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Detection of Herpesviruses and Parvovirus B19 in Gastric and Intestinal Mucosa of Chronic Fatigue Syndrome Patients

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Abstract. *Background: Human herpesvirus-6 (HHV-6), Epstein-Barr virus and parvovirus B19 have been suggested as etiological agents of chronic fatigue syndrome but none of these viruses is consistently detected in all patients. However, active viral infections may be localized in specific tissues, and, therefore, are not easily detectable. The aim of this study was to investigate the presence of HHV-6, HHV-7, EBV and parvovirus B19 in the gastro-intestinal tract of CFS patients. Patients and Methods: Using real-time PCR, viral DNA loads were quantified in gastro-intestinal biopsies of 48 CFS patients and 35 controls. Results: High loads of HHV-7 DNA were detected in most CFS and control biopsies. EBV and HHV-6 were detected in 15-30% of all biopsies. Parvovirus B19 DNA was detected in 40% of the patients versus less than 15% of the controls. Conclusion: Parvovirus B19 may be involved in the pathogenesis of CFS, at least for a subset of patients. The gastro-intestinal tract appears as an important reservoir of infection for several potentially pathogenic viruses.*

Chronic fatigue syndrome (CFS)/myalgic encephalomyelitis is characterized by a severe and debilitating fatigue associated with numerous symptoms including musculoskeletal pain, sore throat, sleep abnormalities and neurocognitive problems (1). The pathogenesis of CFS is still poorly understood, but is probably multifactorial: infections, stress, neuroendocrine dysfunctions may all contribute to the onset and maintenance of the disease (2).

Abbreviations: CFS, chronic fatigue syndrome; HHV, human herpesvirus; EBV, Epstein-Barr virus; PCR, polymerase chain reaction.

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The observation that many cases of CFS begin with a flu-like illness prompted the hypothesis that viral infections were implicated in the disease, and several viruses were suggested as etiological agents for CFS. These viruses include human herpesvirus (HHV)-6, HHV-7 (3-5), Epstein-Barr virus (EBV) (6), cytomegalovirus (5), parvovirus B19 (7-9) and enteroviruses (10).

However, the lack of specificity of these viruses for CFS (most of them are highly prevalent viruses, associated with other pathologies and sometimes even detected in asymptomatic people), as well as the poor consistency between different studies, have created controversy regarding their real implication in the disease (11, 12). One major limitation is the difficulty to detect active, pathological viral infections, or to distinguish the active viruses from the latent ones. Another issue is the tissue in which the virus has to be looked for. Active viral infections will not necessarily be detectable in peripheral blood; other tissues, such as brain or heart, can be the site of active enterovirus, parvovirus or herpesvirus infections (13, 14).

The gastro-intestinal tract is another site where viral infections may persist chronically. EBV can be detected in the gastric epithelium, and is associated with gastritis and gastric carcinoma (15). HHV-6, HHV-7, cytomegalovirus are found in intestinal biopsy specimens of HIV patients and liver transplant recipients (16, 17). Most CFS patients have persistent or intermittent symptoms of intestinal dysfunction, and a significant subset of them present chronic enterovirus infection in the stomach (18). It was therefore suspected that the gastro-intestinal tract could be a site of local viral infections in CFS patients.

The purpose of this study was to investigate whether herpesviruses (HHV-6, HHV-7, EBV) and parvovirus B19 could be detected in gastric and intestinal biopsies of CFS patients. For quantification of viral loads, a new real-time PCR procedure was used where the amplification of a viral DNA sequence was compared with the amplification of an endogenous genomic DNA sequence, used as an internal control. Parvovirus B19 was detected more frequently in

patient biopsies than in control biopsies, suggesting an implication of this virus in the pathogenesis of CFS.

Patients and Methods

Clinical material. A total of 48 CFS patients were analyzed. All were diagnosed for CFS according to the clinical criteria of Fukuda *et al*. (1). Thirty-five non-CFS subjects (healthy people undergoing routine check-up or people suffering from mild gastro-intestinal symptoms) were also included in the study. Seventy-eight percent of the patients, and 66% of the controls, were females. Mean age was 37±13 for the patients, 46±17 for the controls.

