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Determination of Epstein-Barr virus DNA and Its Association in Patients with Inflammatory Bowel Diseases (Crohn's Disease and Ulcerative Colitis) Using PCR, and IgM Antibodies

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ABSTRACT

Introduction: This study investigated the association between Epstein-Barr virus (EBV) infection and inflammatory bowel disease (IBD), including Crohn's disease (CD) and ulcerative colitis (UC).

Methods: This cross-sectional study included 90 participants in three (CD, UC, control) groups, with 30 individuals in each group. Serum samples were analyzed for EBV-IgM antibodies using immunoassay and for EBV-DNA via TaqMan-PCR. Statistical methods included one-way analysis of variances (ANOVA), independent-samples Kruskal-Wallis tests, and receiver operating characteristic (ROC) curves analysis. Odds ratios (ORs) were calculated to assess potential risk factors.

Results: IBD patients exhibited significantly lower EBV-IgM levels than controls ($p < 0.05$). ROC analysis revealed: CD vs. controls: AUC = 0.707 ($p= 0.004$), cutoff ≤4.58 (sensitivity: 83.33%; specificity: 60%); UC vs. controls: AUC = 0.646 ($p= 0.042$), cutoff ≤7.5 (sensitivity: 100%; specificity: 40%). EBV-DNA was detected in only 3 CD patients (5.4% male, 4.3% female). EBV-IgM served as a negative predictor for IBD, while PCR provided no additional diagnostic value over serology. Advanced age was a significant risk factor for IBD (OR: 1.0812; $p< 0.0001$), whereas, increased EBV-IgM serum level is protective against IBD involvement (OR: 0.7739; $p= 0.002$).

Conclusion: Our findings indicate that advanced age constitutes a significant risk factor for IBD. The observed reduction in EBV-IgM levels among IBD patients suggests an impaired acute-phase immune response to EBV infection. However, to establish a definitive association between EBV and IBD, we recommend conducting comprehensive epidemiological studies in Iranian CD and UC populations to better characterize EBV infection status in these patient groups.

Keywords: Epstein-Barr virus, Polymerase chain reaction, Ulcerative colitis, Crohn's disease, Immunoglobulin M

Introduction

Crohn's disease (CD) and ulcerative colitis (UC), collectively termed inflammatory bowel diseases (IBD), are chronic inflammatory disorders of the gastrointestinal tract with incompletely understood pathogenesis (1). IBD increases the risk of gastrointestinal malignancies and exhibits distinct pathological features (1).

CD is characterized by discontinuous transmural inflammation, granuloma formation, and fistulae, typically affecting the small intestine, colon, and perianal region (2, 3). In contrast, UC presents as continuous mucosal inflammation extending proximally from the rectum, often with erosions or ulcers (4-6).

The etiology of IBD involves complex interactions between genetic predisposition and environmental factors, A strong genetic predisposition is evident in IBD, as familial history represents a significant risk factor across all age groups (1, 7, 8). Genome-wide association studies have identified over 200 susceptibility loci associated with CD and UC development (7-10), confirming the substantial genetic contribution to disease pathogenesis.

Emerging evidence also implicates viral infections, particularly herpesviruses, in IBD pathogenesis (11). The epidemiological burden of IBD is substantial, with the U.S. Centers for Disease Control and Prevention (CDC) estimating 1.4 million affected individuals in

2024 and a total prevalence of 2.4–3.1 million nationwide, incurring annual healthcare costs of $8.5 billion (12).

Epstein-Barr virus (EBV), a ubiquitous human double-stranded DNA herpesvirus, establishes lifelong latency in >90% of adults worldwide. EBV is transmitted via close personal contact with body fluids (13). While primary infection is often asymptomatic, EBV employs sophisticated immune evasion strategies through differential gene expression (11, 14, 15). This persistence has prompted investigation into its potential role in chronic inflammatory conditions, including IBD.

Although CD occurs across all ages, peak incidence occurs between 15–35 years (16). Familial predisposition appears weaker in elderly-onset cases (17, 18). Demographic patterns reveal higher IBD incidence in urbanized populations and developed nations (19).

