Original Article

Cytomegalovirus Detection by PCR on Fresh and Formalin-Fixed Colon Tissue Biopsies of Children with Colitis: A Prospective Cohort Study

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What's Known

- The clinical usefulness of different diagnostic methods, particularly polymerase chain reaction (PCR) assay on tissues, for detecting cytomegalovirus (CMV) colitis in children remains controversial.

What's New

- Polymerase chain reaction (PCR) of fresh and formalin-fixed colon biopsies detects CMV in pediatric colitis with high positivity rates, but poor histologic association.
- The CMV immunohistochemistry positivity and high plasma viral DNA levels are associated with viral cytopathic effects on histology.
- Anti-CMV treatment might not be beneficial in CMV-colitis cases with PCR positivity alone but no histologic or IHC evidence of active infection, particularly in IBD patients.

Abstract

Background: Diagnosis of cytomegalovirus (CMV) in biopsies relies on detecting classic viral cytopathic effects (CPE) in tissue. These effects are not always apparent, and confirmatory tests are necessary. This study aimed to compare the results of the different diagnostic tests for CMV detection in colitis, including PCR on fresh and formalin-fixed paraffin-embedded (FFPE) tissue, immunohistochemistry (IHC), histology, and plasma PCR, in association with the clinical course.

Methods: In this prospective study, CMV-PCR was performed on fresh tissue (FT) and FFPE tissue, and IHC was conducted on colon biopsies from 153 children with colitis referred to Children Medical Center Hospital (Tehran, Iran) from 2015 to 2019. The results of different diagnostic methods were evaluated in association with the clinical and histopathological findings.

Results: Fifty out of 153 (32%) cases had positive FT-CMV PCR. Forty of these fifty positive samples and 21 of 103 negative ones had concomitant FFPE biopsy tissue. FFPE-PCR and IHC were positive in 17 (42.5 %) and 2 (5%) out of 40 FT-PCR-positive cases, respectively. The two IHC-positive cases had positive FFPE-PCR and high CMV-DNA plasma levels and showed histologically active colitis and CPE. Remarkably, 14 (35%) cases were identified with positive FT-PCR without any evidence of colitis in histopathology. During follow-up, FT-PCR-positive inflammatory bowel disease (IBD) cases treated with antiviral drugs showed a poorer outcome than the non-treated cases (P=0.03).

Conclusion: A high positive rate was observed for both FT- and FFPE-CMV PCR, with a poor association with histology. IHC positivity was associated with high plasma CMV DNA levels and the presence of CPE. The efficacy of anti-CMV treatment in colitis cases should be verified through randomized controlled clinical trials.

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Introduction

The human cytomegalovirus (CMV), a beta human herpesvirus type 5, belongs to the *Herpesviridae* family with a double-stranded

DNA.[1] Being a prevalent human pathogen, it affects a majority of the general population,[2] with estimates suggesting that up to 80% of individuals are infected with this virus by the age of 35.[3] Even though healthy people are usually asymptomatic, immunocompromised patients may show some symptoms. Following initial infection, CMV establishes lifelong latency but may subsequently reactivate or cause reinfection in susceptible individuals. The virus can affect multiple organ systems, with a particular predilection for the gastrointestinal (GI) tract. GI CMV infection represents a particularly severe complication in immunocompromised hosts, including transplant recipients and patients with congenital or acquired immunodeficiencies.[1, 4]

The colon represents the most frequently involved gastrointestinal site in CMV reactivation. CMV infection has been associated with disease exacerbation in inflammatory bowel disease (IBD) patients, making its treatment a major concern in IBD management.[4, 5] Reported CMV positivity rates in IBD patients vary considerably across studies, with incidence ranging from 0.5% to 100% depending on diagnostic methods, patient selection criteria, and disease phase.[6]

There is uncertainty regarding whether CMV serves as a causative factor for inflammation severity, disease flare-up, and treatment unresponsiveness in IBD colitis. However, CMV infection might develop secondary to inflammation due to the immunosuppressive state induced by immunomodulatory drugs and compromised intestinal mucosal immunity in IBD cases. As immunosuppressants remain the cornerstone of IBD management, distinguishing CMV colitis from a *de novo* IBD exacerbation is crucial.[7]

Fecal calprotectin, a major protein released by activated leukocytes in the inflamed intestinal mucosa, serves as a key biomarker for IBD activity and is routinely used in IBD diagnosis and monitoring.[8, 9] Elevated fecal calprotectin levels might be associated with superimposed CMV colitis in both patients with IBD and immunodeficient patients.

The histopathological diagnosis of CMV infection relies on identifying classic CMV viral inclusions. While these inclusions are usually absent in most clinically suspected CMV cases, immunohistochemistry (IHC) is frequently necessary to detect CMV antigens in tissues. In highly suspicious patients with equivocal IHC results and non-classical staining patterns, CMV DNA detection by polymerase chain reaction (PCR) on formalin-fixed paraffin-embedded (FFPE-PCR) tissue was reported as helpful.[10] The majority of the published studies evaluating PCR on tissue samples were conducted on biopsies from the adult population.[11-13]

Although the significance of CMV infection in pediatric acute severe colitis is not precisely known, the prevalence of CMV detection in colonic biopsies of children with severe refractory colitis was reported to be increased.[14]

In this study, different laboratory diagnostic tests for CMV detection were compared, and their association with the clinical course and outcomes was assessed in pediatric patients who received antiviral treatment. To our knowledge, CMV in colonic biopsies was primarily investigated in adult patients using PCR-based methods, with testing performed either on fresh tissues or FFPE tissues. To date, no study has systematically tested both fresh and FFPE colonic tissue and blood for CMV using PCR in children, or compared IHC results from biopsies with clinical outcomes.