All subjects gave informed consent. For each subject, biopsy specimens were taken in the gastric antrum and in the duodenum (biopsies were obtained by sampling mucosa from two different places of each compartment). For most of the patients, a sample of peripheral whole blood was also collected.

DNA extraction, quantitative PCR conditions. Genomic DNA was extracted from the biopsies or from whole blood using the Blood and Tissue DNA extraction kit (Qiagen, Venlo, Netherlands). Taqman real-time quantitative PCR was performed on the iCycler instrument (BioRad, Hercules, USA); thermal cycling started with a 10-min incubation at 95˚C, followed by 50 cycles of 95˚C for 15 s and 60˚C for 1 min. Reactions were set up in a 30 μL final volume, with the FastStart Taqman Probe Master (Roche Diagnostics, Mannheim, Germany). The sequence and final concentration of primers and probes are shown in Table I. Probes were labeled at the 5'-end with 6-FAM, and linked at the 3'-end with BHQ-1™ quencher. Primers and probes were synthesized by Eurogentec (Seraing, Belgium).

Viral load determination. The strategy for viral load determination was based on the relative quantification of the target sequence (viral DNA) and a reference sequence present in the human genomic DNA (a non-polymorphic region of the human BRCA1 locus, which is present in only two copies in a diploid genome (18)). By comparing the cycle threshold (Ct) of the viral PCR with the cycle threshold of the reference PCR, the ratio "number of viral copies/number of cellular genomes" (Ncop/Ncg), can be calculated according to the formula:

$$Ncop/Ncg = [2^{(Ct[Ref\ PCR]-Ct[Viral\ PCR]+1)}]$$

For convenience reasons the viral loads are rather expressed as "number of viral copies per million cells": $Ncop/Mc=10^6.Ncop/Ncg$.

Calculation of PCR efficiencies. The above calculation is only valid if the efficiencies of the two PCRs (viral and reference) are close to identical. The efficiency of each PCR was determined by amplifying serial dilutions of viral DNA (Advanced Biotechnologies, Columbia, USA) or of DNA samples positives for each of HHV-6A, HHV-6B, HHV-7, EBV and parvovirus B19. The Ct values were plotted against the DNA dilutions, and the efficiencies calculated according to the formula $PCREFF=10^{-(1/curve\ slope)}-1$.

Efficiency of the reference PCR was 86%; efficiencies of the viral PCRs were between 83% and 89%. A 3% difference in efficiency can actually, after 20-25 cycles of amplification, lead to a significant error in the quantification of the viral load (up two two-fold); however this error will be the same for the control samples and the CFS samples, so it will not affect the comparison between the two populations.

HHV-6 A/B typing. The HHV-6 primers shown in Table I will amplify both A and B strains of HHV-6. To differentiate between the two strains, all HHV-6-positive samples were tested again using variant-specific PCR primers and probes. The PCR procedure (cycling parameters, PCR reagents) is as described above; the sequences of primers and probes are shown in Table II.

Results

Results are summarized in Table III. For each virus, the percentage of positive biopsies is indicated; mean values of viral loads (number of viral copies per million cells) are calculated on the positive samples only.

HHV-7. Most biopsies were positive for HHV-7, usually with very high loads (more than 130,000 copies per million cells in some cases). There was a trend for lower prevalence and lower viral loads in controls than in patients however this was not statistically significant. Viral loads were lower in the duodenum than in the stomach (statistically significant for the patient biopsies, p=0.005, not for the control biopsies).

HHV-6. HHV-6 was found in 13-31% of the biopsies, with loads usually under 2000 copies per million cells. There was no significant difference between controls and patients, but again the loads were lower in the duodenum than in the stomach (statistically significant for the patient biopsies, p=0.09, not for the control biopsies).

EBV. EBV was found in 15-31% of the biopsies. There was a trend for lower viral loads in the duodenum than in the stomach but this was not statistically significant. No difference was found between the CFS and the control populations.

Parvovirus B19. The percentage of parvovirus B19 positive biopsies was significantly higher for the CFS patients than for the controls (38-40% *versus* 14%; OR=3.93, p=0.008). Only five controls were positive. There was no difference between stomach and duodenum biopsies.

