JOURNAL OF CLINICAL MICROBIOLOGY, Nov. 1995, p. 3064–3067 Vol. 33, No. 11
0095-1137/95/$04.00+0

Intestinal Cytomegalovirus Disease in Immunocompromised Patients May Be Ruled Out by Search for Cytomegalovirus DNA in Stool Samples

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Received 1 May 1995/Returned for modification 6 July 1995/Accepted 22 August 1995

Cytomegalovirus (CMV) PCR from stool specimens was adopted as a diagnostic tool for patients with suspected CMV colitis. After being established, the method was evaluated in 17 AIDS patients and 19 other immunocompromised patients by comparison of PCR results with clinical, histological, and microbiological or virological data. CMV PCR was positive in 4 symptomatic patients with proven CMV colitis and negative in 15 of 16 patients without characteristic histopathology. Neither CMV immunoglobulin G seropositivity nor intestinal symptoms alone were significantly associated with positive PCR results, but severe active systemic CMV infection may lead to a positive PCR. Absence of CMV DNA in stool samples may prove useful in ruling out CMV related colitis.

One important localization of cytomegalovirus (CMV) disease in human immunodeficiency virus-infected and other immunocompromised patients is the digestive tract (19, 22, 23). The clinical symptoms of intestinal CMV disease, including diarrhea with weight loss, are rather nonspecific and do not permit diagnosis in most cases (9, 13, 19, 24). Differential diagnosis includes symptomatic infection by other intestinal pathogens (9). Because a specific therapy is available that may improve the outcome of disease, early etiologic diagnosis is important (3, 17, 27, 28). CMV serology is not helpful (5, 9), and even diagnosis of an active CMV infection by virus isolation (11), antigen staining (5, 29), or identification of CMV DNA by PCR in urine (8) or blood (31) does not prove the presence of intestinal CMV disease. Furthermore, absence of the virus from these materials does not rule out intestinal CMV disease (5, 13). CMV PCR from blood samples of liver and heart transplant recipients may not be useful for diagnosis of CMV disease, since positive PCR results do not correlate with clinical outcome (7, 10). Thus, the diagnostic value of a method has to be evaluated for every single diagnostic situation.

Demonstration of typical cytomegalic cells in intestinal biopsies is often regarded as the "gold standard" for diagnosis of intestinal CMV disease. However, even in unequivocal cases of intestinal CMV disease, cytomegalic cells may be missed because of a sampling error (12, 14, 33).

Virus detection in feces by isolation, antigen detection, or electron microscopy is well established for typical resistant gastroenteritis viruses such as enteroviruses, rotavirus, adenovirus, or Norwalk virus. Recently, PCR methods have been established for detection of these pathogens in stool samples (1, 6, 15, 21). However, isolation of CMV from stool samples is very uncommon and is an extremely insensitive method, since CMV is easily inactivated and the level of residual infectivity in stool is very low. Today, the definite diagnosis of intestinal CMV disease depends on colonoscopy to obtain biopsies for virological and histological evaluation (4, 14, 16, 19). Obviously, colonoscopy is very stressful for severely ill patients or even impossible in some high-risk patients.

Goodgame et al. (14) claimed the need for a more sensitive test correlated with histopathology but independent of the number of biopsies. We thought that in analogy to typically enteropathogenic viruses, it might be possible that CMV is selectively shed with feces by patients suffering from CMV colitis. Our aim was to establish and evaluate a PCR-aided method for detection of CMV DNA in fecal specimens of immunocompromised patients suffering from intestinal disease.

Thirty-six patients (17 AIDS patients [group IV.C3 according to Centers for Disease Control criteria, 1993] and 19 recipients of allogeneic transplants) suffering from diarrhea of unknown origin or without intestinal symptoms were investigated (Table 1). Intestinal symptoms were defined as diarrhea, abdominal pain, and weight loss, clinically indicative of intestinal CMV (9, 20). Active CMV infection was defined by isolation of the virus from any body fluid or tissue from 4 weeks before until 4 weeks after the time point of stool sampling. CMV disease of the colon in symptomatic patients was diagnosed by characteristic histology and/or immunohistology or by recovery of the virus from intestinal biopsies. Additional virological examinations were performed as described previously (18).

Fecal specimens (5 ml) were collected and transported in 10 ml of hexadecyltrimethylammonium bromide (CTAB)-extraction buffer (100 mM Tris-hydrochloride [pH 8.0], 1.4 M NaCl, 80 mM EDTA [pH 8.0], 2% CTAB [wt/vol], 0.2% [vol/vol] β-mercaptoethanol).