Patients and Methods

Subject Characteristics

This prospective cohort study was conducted over 4 years from 2015 to 2019. The patients, who were referred for colonoscopy to the Children's Medical Center Hospital in Tehran, were screened. The cases who had clinical and colonoscopic diagnoses of colitis were selected. From these patients, inclusion was limited to those who underwent tissue biopsy of the colorectal area with clinical endoscopic suspicion of CMV colitis. Multiple tissue samples were taken from each eligible case, with random allocation for either fresh tissue (FT) PCR processing or fixation in 10% neutral buffered formalin and paraffin embedding (formalin-fixed, paraffin-embedded [FFPE]) for histopathological examination. Following tissue processing for histopathology, the samples with insufficient remaining tissue in paraffin blocks for subsequent IHC and PCR testing were excluded from the study.

Demographic and clinical data, including age, sex, and simultaneous stool calprotectin results (μg/g), and plasma CMV quantitative PCR (qPCR) levels of eligible patients, were collected from the Hospital Information System (HIS). Stool calprotectin levels were measured using the BUHLMANN fecal® ELISA Calprotectin test (Switzerland) according to the manufacturer's instructions, with results interpreted as: normal (<50 μg/g), mild organic disease (50-200 μg/g), and active organic disease (>200 μg/g).

The study was approved by the Research Ethics Committee of the National Institute for Medical Research Development (NIMAD, code: IR.NIMAD.REC.1398.225). All study procedures

were conducted in accordance with the ethical principles of the 1964 Helsinki Declaration and its later amendments. Written informed consent was obtained from the parents or legal guardians of all participants prior to their enrollment in the study.

Human Cytomegalovirus Polymerase Chain Reaction

Four 5-µm-thick sections were cut from each paraffin block, and DNA extraction was performed using QIAamp®DNA FFPE Tissue Kit (QIAGEN, Germany) following deparaffinization. For fresh tissue, DNA extraction was conducted using the QIAamp®Fast DNA Tissue Kit (QIAGEN, Germany). Qualitative CMV PCR (GeneProof Cytomegalovirus PCR Kit, Czech Republic) was performed using a LightCycler® 96 System (Roche, Switzerland) according to the manufacturer's instructions. The results of the PCR test were reported as positive or negative based on comparison with positive and negative controls. Plasma CMV DNA extraction was performed using the QIAamp®DNA blood Kit (QIAGEN, Germany), and CMV real-time qPCR was conducted using the artus®CMV RG PCR Kit (QIAGEN, Germany) on a Rotor-Gene Q instrument (QIAGEN, Germany).

Cytomegalovirus Immunohistochemistry

Sections measuring 3-µm in thickness were cut from FFPE blocks and mounted on silane-coated slides. Following deparaffinization and antigen retrieval, tissue sections were incubated with mouse anti-CMV of monoclonal antibody (Clone DDG9/CCH2; Master Diagnostica, Spain). The EnVision detection kit (Master Diagnostica, Spain) with 3,3'-diaminobenzidine (DAB) chromogen was used for visualization.

Histopathology

All slides were independently reviewed by two pathologists who were experts in GI pathology. Histopathological features of CMV colitis were systematically documented, including inflammatory cell composition, cryptitis, crypt abscess, and crypt destruction, presence of mucin depletion, apoptosis, Paneth cell metaplasia, erosion/ulceration, plump endothelial cells, vasculitis, and viral cytopathic effects (CPE) as nuclear enlargement and viral-type nuclear inclusion bodies. For each biopsy, the degree of inflammation was scored as (0) inactive/absent, (1) mild, (2) moderate, and (3) severe, according to Gupta and colleagues' criteria.[15] The Geboes histological scoring system was applied for standardized inflammation grading.[16]

Follow-up

The participants were followed to assess the clinical course and disease outcomes, including colectomy requirements and mortality. Outcomes were evaluated through a review of medical records at discharge time, outpatient clinic visits, or telephone follow-up.

Statistical Analysis

Descriptive data were presented as frequencies and percentages. Parametric variables were expressed as mean±SD, and nonparametric variables were reported as median with interquartile range. All statistical analyses were performed using SPSS software (version 24, IBM Corp., USA). The Mann-Whitney U test was used to compare nonparametric continuous variables. Categorical variables were analyzed using the Chi square test. Inter-method agreement for CMV detection methods was assessed using the Kappa statistic. $P<0.05$ was considered statistically significant.

Results

Demographic Data

During the study period, fresh biopsies from 153 patients who were clinically suspicious of CMV-induced colitis were submitted for CMV PCR testing, out of which 50 samples had positive FT PCR results. The FFPE blocks of 40 out of 50 PCR-positive samples and 21 out of 103 PCR-negative samples were available for further testing. Histopathological examination, IHC, and PCR were performed on the 40 FFPE blocks from 38 patients (2 cases had two separate times of biopsy sampling). The 21 PCR-negative samples with available FFPE tissue underwent histologic evaluation. The age range of the patients with positive FT PCR was from 3 months to 15 years (mean age=7.6±4.6) years, with equal sex distribution (19 boys, 19 girls).