Recent studies have explored herpesvirus contributions to IBD, with EBV, cytomegalovirus (CMV), and herpes simplex virus (HSV) detected in intestinal lesions (20-22). Notably, Wang et al. reported EBV positivity in 79.4% and CMV in 34.5% of Chinese IBD patients' mucosal samples (23) suggesting potential pathogenic involvement.

The coexistence of EBV infection complications in patients with IBD has been reported in several studies (24, 25). However, there is not a clear association between EBV infection and IBD involvement. Given the importance of IBD as the unknown condition with unknown causative infectious factor, identifying an infectious agent can lead to several achievements in the therapeutic and preventive approaches. In addition, due to the lack of a control center and appropriate reporting system, there are not enough evidences about the importance of EBV status in IBD patients. Accordingly, this case-control study was designed to investigate active EBV infection in a group of Iranian patients with IBD compared with healthy controls. Furthermore, this study achieved valuable information about the EBV infection status, and relevance of advanced age in IBD patients.

Materials and Methods

Ethical Statement

This study was reviewed and approved by the Ethics Committee of the Research Institute for Gastroenterology and Liver Diseases, Tehran University of Medical Sciences (Ethics Code: **IR.TUMS.DDRI.REC.1395.11**).

All laboratory specimens used in this study were obtained from routine clinical evaluations ordered by physicians; no additional samples were collected specifically for this research. Prior to inclusion, the study objectives were explained to all participants. Only specimens from individuals who provided written informed consent were analyzed.

Sampling, study type, setting, and inclusion criteria

This cross-sectional study enrolled 90 participants equally divided into three groups (n=30 per group): CD, UC and healthy controls. Participants were selected through field sampling from the Digestive Disease Research Institute at Shariati Hospital (Tehran, Iran). All IBD cases were clinically confirmed by gastroenterologists through comprehensive medical records review. We included patients with confirmed intestinal inflammation meeting established IBD diagnostic criteria. Exclusion criteria included: (1) comorbid irritable bowel syndrome, and (2) lack of signed informed consent for research use of clinical specimens. Prior to laboratory analysis, all samples were de-identified to ensure blinded evaluation. Venous blood samples were collected in the hospital's clinical laboratory following standard phlebotomy procedures. Serum was isolated by centrifugation at 3,000 × g for 10 minutes and stored at -80°C until analysis. Only samples from fully consented participants underwent laboratory assessments.

Anti-EBV immunoglobulin M (EBV-IgM)

Serum EBV-IgM levels were quantified using a commercial indirect enzyme-linked immunosorbent assay (ELISA) kit (Vircell Inc., Granada, Spain). Prior to analysis, all samples were equilibrated to room temperature and processed according to the manufacturer's protocol. Optical density measurements were performed at 405 nm with a reference wavelength of 630 nm using a Stat Fax 4200 microplate reader (Awareness Technology, Inc., USA). Results were interpreted per manufacturer specifications: negative: <9 IU/mL, equivocal: 9-11 IU/mL, and positive (indicating active EBV infection): >11 IU/mL. EBV-IgG testing was not performed, as all participants exhibited elevated

baseline IgG levels, and single measurements of EBV-IgG have limited diagnostic utility in this context.

EBV DNA extraction and real-time PCR analysis

The viral DNA extraction kit was used to extract DNA from serum samples, according to the manufacturer instruction (FAVORGEN Biotech Corp. Ping Tung Biotechnology Park, Taiwan). We used the primers and probe sequences that were introduced previously by Jebbink et. al, (the forward primer sequence was 5′-AAACCTCAGGACCTACGCTGC-3′ and the revers sequence was 5′-AGACACCGTCCTCACCAC-3′). the TaqMan probe sequence was 5′-TAGAGGTTTTGCTAGGGAGGAGACGTGTG-3′ (26). For running the Real-time PCR amplification, 5 µL of the extracted DNA was added to the MasterMix (Yekta Tajhiz Azma Co.; Iran) solution. The kit insert had a positive control specimen, whereas for negative control, deionized distilled water was added to the related microtubes. After mixing the reaction mixture, amplification program was started as follows: 95°C for 15 minutes, followed by 50 cycles of 60°C for 0.5 minutes. We have used a Rotor-Gene 1.7.87 real-time PCR machine (Qiagen NV, Netherlands. Hulsterweg). Positive and negative controls were tested in each run.