Co-infections. Not considering HHV-7, co-infections (presence of at least two different viruses in the same biopsy) were seen in 8 out of 48 patients, and 6 out of 35 controls (about 17% in both populations).

HHV-6 typing. In all cases the variant present in the biopsies was HHV-6B.

Detection of viruses in peripheral blood. Peripheral whole blood samples from 32 of the patients were tested using the same PCR procedure. Parvovirus B19 could not be detected in any of those samples, although 11 of them

Table I. *Primers and probes used for viral PCR.*

		Sequence	Final conc.	Reference
Genomic DNA reference	For. primer	5'-aaacatgttcctcctaaggtgcttt-3'	400 nM	(19)
	Rev. primer	5'-atgaaaccagaagtaagtccaccagt-3'	400 nM	
	Probe	5'-FAM-ccttcacacagctaggacgtcatcttt-BHQ1-3'	50 nM	
HHV-6	For. primer	5'-caaagccaaattatccagagcg-3'	500 nM	(20)
	Rev. primer	5'-cgctaggttgaggatgatcga-3'	500 nM	
	Probe	5'-FAM-caccagacgtcacacccgaaggaat-BHQ1-3'	150 nM	
HHV-7	For. primer	5'-tttcctgtgacaaaagaagcagtta-3'	800 nM	(21)
	Rev. primer	5'-atcccacacgctttacggg-3'	800 nM	
	Probe	5'-FAM-ttcctgcgcaataaagtgaaaactgttagcatt-BHQ1-3'	100 nM	
EBV	For. primer	5'-tcatcatcatccgggtctcc-3'	600 nM	(22)
	Rev. primer	5'-cctacagggtggaaaaatggc-3'	600 nM	
	Probe	5'-FAM-cgcaggccccctccaggtagaa-BHQ1-3'	150 nM	
Parvovirus B19	For. primer	5'-ggcagcatgtgttaaagtggat-3'	200 nM	(23)
	Rev. primer	5'-cctgctacatcattaaatggaaagtt-3'	200 nM	
	Probe	5'-FAM-accatgccatatactggaacacttttagca-BHQ1-3'	100 nM	

Table II. *Primers and probes used for HHV-6 A/B typing.*

		Sequence	Final conc.	Reference
HHV-6A	For. primer	5'-aggaaccatcttgttctgtccctt-3'	300 nM	
	Rev. primer	5'-gtacagcctcagtgacagatctg-3'	300 nM	
	Probe	5'-FAM-ttgaactccatcagcggcctccagagttgt-BHQ1-3'	200 nM	(24)
HHV-6B	For. primer	5'-ggtcatacaaggaagcgtttcg-3'	300 nM	
	Rev. primer	5'-gtacagcctcagtgacagatctg-3'	300 nM	
	Probe	5'-FAM-cagccccgataaaaggtcacagacaaaaga-BHQ1-3'	200 nM	

Table III. *Viral loads in gastric and duodenal biopsies.*

	HHV-7		HHV-6		EBV		Parvovirus B19	
	% pos.	Viral load	% pos.	Viral load	% pos.	Viral load	% pos.	Viral load
CFS stomach	92%	21340±5100	31%	1650±500	21%	2700±2300	38%	1580±630
Controls stomach	83%	13400±5010	29%	1070±440	17%	2260±1380	14%	1430±910
CFS duodenum	85%	5650±1520	13%	250±110	31%	940±540	40%	1310±280
Controls duodenum	66%	3950±1160	22%	330±130	15%	1310±860	14%	910±400

corresponded to patients with parvovirus-positive biopsies. Similarly, HHV-6 was not detected in any of the blood samples, although 13 of these samples corresponded to patients with HHV-6-positive biopsies. EBV was detected in three blood samples; these three patients were also EBV-positive in the stomach and/or duodenum. However there were 11 cases of biopsy-positive patients whose blood tested negative. HHV-7 was detected in 12 of the blood samples. There was no correlation between the load of HHV-7 in the biopsy, and the presence of viral DNA in the blood.