The FT PCR-negative group included 7 (33.3%) girls and 14 (66.7%) boys, with a mean age of 9.3±4.0 (range=1.4 to 16.5 years). No significant differences were observed in age or sex distribution between FT PCR-positive and FT PCR-negative groups. The underlying diseases of FT CMV PCR-positive patients are presented in table 1.

Histopathology

The inflammatory infiltrate was predominantly composed of lymphocytes and plasma cells, with patchy foci of neutrophils and eosinophils (table 2). Specimens with clinical suspicion of CMV colitis and positive FT-PCR results showed significantly greater histologic activity (diagnosed as chronic active colitis on tissue biopsy) than negative cases (57.5% vs. 19%, P=0.01).

Table 1: Underlying diseases of the study cases with clinical suspicion of CMV colitis and positive FT CMV-PCR (n=40)

Disease category and diagnosis	Frequency, n (%)
IBD	23 (57.5)
UC	14 (35)
CD	9 (22.5)
ID	7 (17.5)
CVID	1 (2.5)
ID NOS*	1 (2.5)
SCID	1 (2.5)
XLA*	1 (2.5)
MHC II deficiency	1 (2.5)
HSCT/MT/GI GVHD	1 (2.5)
HSCT/FA/GI GVHD	1 (2.5)
Allergic disease	2 (5)
Food allergy/ EGE	1 (2.5)
EGE	1 (2.5)
AI diseases	3 (7.5)
AIH+PSC	1 (2.5)
AI Enteropathy	2 (5)
Others	5 (12.5)
TB	1 (2.5)
Storage disease	1 (2.5)
Clinical information NA	3 (7.5)

IBD: Inflammatory bowel disease; UC: Ulcerative colitis; CD: Crohn's disease; ID: Immunodeficiency; ID NOS: Immunodeficiency not otherwise specified; CVID: Common variable immunodeficiency; SCID: Severe combined immunodeficiency; XLA: X-linked agammaglobulinemia; MHC II: Major histocompatibility complex class II; EGE: Eosinophilic gastroenteritis; AI: Autoimmune; AIH: Autoimmune hepatitis; PSC: Primary sclerosing cholangitis; HSCT: Hematopoietic stem cell transplantation; MT: Major thalassemia; GI: Gastrointestinal; GVHD: Graft versus host disease; FA: Fanconi anemia; TB: Tuberculosis; NA: Not available. *Cases finally diagnosed as CMV colitis.

Table 2: The relationship between histopathologic features and FT CMV-PCR positivity in the studied cases

Histopathological feature		FT CMV-PCR		P value
		Positive no. (%)	Negative no. (%)	
Lymphocytic Infiltration	Absent/Mild	8 (20)	10 (47.6)	0.05
	Moderate	29 (72.5)	11 (52.4)	
	Severe	3 (7.5)	0 (0)	
Neutrophils infiltration	None	0 (0)	2 (9.5)	0.12
	Mild	21 (52.5)	9 (42.9)	
	Moderate	13 (32.5)	9 (42.9)	
	Severe	6 (15)	1 (4.7)	
Eosinophils infiltration	Mild	13 (32.5)	8 (38.1)	0.73
	Moderate	18 (45)	10 (47.6)	
	Severe	9 (22.5)	3 (14.3)	
Crypt distortion	None	8 (20)	12 (57.1)	0.02
	Mild	21 (52.5)	8 (38.1)	
	Moderate	9 (22.5)	1 (4.8)	
	Severe	2 (5)	0 (0)	
Vasculitis	Absent	36 (90)	20 (95.2)	0.48
	Focally present	4 (10)	1 (4.8)	
CMV CPE	Absent	38 (95)	21 (100)	0.54
	Present	2 (5)	0 (0)	
Apoptotic figures	Absent	18 (45)	15 (71.4)	0.04
	Present	22 (55)	6 (28.6)	
Cryptitis	Absent	11 (27.5)	10 (47.6)	0.12
	Present	29 (72.5)	11 (52.4)	
Crypt abscess	Absent	17(42.5)	15 (71.4)	0.03
	Present	23 (57.5)	6 (28.6)	
Ulcer	Not Seen	33(82.5)	19 (90.5)	0.40
	Present	7 (17.5)	2 (9.5)	

FT: Fresh tissue; CMV: Cytomegalovirus; PCR: Polymerase chain reaction; CPE: Cytopathic effect; FFPE: Formalin-fixed paraffin-embedded; IHC: Immunohistochemistry; Chi square test; $P<0.05$ was considered statistically significant.

CMV PCR positivity in fresh tissue correlated with the degree of crypt distortion (P=0.02), crypt abscess formation (P=0.03), and apoptotic bodies (P=0.04). There was no significant association between the histologic findings and PCR positivity in FFPE specimens.

Only two cases (5%) showed viral CPE as homogenously enlarged nuclei with prominent nuclear inclusion bodies (with or without perinuclear halos creating an "owl's eye appearance"). These findings were present in stromal, endothelial, and epithelial cells (figure 1). Both cases demonstrated positive IHC results and elevated plasma CMV viral loads.

Tissue PCR and IHC Results

Among the 50 CMV PCR-positive FT biopsies, 40 had corresponding FFPE samples available from the same procedure. Of these, 17 FFPE samples (42.5%) were PCR-positive. Only 2 (11.7%) of the 17 PCR-positive FFPE cases demonstrated positive IHC staining, both of which also exhibited CPE on histopathological examination (figure 2).