Statistical Analysis

Statistical analyses were performed using SPSS version 24 (IBM Corp., Armonk, NY, USA) and MedCalc® Statistical Software version 22.009 (MedCalc Software Ltd, Ostend, Belgium). Continuous variables were compared between groups using analysis of variance (ANOVA) or the independent-samples Kruskal-Wallis test, as appropriate. Receiver operating characteristic (ROC) curve analysis was conducted to assess the diagnostic value of serum EBV-IgM levels in CD and ulcerative colitis UC patients. Additionally, logistic regression analysis was performed to calculate odds ratios (ORs) with 95% confidence intervals. A two-tailed p-value <0.05 was considered statistically significant.

Results

Demographic characteristics

The study population comprised control, CD, and UC groups with 14, 10, and 13 female participants and 16, 17, and 20 male participants, respectively. The ethnicity of most studied subjects was Fars (56 individuals; 62.2%), followed by Tork (17 individuals; 18.9%) and Lor (8 individuals (8.9%). The age of individuals in control group (mean±SD= 22.87±18.04 years) was lower than CD (mean±SD= 38.20±11.73 years) and UC (mean±SD= 39.10±12.17 years). There was a significant difference between control group age mean when compared with Crohn's disease ($p< 0.001$) or ulcerative colitis ($p<0.001$). But there was not any statistically significant difference among persons in Crohn's disease and ulcerative colitis groups for the age variable ($p< 0.968$).

Age as a Risk Factor for IBD

Given that participants in the control group were significantly younger than IBD patients, advanced age may be a risk factor for IBD. This is supported by an odds ratio (OR) of 1.0812 (95% CI: 1.0405–1.1234; $p < 0.0001$) (**Figure 1**), indicating that older individuals have a higher likelihood of IBD involvement compared to younger ones.

EBV-IgM serum levels

The Kruskal-Wallis test revealed significant intergroup differences in EBV-IgM antibody levels ($p= 0.012$); EBV-IgM levels mean±SE were equal to 3.415±0.27896 IU/mL, 4.0517±0.35917 IU/mL, and 15.1140±6.64343 IU/mL in CD, UC, and control individuals, respectively. Pairwise comparisons demonstrated No significant difference between CD and UC patients ($p=0.252$). A non-significant trend toward lower levels in UC versus controls ($p=0.071$). Significantly elevated levels in controls compared to CD patients were seen ($p=0.003$) (**Figure 2**). Notably, 10 control subjects (33.3%) exhibited detectable EBV-IgM (9 positive, 1 equivocal). One control subject showed markedly elevated IgM (>200 IU/mL), while maximum concentrations in CD and UC patients were 6.75 IU/mL and 7.5 IU/mL, respectively.

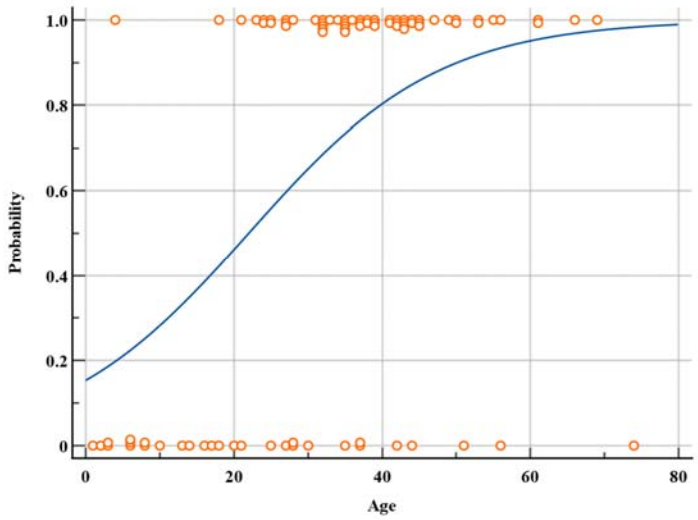

Figure 1. Logistic regression analysis demonstrates a positive association between age and IBD involvement, suggesting that advanced age is a risk factor for IBD.