Discussion

The real-time PCR approach used in this study proved to be perfectly suitable for the quantification of viral loads in solid, cellular samples such as biopsies. Absolute quantification using a standard curve would only make sense if absolute viral counts per volume, or weight, of the biopsied tissue were required, but neither volume nor weight can be accurately measured (very small amount of tissue available). Therefore, relative quantification and expression of the result as a viral load per cell is a much more relevant approach.

One objective of this study was to investigate whether the presence of HHV-6, HHV-7 and/or EBV in the gastro-intestinal tract could be associated with CFS. All three viruses could be detected in both gastric and intestinal biopsies. However, they could be detected at similar frequencies, and usually similar loads, in biopsies from both CFS patients and non-CFS controls. Only in the case of HHV-7 could a possible trend towards higher loads be observed in the CFS samples compared to the controls, however the difference was not statistically significant. The pathogenic potential of HHV-7 is anyway questionable: it was detected in most control samples; titers reaching several tens of thousands viral copies were observed in subjects that did not suffer from any particular pathology. Higher loads of HHV-7 in CFS *versus* control samples, if confirmed, could be a consequence of impaired mucosal immunity rather than a real cause of the disease.

Regarding HHV-6 and EBV, no significant difference could be found between the CFS patients and the controls: the frequency of positive samples and the mean viral loads in these positive samples were similar in both populations. Therefore, the results do not seem to support an implication of these viruses in the pathogenesis of CFS. This implication, however, cannot be totally excluded: HHV-6 is known to induce a selective immune suppression which could have, in some of the positive patients, favored the development of other intracellular pathogens, viruses or mycobacteria (25). Also, persistence of herpesviruses could cause a chronic inflammation of the gastro-intestinal tract, which could still, in certain predisposed people, contribute to the disease (chronic activation of immune function).

Nevertheless, the presence of these potentially pathogenic viruses in the gastrointestinal tract of apparently healthy people is a clinically relevant observation. HHV-6, HHV-7 and EBV can reactivate and cause severe complications in immunocompromised patients (26, 27). Obviously the gastrointestinal tract is a major reservoir from which herpesviruses could spread in case of impaired immunity. This could be an important mechanism of viral reactivation in post-transplant patients.

A most important finding is the higher frequency of parvovirus B19 positive biopsies in the CFS population, compared to the controls (38-40% in CFS duodenum and stomach biopsies, *versus* less than 14% in the controls). This difference suggests that parvovirus B19 may be involved in the development and maintenance of CFS, at least for a subset of patients.

Development of CFS following parvovirus B19 infection had previously been reported (7-9). In one of these reports CFS was associated with a persistent, low-grade viremia (9) but in the other cases there was no evidence that the virus was persisting in the host following the acute infectious phase. Viral DNA disappeared from the serum after 4-5 months, and antibody titers also decreased rapidly. In contrast to herpesviruses, parvovirus B19 is not thought to establish latency or integrate into the host genome (8). A causal link between parvovirus B19 and maintenance of the CFS symptoms was therefore difficult to establish.

The gastro-intestinal tract has now been identified as a reservoir where parvovirus B19 may persist in the host following primary infection. It is noteworthy that no viral DNA was detectable in the blood samples isolated from the same patients. Chronic intestinal infections may remain undetected, and yet elicit numerous symptoms in the affected patients. Acute parvovirus B19 infections are accompanied by increased levels of various cytokines (IL-1β, IL-6, TNF-α, IFN-γ); raised levels of TNF-α and IFN-γ persist in those patients that develop CFS (28). These altered cytokine levels may contribute to the immune dysregulations associated with CFS.

Persistence of parvovirus B19 infections in certain individuals may be determined by genetic factors (29), as well as by pre-existing immune dysfunction in the intestinal mucosa. Interestingly, a significant association was recently reported between psychological stress, which has a demonstrated effect on intestinal function, and the development of chronic fatigue following acute parvovirus infection (30).

These results provide further support for an implication of gastro-intestinal dysfunction in the pathogenesis of chronic fatigue syndrome.

References

1 Fukuda K, Straus SE, Hickie I, Sharpe MC, Dobbins JG and Komaroff A: The Chronic Fatigue Syndrome: a comprehensive approach to its definition and study, International Chronic Fatigue Syndrome Study Group. Ann Intern Med *121*: 953-959, 1994.