DNA was extracted according to a modified method of Saghai-Maroof et al. (30). Briefly, 3 ml of the stool-CTAB

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TABLE 1. Clinical, histological, virological, and microbiological data of the study patients[a]

Patient	Diagnosis	Intestinal symptoms[b]	Histology		CMV DNA in stool	Active CMV infection[d]	Patient's CMV status[e]	CMV retinitis	Antiviral therapy[f]	Other intestinal pathogen(s)[g]
			Intestinal GvHD (BMT patients)	Intestinal CMV disease[c]						
1	AIDS	+		+	++	+	+	−	ACV-GCV	−
2	AIDS	+		+	−+	+	+	+	GCV	−
3	AIDS	+		+	++	+	+	−		−
4	AIDS	+		+	++	+	+	−	GCV	−
5	AIDS	+		+	n.a.	∅	+	−		−
6	AIDS	+		+	n.a.	∅	+	−		−
7	BMTa-CML	+	+	−	++	+[h]	+	−	ACV	−
8	BMTa-CML	+	n.a.	n.a.	−	+[h]	+	−		−
9	BMTc-PNP	+	−	−	−	+[h]	+	−	ACV-GCV	Adenovirus
10	BMTc-SCID	+	−	−	−	+[h]	+	−		−
11	AIDS	+		∅	−	++[h]	+	+		−
12	AIDS	+		∅	−	+[h]	+	+	GCV	−
13	AIDS	+		∅	−	∅	+	+	PFA	*Clostridium difficile*
14	AIDS	+		−	−	∅	+	+	PFA	*Salmonella* sp.
15	AIDS	+		−	−	∅	+	−		*Cryptosporidium* sp.
16	AIDS	+		−	−	∅	+	−		*Microsporidium* sp.
17	AIDS	+		−	−	∅	+	−	PFA	*Clostridium difficile*
18	BMTc-WAS	+	−	−	−	−	+	−	ACV	Rotavirus
19	BMTc-CVID	+	−	−	−	−	−	−		Rotavirus
20	BMTc-WAS	−	n.a.	n.a.	++	++[h]	+	−	GCV-PFA	−
21	BMTc-WAS-2	−	n.a.	n.a.	++	++[h]	+	−	GCV-PFA	−
22	AIDS	−		n.a.	−+	++[h]	+	+	∅	∅
23	AIDS	−		n.a.	−	+[h]	+	+	GCV	∅
24	BMTa-AML	−	n.a.	n.a.	−	+[h]	+	−	ACV	∅
25	BMTc-OS	−	n.a.	n.a.	−	++[h]	+	−	ACV-GCV	−
26	BMTc-SCID	−	−	−	−	+[h]	+	−	GCV	Adenovirus
27	NTx	−		n.a.	−	+[h]	+	−		−
28	NTx-2	−		n.a.	−	+[h]	+	−	GCV	∅
29	AIDS	−		−	−	∅	+	−		∅
30	BMTc-TP	−	n.a.	n.a.	−	−	+	−		∅
31	BMTc-AML	−	−	−	−	−	n.a.	−	ACV	∅
32	BMTc-SCID	−	−	−	−	−	n.a.	−		∅
33	BMTc-SCID	−	−	−	−	−	n.a.	−	GCV	∅
34	BMTa-CML	−	−	−	−	−	−	−		∅
35	BMTc-ALL	−	−	−	−	−	−	−		Adenovirus
36	AIDS	−		n.a.	−	−	−	−	ACV	∅

[a] Symbols and abbreviations: +, positive (PCR, ++, first and seminested PCR positive; −+, only seminested PCR positive); −, negative; n.a., result not available due to technical (PCR inhibitors), clinical (immunoglobulin substitution), or ethical (asymptomatic patients or high-risk bone marrow transplant [BMT] patients) problems; ∅, unknown; GvHD, graft versus host disease; BMTa, adult bone marrow recipient; BMTc, child bone marrow recipient; NTx, kidney transplant recipient; ALL, acute lymphatic leukemia; AML, acute myelogenous leukemia; CML, chronic myelogenous leukemia; CVID, common variable immunodeficiency; OS, Ommen's syndrome; PNP, purine nucleoside phosphorylase deficiency; SCID, severe combined immunodeficiency; TP, thrombocytopathia; WAS, Wiscott-Aldrich syndrome; ACV, acyclovir; GCV, ganciclovir; PFA, foscarnet.

[b] Diarrhea, abdominal pain, and weight loss.

[c] Cytomegalic inclusions in intestinal biopsy or virus isolation from intestinal biopsy.

[d] CMV antigenemia and/or virus isolation within ± 4 weeks of stool sampling.