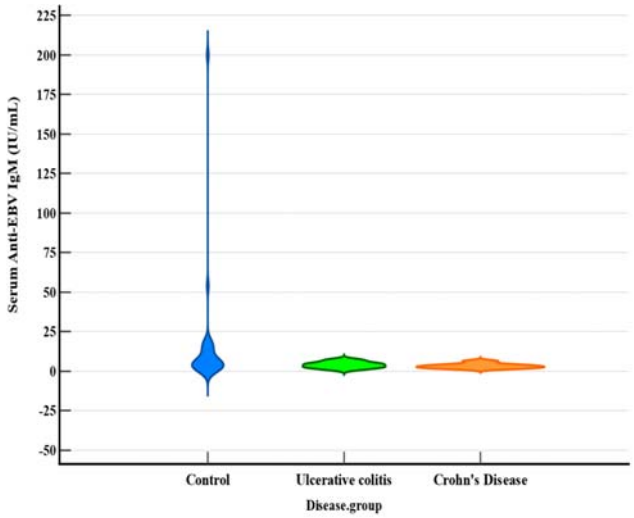

Figure 2. Comparative violin plot of serum EBV-IgM levels among control individuals, ulcerative colitis, and Crohn's disease groups. Serum EBV-IgM levels were meaningfully higher in control group (mean±SE= 15.114±6.643; range= 0.83-200 IU/mL) than individuals with Crohn's disease (mean±SE= 3.415±1.527; range= 1.25-6.75), or Ulcerative colitis (mean±SE= 4.051±0.359; range= 0.83-7.50).

Diagnostic value of serum EBV-IgM in IBD patients

To evaluate the diagnostic utility of EBV-IgM in inflammatory bowel disease, we performed receiver operating characteristic (ROC) curve analysis (**Figure 3).** For CD versus control individuals, an area under curve (AUC): 0.707 (p= 0.004), optimal cut-off ≤ 4.58 with sensitivity: 83.33%, specificity: 60%, positive predictive value (PPV): 67.6%, negative predictive value (NPV): 78.3% were obtained. These results suggest EBV-IgM may serve as a potential negative predictor for CD, with significantly lower levels observed in CD patients compared to controls (p=0.003).

For UC versus control individuals, an AUC: 0.646 (p= 0.042), optimal cut-of) ≤7.5, sensitivity: 100%, Specificity: 40%, PPV: 62.5%, NPV: 100%, were obtained. The EBV-IgM assay demonstrated excellent rule-out capability for UC at this threshold.

For CD versus UC patients, AUC: 0.600 (p= 0.183), optimal cut-off ≤4.0, sensitivity: 73.33%, specificity: 53.33%, PPV: 61.1%, and NPV: 66.7%, showed limited discriminatory value for distinguishing CD from UC.