Among the IBD cases, 10 out of 23 samples (43.5%) were positive for CMV PCR on FFPE tissue, including three out of nine (33.3%) Crohn's disease (CD) cases and 7 out of 14 (50%) ulcerative colitis (UC) cases. None of the IBD cases showed positive CMV in IHC.

Agreement of PCR and IHC Results on FFPE Tissue

There was a significant agreement between IHC and FFPE PCR results in CMV detection in immune-deficient patients (Kappa=0.714, P<0.001). However, no agreement was observed in IBD patients and total cases (Kappa=0.13, P=0.09). Considering histopathology combined with IHC as the gold standard method, CMV PCR on FFPE tissue demonstrated 100% sensitivity, 60% specificity, 12% positive predictive value (PPV), and 100% negative predictive value (NPV) for diagnosing CMV colitis.

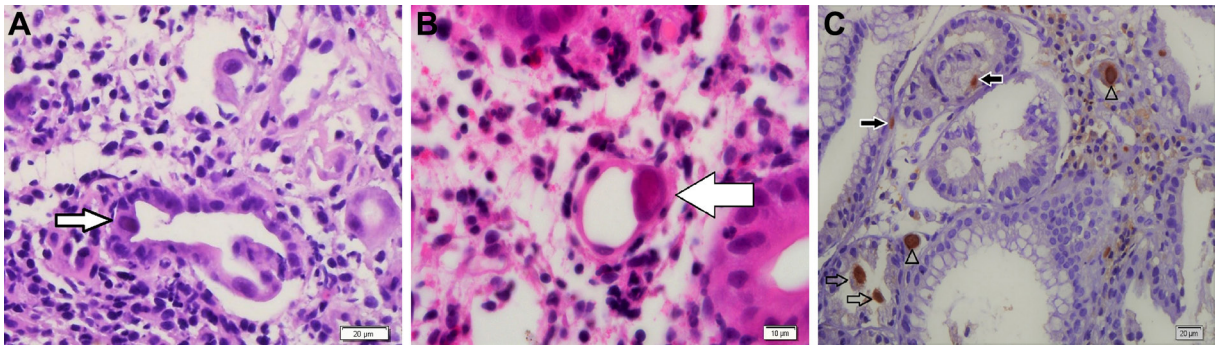

Figure 1: A: Hematoxylin and Eosin staining shows CMV CPE in the epithelial cells (×600). B: Hematoxylin and Eosin staining shows CMV CPE in the endothelial cells (×1000), characterized by large intra-nuclear eosinophilic inclusions and fine perinuclear halo (arrows). C: Immunohistochemical staining shows CMV antigens in the epithelial cells (empty arrow), endothelial cells (arrowhead), and stromal cells (solid arrow) of the colon mucosa (×250).

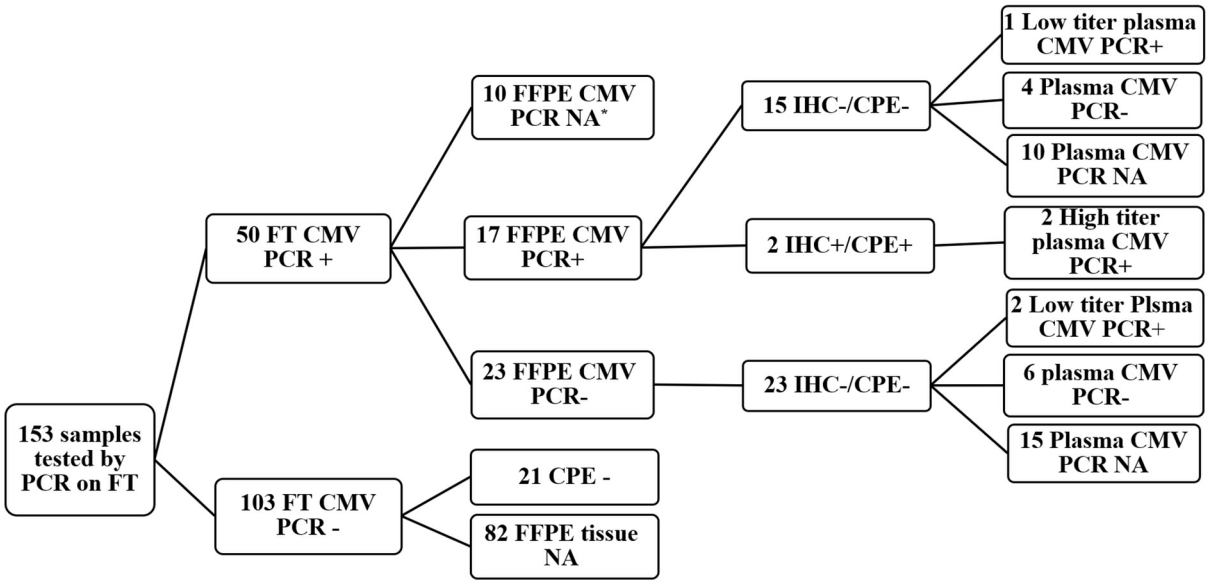

Figure 2: Results of different CMV tests are shown in the studied samples. PCR: Polymerase chain reaction; FT: Fresh tissue; CMV: Cytomegalovirus; CPE: Cytopathic effect; FFPE: Formalin-fixed paraffin-embedded; IHC Immunohistochemistry; NA: Not adequate/Not available.