[e] +, patients with primary CMV infection; −, patients without CMV infection.

[f] At least 4 days before and at the time of stool sampling.

[g] Within ±4 weeks of stool sampling.

[h] Semiquantitative result of antigenemia test. +, <100 positive cells; ++, >100 positive cells per 2×10^5 leukocytes.

suspension was diluted with 10 ml of CTAB-extraction buffer and shaken for 30 min at 60°C. After the mixture cooled to room temperature, 10 ml of chloroform was added, followed by 5 min of mixing. After centrifugation at 6,000 × *g* for 15 min, the liquid phase was transferred to a new tube, 10 ml of isopropanol was added, and the nucleic acids were precipitated for 20 min at −20°C, followed by centrifugation at 6,000 × *g* for 10 min. The pellet was washed with 70% ethanol, dried at room temperature, and resuspended in 500 μl of distilled water with 2 μg of RNase A per ml. After incubation at 37°C for 10 min, extraction with phenol and chloroform was performed. The DNA was dissolved in 100 μl of distilled water overnight at 4°C.

Amplification of human pyruvate dehydrogenase (PDH; PDH+, 5′-GGT ATG GAT GAG GAC CTG GA-3′, and PDH−, 5′-CTT CCA CAG CCC TCG ACT AA-3′ [185 bp]) was performed in each experiment to confirm that DNA was extracted from the specimen and to detect inhibitors of *Taq* polymerase.

Amplification of CMV DNA was done by using one primer pair and one additional inner primer for a second, seminested PCR selected from the major immediate early gene (32) (outer primer set, MIE-P, 5′-ACA TAC ATG TCA ACA GAC TTA CCG-3′, and MIE-M, 5′-AAC AGA CTG TTC CTT TCC ATG GG-3′ [444 bp]; inner primer set, MIE-P and IE+, 5′-CGT CCT TGA CAC GAT GGA GTC-3′ [404 bp]).

One microgram of DNA in 5 μl of water was added to 45 μl of reaction mixture containing 50 mM Tris hydrochloride (pH 8.3), 50 mM KCl, 1.5 mM $MgCl_2$, 200 μM (each) deoxynucleoside triphosphates (Pharmacia, Uppsala, Sweden), 25 pmol of

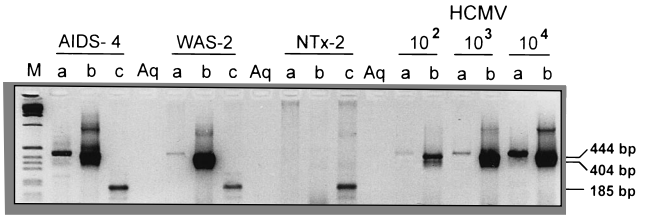

FIG. 1. Detection of human CMV (HCMV) DNA by PCR in stool specimens. Agarose gel electrophoresis was performed with 10 μl of amplified DNA from stool samples derived from patients AIDS-4, WAS-2 (Wiscott-Aldrich syndrome), and NTx-2 (kidney recipient). Lanes a, CMV PCR with 1 μg of extracted DNA and the outer primer pair results in 444-bp fragments; lanes b, seminested CMV PCR was performed with 2 μl of the first PCR, (resulting in 404-bp fragments); lanes c, human PDH was amplified as a control for successful extraction and amplification of DNA; lanes Aq, reaction mix without DNA; HCMV, semiquantification with HCMV standards (10^2 to 10^4 genome equivalents); lane M, DNA size marker (Gibco BRL).

each primer, 0.01% (wt/vol) gelatin, 0.2% Tween 20, and 2.5 U of thermostable *Taq* DNA polymerase (Perkin-Elmer Cetus, Norwalk, Conn., or MBI Fermentas, Vienius, Lithuania). The mixture was covered with 50 μl of mineral oil. After an initial step of denaturation for 2 min at 94°C, 35 cycles of amplification (first PCR) were carried out at 94°C for 45 s, 58°C for 50 s, and 72°C for 50 s. After the first amplification, 2 μl of the sample was transferred to a mixture containing the primers for the seminested PCR, and 30 cycles of amplification were performed. As a positive control and for semiquantification, CMV DNA standards were used (Fig. 1). While the method was being established, the specificity of the amplified fragments was additionally confirmed by Southern blotting and hybridization with an internal probe (IE-probe, 5′-CGT CGT GGC CTT GGT CAC GGG TGT C-3′).