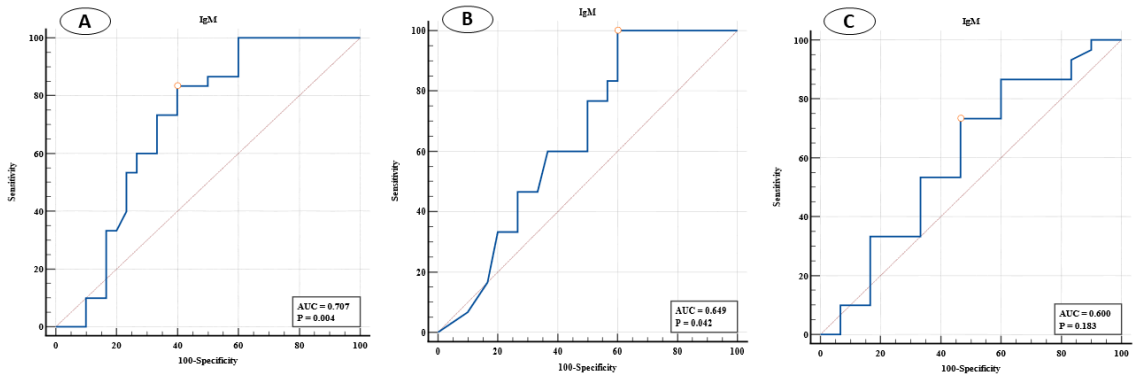

Figure 3. ROC curves plotted to determine differential diagnosis value of measuring the serum EBV-IgM levels in CD and UC patients; **A**: Crohn's disease and control individuals; **B**: Ulcerative colitis and control individuals; **C**: Crohn's disease and ulcerative colitis. EBV-IgM levels were valuable for reverse differential diagnosis and rule-out of patients with CD (Graph **A**) or UC (Graph **B**). In fact, individuals without CD/UC involvement had higher levels of EBV-IgM than patients with CD/UC, meaningfully. However, EBV-IgM levels were not useful in differentiating CD from UC (Graph **C**).

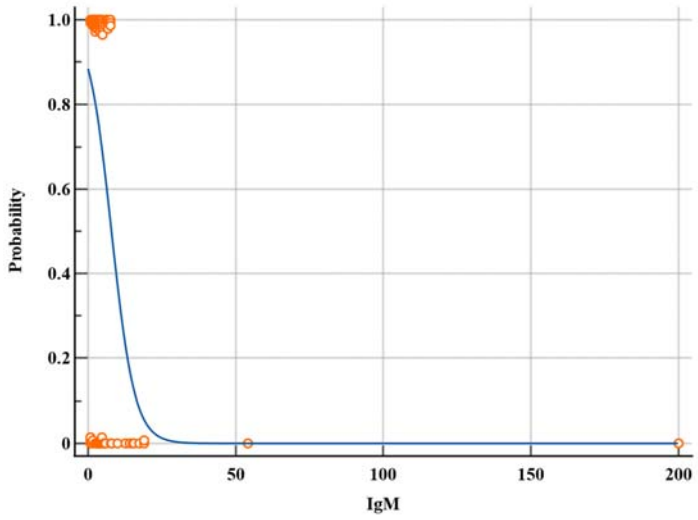

Figure 4. Logistic regression analysis indicates an inverse relationship between EBV-IgM levels and IBD involvement, with higher EBV-IgM levels correlating with a reduced likelihood of IBD.

EBV Primary Infection and IBD Risk

Primary EBV infection does not appear to be a risk factor for IBD. Instead, increased EBV-IgM serum level is protective against IBD involvement (OR: 0.7739; 95% CI: 0.6577–0.9107; p = 0.002) (**Figure 4**).

Rare EBV-positive detection by PCR

PCR analysis revealed EBV DNA positivity in only 3 of 90 evaluated subjects (3.3%). The EBV-positive cases comprised: 2 male patients (5.4% of male participants) and 1 female patient (4.3% of female participants). All EBV-positive individuals belonged to the CD group. The remaining 87 samples (96.7%) tested negative for EBV-DNA by PCR (**Figure 5**).

Discussion

Association between IBD and age

In the present study, advanced age was identified as a potential risk factor for IBD. Patients with IBD were significantly older than those in the control group, suggesting that the likelihood of CD and UC increases with age.

Association between EBV infection and age

Upon primary exposure to an infectious agent, the acquired immune system produces IgM antibodies, followed by IgG production days or weeks later. In cases of reinfection, memory B-lymphocytes enhance IgG production, leading to elevated serum IgG levels, while IgM levels remain largely unchanged.

No.	Colour	Name	Type	Ct	Given Conc (Copies)	Calc Conc (Copies)	% Var
1		ntc	Unknown				
2		pc	Unknown	26.24			
3		786	Unknown	37.93			
4		839	Unknown	48.22			
5		862	Unknown				
6		952	Unknown				
19		818	Unknown				
20		831	Unknown				
21		817	Unknown				
22		789	Unknown				
23		788	Unknown				
24		845	Unknown	34.44			