Plasma CMV-DNA PCR

Plasma levels of CMV DNA were available for 15 patients. Ten patients tested negative, three showed low-level CMV DNAemia (<500 copies/L), and two demonstrated high levels of plasma CMV viral load (>1000 copies /L). The latter two patients were immune-deficient and also exhibited positive CMV results in IHC, FFPE-PCR, and histologic evidence of active colitis. Among the three low-level CMV cases, only one was FFPE-PCR positive, and none showed active crypt-destructive colitis on histologic examination.

Plasma CMV PCR was negative in all IBD patients, while five out of eight non-IBD cases (62.5%) showed a positive plasma CMV PCR (P=0.03). The prevalence of CMV positivity in the non-IBD group was 41.2% (7/17) in FFPE-PCR and 11.8% (2/17) in IHC.

A moderate agreement was observed between the results of plasma CMV DNA positivity and IHC reactivity for CMV (Kappa=0.47, P=0.03), while no significant agreement existed with FFPE-PCR results (kappa= 0.18, P=0.46). Notably, CMV IHC positivity in colon tissue showed a significant association with the copy number of CMV in the plasma (P=0.009).

Stool Calprotectin Level

Thirty cases had simultaneous stool calprotectin levels at colonoscopy. Stool calprotectin level was elevated in all measured cases (median=775.3 µg/g, IQR=456.7-841.2 µg/g, range=77-850 µg/g). Two cases with immune deficiency who had FFPE-PCR-confirmed CMV colitis had relatively lower calprotectin levels (77 and 93 µg/g). One of these two cases showed high CMV DNAemia and positive CMV IHC, while the other (without plasma PCR testing) was IHC-negative. All remaining patients exhibited calprotectin levels >200 µg/g.

Fecal calprotectin levels showed no association with either plasma CMV viral load or CMV PCR positivity. However, calprotectin levels significantly correlated with histologic disease activity (P= 0.04), demonstrating higher median values in active colitis (800.0 µg/g) than those with non-crypt-destructive chronic colitis (515.0 µg/g) or normal histology (436.5 µg/g). ROC analysis revealed analysis identified 500 µg/g as the optimal cutoff for predicting histologic activity (AUC=0.727, P=0.04, figure 3).

Cryptitis was observed in 17 out of 22 (77%) patients with calprotectin level>500 µg/g, while only one out of 18 (12.5%) of those with calprotectin level of <500 µg/g exhibited active colitis in histology (P<0.001).

Patients' Follow-Up and Clinical Course

Outcomes were evaluated in 40 cases with positive FT CMV-PCR that belonged to 38 patients (two IBD patients experienced recurrent episodes of CMV-suspected colitis at 7-month and 2-year intervals, respectively). Follow-up data were available for 34/38 patients (mean duration: 3.0±1.5 years). They were evaluated for well-being, colectomy rates, and any disease-related surgical interventions. In total, seven deaths and four colectomies took place. The patients' outcomes stratified by treatment status are presented in figures 4 and 5.

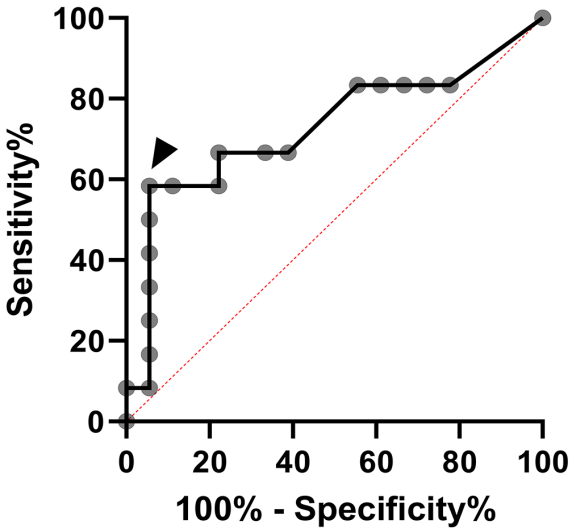

Figure 3: ROC curve analysis identified a cutoff value of 500 µg/g for stool calprotectin level (marked by arrowhead) in predicting disease activity in pediatric colitis, with 58.3% sensitivity and 94.4% specificity.

Among 19 patients with only FT-positive PCR, three deaths and one colectomy occurred (complication rate=20.9%). The 14 patients with both FT- and FFPE-PCR positivity experienced two deaths and two colectomies (complication rate=28.5%). Both patients who tested positive by all CMV assays with high plasma CMV DNA levels had poor prognoses (100% complication rate=one death and one colectomy). One out of two samples with FT PCR positivity and low plasma CMV viral load died (50% complication rate). Treated IBD patients (10/21) showed a complication rate of 33.3%, while none of the untreated patients showed complications (P=0.03).

Among 17 non-IBD colitis patients, nine received antiviral treatment. Treated cases experienced three deaths and two colectomies (55.5% complication rate), whereas three deaths were seen (37.5%) in the non-treated patients (P=0.55). The immune-deficient patients with positive FT CMV PCR had a higher rate of complications (death or colectomy) than IBD children with positive FT CMV PCR (85.7% vs. 13%, P<0.001). In addition, a positive plasma level of CMV DNA was associated with worse outcomes in immune-deficient patients (P<0.001).