To establish a reproducible method for extraction of CMV DNA from stool specimens, several methods were tested (proteinase K digestion, polyethylene glycol precipitation, heat-treatment, alkaline lysis, sodium dodecyl sulfate treatment, and direct phenol extraction). Reproducible PCR signals were only obtained with CTAB. It has been shown that this cationic detergent removed inhibitory factors and significantly improved the sensitivity of DNA detection (21, 25, 26, 34). Stool specimens of CMV immunoglobulin G-seronegative individuals were spiked with different amounts of CMV (from an AD169-infected cell culture) ranging from 10^3 to 10^7 genome equivalents per ml. The samples were extracted either immediately or after incubation for 6, 24, or 48 h at −20°C, 4°C, or room temperature to simulate storage and transport conditions. CMV DNA was detectable in all samples, although high-molecular-weight DNA (human and bacterial) was degraded with increasing time (data not shown). The limits of detection for viral DNA in stool specimens were 2×10^4 CMV genome equivalents per ml when only the outer primers were used and about 10^2 to 10^3 genome equivalents per ml when the seminested PCR was performed additionally (Fig. 1).

Clinical, histological, virological, and microbiological data from all patients are summarized in Table 1.

Amplification of the cellular control DNA (PDH) was possible in 34 of the 36 samples (94%). With patients 5 and 6 (Table 1), no PDH or CMV DNA signals were obtainable even after several extraction attempts, but this would not have led to a false-negative diagnosis, since the control DNA of the cellular PDH gene could not be amplified either. The reason for this is not known, but this clearly indicates that our method can only be evaluated diagnostically when a cellular single-copy DNA is extracted and amplified as a control.

Nineteen patients (13 AIDS patients and 6 bone marrow transplant patients) suffered from intestinal symptoms as defined previously. From 15 of these 19 patients, colon biopsies were taken, and 6 were found positive for CMV. In stool samples from four of these six patients (patients 1 to 4), CMV DNA was detectable. In the ganciclovir-treated patient (patient 2), the viral DNA could only be detected by the seminested PCR.

Only one additional bone marrow transplant patient with intestinal symptoms (patient 7) was clearly positive for CMV DNA in stool samples. However, in intestinal biopsies taken from this patient, CMV disease could not be diagnosed but intestinal graft versus host disease was detected. Several weeks later, he died from generalized severe CMV disease; detection of CMV DNA as well as virus isolation was possible from several organs. In this patient, assumption of CMV colitis was plausible but could not be proven according to the definition used.

Interestingly, in 7 of the 12 symptomatic patients without proven CMV colitis and/or detection of CMV DNA, other enteropathogenic microbes or rotaviruses could be identified (patients 13 to 19). Adenovirus was not considered to be an enteropathogen, since it was also found in asymptomatic patients, which is a well-known fact. In one patient (patient 8), severe graft versus host disease was observed, and involvement of the intestine was suspected. However, endoscopy could not be performed.

The diagnostic specificity of the PCR is influenced by the fact that CMV DNA could be detected in stool samples of 3 of the 17 asymptomatic immunocompromised patients (patients 20 to 36). These patients (patients 20 to 22) suffered from a severe active systemic CMV infection with antigenemia (Wiscott-Aldrich syndrome or AIDS). CMV was isolated repeatedly from various body sites of these patients. Therefore, we assume that severe active systemic CMV infection with large amounts of viral DNA in blood cells might lead to detectable numbers of CMV genomes in feces by minimal intestinal hemorrhage in the absence of visible blood. It is possible, on the other hand, that these patients had a minor intestinal CMV infection that did not lead to clinical symptoms. These patients did not suffer from intestinal symptoms, and colonoscopy was not performed.

These 3 patients (patients 20 to 22) also have to be connected with the 14 patients (patients 8 to 12, symptomatic; patients 20 to 28, asymptomatic) who suffered from active systemic CMV infection but were not diagnosed with CMV colitis and/or were asymptomatic (21%).

Despite the high level of sensitivity of the PCR in general, we have no data indicating that our PCR method does detect CMV DNA in stools of only latently infected individuals. This agrees with the findings of other authors (2).

It should be mentioned that CMV colitis was a rare event in the bone marrow transplant patients studied compared with the AIDS patients, although more transplant recipients suffered from active and even more severe CMV infections.

In conclusion, we consider that PCR in stool samples may prove helpful for the noninvasive ruling out of intestinal CMV disease in patients at high risk, whereas positive PCR may help define patients at high risk for CMV disease of the colon. For the final evaluation of this diagnostic method, more information is needed, and therefore more patients have to be tested in different centers taking care of large numbers of AIDS patients.

This work was supported in part by a grant from the Ministerium für Wissenschaft und Forschung Baden-Württemberg no. 7532.292-1/5. E. Marre received support from the II. Hochschulsonderprogramm.

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