D. C.

SHUSTER, V; MATZ, B.; WIEGAND, H.; TRAUB, B.; KAMPA, D. & NEUMAN-HAFELIN, D., 1986. Detection of human cytomegalovirus in urine specimens by DNA-DNA and RNA-DNA hybridization. *J. Inf. Dis., 154:* 309-314.

SNYDMAN, S. R., 1988. Ganciclovir therapy for Cytomegalovirus disease associated with renal transplants. *Rev. Inf. Dis., 10:* 554-562.

SPECTOR, S. A.; RUA, J. A.; SPECTOR, D. H. & Mc MILLAN, R., 1984. Detection of human cytomegalovirus in clinical specimens by DNA-DNA hybridization. *J. Infec. Dis., 150: 1*21-126.

SWENSON, P. D. & KAPLAN, M. H., 1985. Rapid detection of cytomegalovirus in cell cultures by indirect immunoperoxidase stainning with monoclonal antibody to an early antigen. *J. Clin. Microbiol., 21:* 669-673.

TYMS, A. S.; TAYLOR, D. L. & PARKIN, J. M., 1989. Cytomegalovirus and the acquired immunodeficiency syndrome. *J. Antimicrob. Chemotherapy., 23:* 89-105.