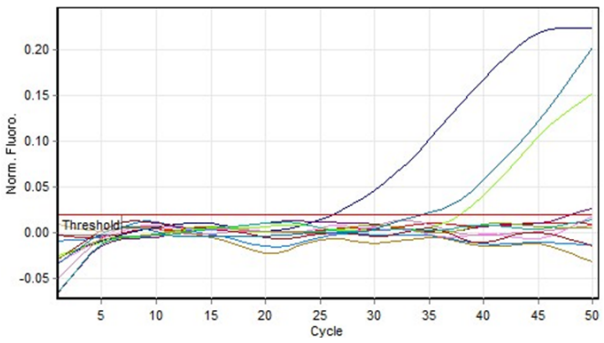

Figure 5. Table and expression graph of TaqMan real time-PCR for selected samples, control negative (nc) and control positive (pc) specimens.

In our study, control individuals were significantly younger and exhibited higher IgM serum levels compared to IBD patients. Elevated IgM levels in younger participants suggest recent EBV infection. However, IgG levels were not measured in any participants, limiting our ability to assess reinfection. Confirming reinfection requires two separate IgG measurements taken at least two weeks apart, with a significant rise in IgG levels indicating reactivation or reinfection. Unfortunately, our study lacked EBV-IgG data, and a single IgG measurement would not have been diagnostically meaningful. Nevertheless, PCR results confirmed the presence of EBV-DNA in circulation. Only three CD individuals tested positive for EBV-DNA.

Considering EBV-IgM results, our findings, align with previous studies (27, 28), reporting a higher prevalence of EBV infection in younger individuals (25, 26). For instance, Rochford (27) and Leung et al. (29) demonstrated a high EBV seroprevalence in children and adolescents.

Risk Factors for IBD

Established risk factors for IBD include smoking, psychological stress, diet, lifestyle, family history, ethnicity, infections, antibiotic use, and urban residence (7, 10, 19, 30, 31). Hadithi et al. (32) noted that while IBD can develop at any age, the peak onset occurs between 15 and 30 years. They also suggested that colitis in elderly patients (aged >60 years) may present as a broad colonic disease, including infections, carcinoma, microscopic or ischemic colitis, IBD, or vasculitis. Consequently, diagnosing IBD in older adults can be challenging due to overlapping clinical presentations with other forms of colitis.

EBV serological and molecular detection results

Serological analysis revealed significantly elevated EBV-IgM levels in control subjects compared to CD patients (p=0.003), with a non-significant trend toward higher levels versus UC patients (p=0.071). Collectively, IBD patients demonstrated markedly lower EBV-IgM concentrations than non-IBD controls, suggesting potential utility as a negative predictor for IBD diagnosis.

Association of EBV with IBD

Previous studies have implicated viral pathogens, including cytomegalovirus (CMV) and Epstein-Barr virus (EBV), in the pathogenesis of Crohn's disease (CD) and ulcerative colitis (UC). Notably, EBV infection in IBD patients has been associated with more severe clinical complications and worse disease outcomes (33). In our study, we employed TaqMan real-time PCR for EBV-DNA detection, which identified only 3 EBV-positive cases exclusively within the CD group. In contrast, EBV-IgM serological testing detected 9 positive individuals, all of whom were in the control group. No UC patients tested positive for either EBV-DNA or EBV-IgM, revealing a distinct pattern of EBV detection between diagnostic modalities and patient groups.

Several studies have reported elevated viral infection rates in IBD patients, potentially attributable to immunosuppressive therapy (34-36). However, as Xu and colleagues noted, these studies exhibit methodological inconsistencies related to study design, patient populations, and detection techniques (37). Using PCR-based EBV-DNA detection in UC patients, Xu et al. (37) reported suboptimal test performance (sensitivity: 76.5%; specificity: 68.5%).

Similarly, Mehrabani Khasraghi et al. (38) documented EBV-DNA detection rates of 60% in Crohn's disease patients versus 37.7% in healthy controls. In contrast, our study identified only 3 PCR-positive cases (3.3%) in CD group, and 9 IgM-positive control individuals, substantially lower than previously reported rates by Mehrabani Khasraghi and coworkers. These discrepancies may reflect technical differences in detection methodologies, geographic variation in EBV epidemiology, seasonal fluctuations in viral prevalence, particularly during summer and autumn months (39, 40).