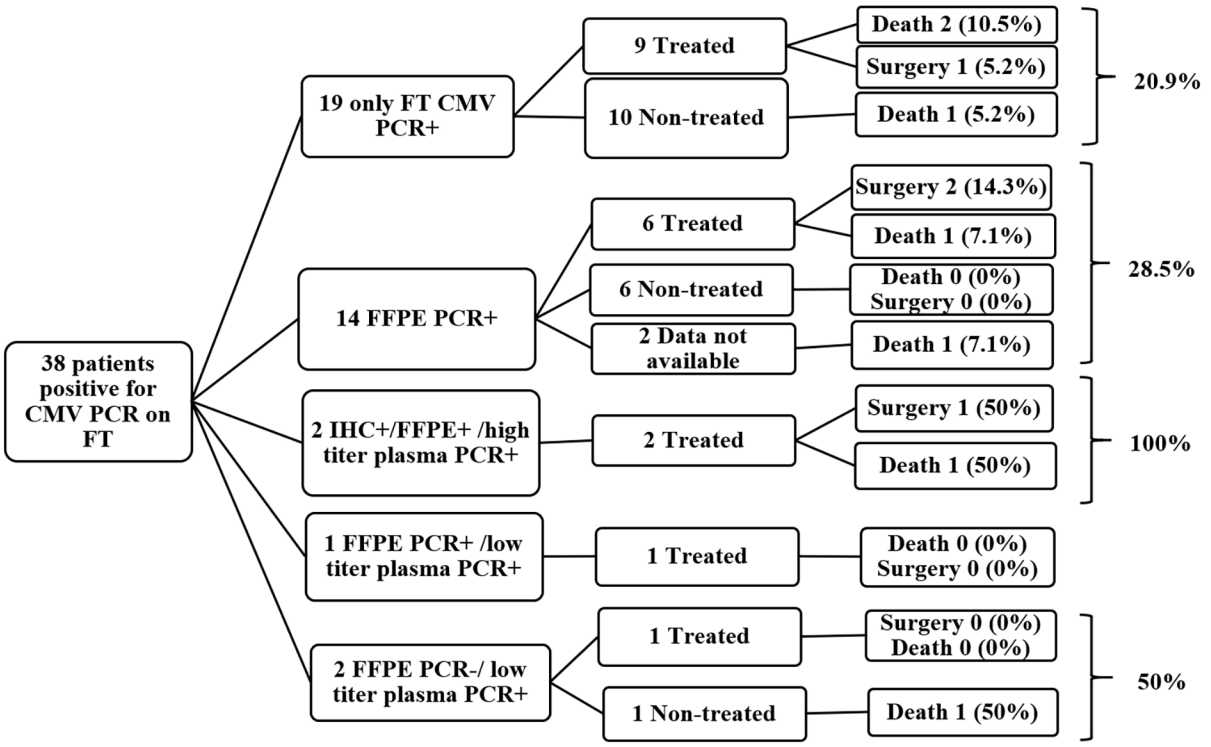

Figure 4: Clinical outcomes are stratified by test positivity and antiviral therapy status. Complication rates increased with the number of positive test results. PCR: Polymerase chain reaction; FT: Fresh tissue; CMV: Cytomegalovirus; FFPE: Formalin-fixed paraffin-embedded; IHC: Immunohistochemistry.

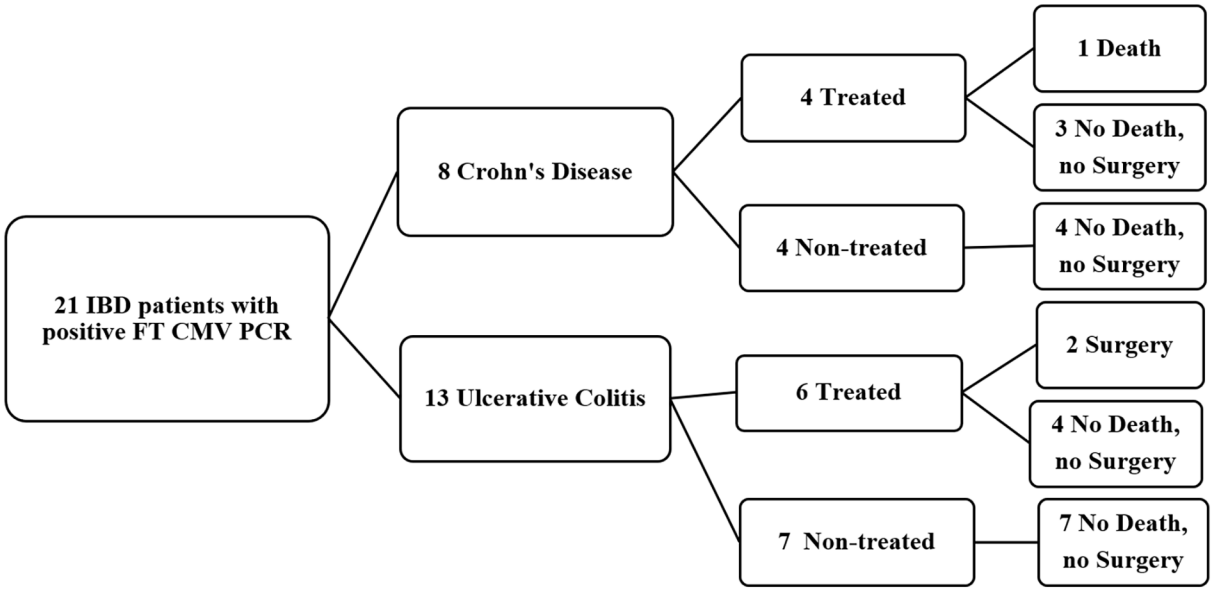

Figure 5: Clinical outcomes by test positivity and antiviral therapy status are shown among the IBD group. IBD: Inflammatory bowel disease; FT: Fresh tissue; CMV: Cytomegalovirus; PCR: Polymerase chain reaction

Discussion

We found a high rate of false positivity in CMV detection by tissue PCR, which was neither associated with histopathological cytopathic changes nor with CMV DNA plasma levels. Among all PCR-positive cases in fresh tissue, CMV positivity rates were 33.3% (5/15) when detected by plasma CMV PCR, compared to only 5% (2/40) identified through IHC and histopathologic examination. Few studies addressed the significance of PCR in intestinal biopsies for diagnosing CMV infection in pediatric patients with colitis.[14, 17]

Previous studies on colon biopsies in both pediatric and adult populations also demonstrated that CMV positivity was more prevalent in PCR than in IHC and histologic studies.[18, 19]

The findings of the present study indicated that marked tissue lymphocyte and neutrophil infiltration were associated with FT CMV PCR positivity. Recruited inflammatory cells carrying CMV virus particles (or DNAs) may contribute to this higher CMV PCR positivity rate than the IHC, leading to false-positive results. A high positivity rate in tissue PCR may indicate a dormant phase of the virus within colon tissue cells, and a positive PCR result does not necessarily imply an active viral replicative state. Yadegarynia and others detected CMV DNA by PCR in fresh colon tissues of 7% of 86 adult patients with UC, with a viral load exceeding 250 copies/mg, despite the absence of inclusion bodies on IHC staining.[19] We observed that FT-PCR yielded a higher positivity rate than FFPE-PCR for CMV DNA. This means a higher sensitivity of FT CMV PCR than FFPE tissue CMV PCR for detecting CMV genome, without necessarily indicating the presence of an actively replicating virus, as previously reported.[20] PCR on FFPE biopsies offers an advantage over fresh samples by enabling histologic examination, which allows for the selection of the most suitable areas for PCR and IHC testing. In addition, retrospective studies could also be performed on FFPE while conducting adjunct IHC.[21] It is not surprising that FFPE processing may adversely affect both DNA quality and quantity, due to formalin-induced DNA degradation during tissue processing. The quantification of extracted DNA from FFPE tissues yielded lower results than in fresh materials, particularly for long DNA products in PCR.[22]

There was poor agreement between IHC and FFPE PCR results, except in immune-deficient samples. Furthermore, during follow-up, the majority of tissue CMV PCR-positive cases did not exhibit a poor clinical course. In contrast, all IHC-positive cases demonstrated poor outcomes despite anti-viral treatment.

CMV-PCR on tissue biopsies is considered a useful diagnostic marker, particularly in the early phases of CMV infection, with a sensitivity and reliability comparable to IHC.[12, 23, 24] However, the false positivity rate of tissue PCR may lead to unnecessary antiviral treatment.[17, 20]

Two cases demonstrated plasma CMV-DNA levels of more than 1000 copies/mL, with both showing active colitis and viral inclusions in histology and IHC. In our study, high plasma CMV viral load (>1000 copies/mL) was associated with IHC positivity and poorer clinical outcomes, while showed no association with either FFPE-PCR or FT-PCR results. Approximately half of the patients with plasma CMV-DNA levels less than 500 copies/mL showed no significant histologic activity in biopsies, and none showed CMV CPE on histologic examination.

The UC patients with positive blood CMV PCR results showed a significantly higher probability of CMV colitis than blood CMV PCR-negative patients.[25] Nevertheless, blood CMV PCR might lack sufficient sensitivity to replace either IHC or tissue PCR for documenting CMV reactivation in IBD patients.[26]

Buck and colleagues found an incidence rate of as low as 0.6% for CMV colitis in pediatric patients with colitis when using routine histology alone.[27] Our data implied that histopathologic findings (excluding CMV CPEs) could not reliably differentiate between CMV-positive and CMV-negative cases. However, the classic viral CPE (intranuclear inclusion) might be missed or potentially confused with prominent nuclei in the context of regenerative changes within severely inflamed colonic mucosa.

We observed that fecal calprotectin levels >50 µg/g were associated with active intestinal inflammation, though this finding was not specific to IBD. Studies suggested a cutoff level of <50 µg/g for normal fecal calprotectin levels in healthy children, consistent with the threshold established for adults.[28] Fecal calprotectin has been proposed as a noninvasive marker of disease activity in pediatric IBD, demonstrating high sensitivity and acceptable specificity.[8, 9, 29] In the present study, histologically active disease occurred significantly more frequently when stool calprotectin level exceeded 500 µg/g. However, some of the immune-deficient cases failed to demonstrate a proportionate increase in fecal calprotectin levels relative to their degree of colitis activity. This was particularly evident in one agammaglobulinemia case that showed only a mild calprotectin elevation (<200 µg/g), despite considerable histopathologic disease activity. Based on our findings, we propose 500 µg/g as the optimal cutoff value for predicting histologic disease activity using stool calprotectin.