CD patients with detectable EBV-DNA showed negative EBV-IgM results (<9 IU/mL), suggesting an impaired immune response to active infection. This immune dysfunction in Crohn's disease patients may be attributed to anti-inflammatory or immunomodulatory therapies. Common medications for CD and UC treatment include aminosalicylates (e.g., mesalazine and sulfasalazine), corticosteroids (e.g., prednisone and methylprednisolone), immunomodulators (e.g., azathioprine and 6-mercaptopurine), and biologic agents (e.g., adalimumab, infliximab, vedolizumab, and ustekinumab) (41). No EBV-DNA was detected in UC patients or control group individuals. To further elucidate these findings, larger-scale studies and epidemiologic investigations in CD and UC populations are warranted.

EBV antibody dynamics

The humoral immune response to EBV primarily targets the viral capsid antigen (VCA) and nuclear antigen (EBNA). Among these, VCA-specific IgM antibodies demonstrate the greatest diagnostic utility, appearing within 7 days post-infection and persisting for up to 3 months (42). In contrast, VCA-IgG emerges within 7 days while EBNA-IgG develops after approximately 3 weeks; both IgG isotypes typically persist lifelong (42, 43). Elevated VCA antibody titers provide strong serological evidence of active EBV infection. Notably, since EBNA antibodies develop 4-6 weeks post-infection, their detection during acute illness suggests alternative diagnoses to infectious mononucleosis. The presence of VCA-IgG indicates prior EBV exposure (42-44). In the current study, we focused exclusively on IgM detection based on prior evidence demonstrating universally elevated EBV-IgG levels in our patient population, which would preclude meaningful interpretation of IgG results.

ROC analysis demonstrated that the EBV-IgM test shows potential for differentiating CD and UC patients from non-IBD individuals, exhibiting acceptable sensitivity, specificity, and NPV. These findings suggest the EBV-IgM test may be clinically useful for ruling out non-CD/UC cases. Specifically, patients presenting with IBD symptoms and EBV-IgM levels ≤4.58 IU/mL showed increased likelihood of CD, while those with levels ≤7.5 IU/mL demonstrated higher probability of either CD or UC involvement. However, the EBV-IgM test showed no significant PPV or NPV in distinguishing between CD and UC cases.

As IBD patients may use anti-inflammatory medicines, an immunosuppression event is suspected for such patients (30, 45). Therefore, if an IBD patient has a concurrent EBV infection, irresponsive immunologic status cannot result in properly IgM production. Therefore, lower EBV-IgM levels in CD or UC patient could be logical considering usage of immunosuppressive drugs. Furthermore, Di Sabatino and colleagues have shown that IgM memory B cells become decreased in IBD patients (46). Collectively, IBD patients have a natural decrease in IgM producing B-cells and this circumstance could be worsened if they use immunosuppressive anti-inflammatory medicines. So, decreased levels of EBV-IgM antibody in our IBD patients is confirmed by these evidences.

Higher EBV-IgM level accompanied with lower IBD involvement likelihood

Since IgM antibodies are elevated during acute infection, these findings suggest that recent EBV infection may not contribute to IBD development and could even have a protective effect. However, further studies with larger sample sizes and comprehensive assessments of natural and humoral immunity are needed to validate this hypothesis.

Study Limitations

This study has several limitations. First, the relatively small sample size, due to resource constraints, hindered optimal age and gender matching. Second, EBV-IgG serum levels were unavailable, as ordering physicians often omit this test due to its limited diagnostic utility in IBD management.

Conclusion

This study found no significant association between Epstein-Barr virus (EBV) infection and inflammatory bowel disease (IBD). Furthermore, our findings highlight the clinical relevance of advanced age and EBV serum IgM levels in IBD patients. However, higher levels of EBV serum IgM are valuable for rule-out of CD or UC. The diminished EBV-IgM in IBD patients can be due to decreased lymphocyte-B population or usage of anti-inflammatory drugs. As IgG serum levels have a good lifelong than IgM antibody, EBV vaccination of populations with a high risk of infection is suggested.

Declaration of conflict of interest

Authors declare there is no conflict of interest.

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