During follow-up, we investigated whether antiviral treatment is beneficial for patients with suspected CMV colitis. A key limitation was the presence of different underlying diseases that could independently affect patients' outcomes. When focusing specifically on IBD cases, it was observed that patients who tested positive for CMV only by PCR (in both fresh and FFPE tissues) showed no clinical benefit from antiviral therapy. On the contrary, looking at the outcomes of the 34 cases during follow-up, treated patients demonstrated worse outcomes than untreated ones. We hypothesized that a higher complication rate in the treated group might reflect a more severe underlying active

disease that was refractory to the treatment. Our findings suggested that CMV positivity by tissue PCR alone did not necessarily indicate clinically significant infection that contributed to inflammation, and therefore did not justify antiviral therapy in the absence of IHC evidence of active viral protein production or histologic findings of viral cytopathic effects (viral CPE).

The impact of antiviral treatment for CMV on IBD disease course remains controversial in CMV-positive patients. Several studies demonstrated no detrimental effect of CMV reactivation on disease severity, IBD flare progression, or time to colectomy,[30, 31] consequently suggesting no therapeutic benefit from antiviral treatment.[12, 32, 33] In contrast, other studies reported that CMV reactivation adversely affects IBD prognosis and that early antiviral intervention might reduce colectomy rates.[5, 34, 35] Criscuoli and others recommended antiviral therapy for CMV in cases of active and severe IBD when CMV was detected in colonic tissue samples concurrently with the presence of antigenemia.[6] According to Roblin and colleagues, a viral load of >250 copies/mg of tissue is predictive of poor response to treatment.[36] Similarly, Ciccocioppo and colleagues found that a mucosal viral load greater than 10^3 copies/10^5 cells was associated with poor treatment outcomes.[37] Several limitations in these studies preclude definitive conclusions: retrospective design, small sample sizes, variability in detection methods with differently defined cut-offs for tissue and plasma PCR CMV positivity, and inconsistent tissue types (fresh vs. FFPE). To establish the true efficacy of antiviral treatment in CMV colitis, prospective randomized clinical trials are warranted.

The present study had several limitations: (1) unavailable paired FFPE-PCR and IHC results for all cases, and (2) the absence of a non-colitis control group with normal endoscopic and histologic findings. The first limitation was addressed through the employment of multiple diagnostic modalities, including plasma quantitative nucleic acid testing, with these results being correlated with clinical and histological parameters. In addition, while plasma CMV DNA data were available for some cases (predominantly immunocompromised patients who were more susceptible to disseminated CMV disease), positive plasma CMV PCR results were not demonstrated in any IBD cases-preventing assessment of plasma quantitative PCR's diagnostic and prognostic value in this populatoin.

Compartmentalized CMV infection, including CMV colitis, without systemic manifestations might remain undetectable using blood CMV PCR. A negative plasma CMV PCR result does not necessarily exclude localized CMV disease. Some patients with organ-specific CMV involvement may demonstrate either: (1) transient viral DNA detection in blood, or (2) very low plasma viral loads below conventional assay thresholds.

The qPCR is a highly precise and efficient molecular tool that might facilitate the prompt identification of CMV infection in blood. It might be useful for small tissue biopsies, cases with equivocal IHC or qualitative PCR results, or patients with negative results but strong clinical suspicion of CMV colitis. Nevertheless, tissue qPCR poses challenges, such as defining the cut-off values and standardizing reporting units (e.g., copies per weight of tissue sample, copies per number of cells, copies per volume of tissue, etc.).[38-40] These units of measurement may lack sufficient clinical relevance, as biopsied tissue mass or volume might contain heterogeneous components, including inflammatory cells, stromal cells, endothelial/epithelial cells, extra-cellular matrix, fibrins, mucus, and water. Besides, expressing viral load in copies per concentration of extracted DNA has also been suggested. However, this index has limitations due to the presence of recruited inflammatory cells from the blood while having dormant CMV DNA in their nuclei. Consequently, the absence of standardized assays leads to interlaboratory variability, making the establishment of clinically meaningful qPCR cut-off values for CMV in biopsies particularly challenging.

Conclusion

A low rate of CMV positivity (about 5%) was detected by IHC in pediatric patients with colitis. High detection rates of CMV were observed by PCR in both fresh tissue (FT) and FFPE samples, though no correlation with histologic findings was found. Substantial agreement between CMV IHC and FFPE PCR results was identified specifically in immune-deficient patients. Furthermore, CMV IHC positivity was more likely to be associated with high plasma CMV viral loads, which were found to have prognostic significance in immunocompromised children with colitis. Stool calprotectin levels were strongly related to histopathologic disease activity but were not considered specific to IBD. Based on these findings, it is suggested that positive tissue PCR results alone should not be used as the sole criterion for initiating CMV treatment in colitis patients. A randomized trial with large sample size is recommended to determine the most appropriate treatment

strategy for colitis cases with CMV-DNA detected by quantitative PCR in colonic mucosa.

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Authors' Contribution

H.A., F.M., M.P., and P.R.: Participated in case recruitment, data acquisition, clinical management, patient follow-up, and manuscript editing. F.H.G.: Participated in PCR performance, data collection, and manuscript writing. Z.N.: Performed data gathering and wrote the manuscript draft. M.S.: Participated in study design, interpretation of the histopathology and IHC slides, and editing of the manuscript. S. M.: Contributed to data gathering, performing the statistical analysis, and writing and editing the paper. M.V.: Designed the study interpreted the histopathology and IHC slides and wrote the paper. All authors critically reviewed and approved the final article and agreed to be accountable for all aspects of the work in ensuring that questions related to the accuracy or integrity of any part of the work are appropriately investigated and resolved.

Conflicts of Interest: None declared.

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