2 Devanur LD and Kerr JR: Chronic Fatigue Syndrome. J Clin Vir *37(3)*: 139-150, 2006.

3 Ablashi DV, Eastman HB, Owen CB, Roman MM, Friedman J, Zabriskie JB, Peterson DL, Pearson GR and Whitman JE: Frequent HHV-6 reactivation in multiple sclerosis (MS) and chronic fatigue syndrome (CFS). J Clin Virol *16(3)*: 179-191, 2000.

4 Chapenko S, Krumina A, Kozireva S, Nora Z, Sultanova A, Viksna L and Murovska M: Activation of human herpesviruses 6 and 7 in patients with chronic fatigue syndrome. J Clin Virol *37(S1)*: S47-S51, 2006.

5 Chia JK and Chia A: Diverse etiologies for chronic fatigue syndrome. Clin Infect Dis *36(5)*: 671-672, 2003.

6 Lerner AM, Beqaj SH, Deeter RG and Fitzgerald JT: IgM serum antibodies to Epstein-Barr virus are uniquely present in a subset of patients with the chronic fatigue syndrome. In Vivo *18(2)*: 101-106, 2004.

7 Kerr JR, Bracewell J, Laing I, Mattey DL, Bernstein RM, Bruce IN and Tyrrell DA: Chronic fatigue syndrome and arthralgia following parvovirus B19 infection. J Rheumatol *29(3)*: 595-602, 2002.

8 Seishima M, Mizutani Y, Shibuya Y and Arakawa C: Chronic fatigue syndrome after human parvovirus B19 infection without persistent viremia. Dermatology *216(4)*: 341-346, 2008.
9 Jacobson SK, Daly JS, Thorne GM and McIntosh K: Chronic parvovirus B19 infection resulting in CFS: case history and review. Clin Infect Dis *24(6)*: 1048-1051, 2007.
10 Chia JK: The role of enterovirus in chronic fatigue syndrome. J Clin Pathol *58*: 1126-1132, 2005.
11 Reeves WC, Stamey FR, Black JB, Mawle AC, Stewart JA and Pellett PE: Human herpesviruses 6 and 7 in chronic fatigue syndrome: a case-control study. Clin Infect Dis *31(1)*: 48-52, 2000.
12 Soto NE and Straus SE: Chronic fatigue syndrome and herpesviruses: the fading evidence. Herpes *7(2)*: 46-50, 2000.
13 Kühl U, Pauschinger M, Seeberg B, Lassner D, Noutsias M, Poller W and Schultheiss HP: Viral persistence in the myocardum is associated with progressive cardiac dysfunction. Circulation *112*: 1965-1970, 2005.
14 Fotheringham J, Akhyani N, Vortmeyer A, Donati D, Williams E, Oh U, Bishop M, Barrett J, Gea-Banacloche J and Jacobson S: Detection of active human herpesvirus-6 infection in the brain: correlation with polymerase chain reaction detection in cerebrospinal fluid. J Infect Dis *195(3)*: 450-454, 2007.
15 Yanai H, Takada K, Shimizu N, Mizugaki Y, Tada M and Okita K: Epstein-Barr virus infection in non-carcinomatous gastric epithelium. J Pathol *183(3)*: 293-298, 1997.
16 Gautheret-Dejean A, Agut H, Nicolas JC and Beaugerie L: Roseolovirus DNA in the colonic mucosa of HIV-seropositive patients with diarrhea. Clin Infect Dis *36(10)*: 1348-1349, 2003.
17 Halme L, Arola J, Höckerstedt K and Lautenschlager I: Human herpesvirus 6 infection of the gastroduodenal mucosa. Clin Infect Dis *46(3)*: 434-439, 2008.
18 Chia JK and Chia AY: Chronic fatigue syndrome is associated with chronic enterovirus infection of the stomach. J Clin Pathol *61(1)*: 43-48, 2008.
19 Haque KA, Pfeiffer RM, Beerman MB, Struewing JP, Chanock SJ and Bergen AW: Performance of high-throughput DNA quantification methods. BMC Biotechnology *3(20)*: doi: 10.1186/1472-6750-3-20, 2003.
20 Locatelli G, Santoro F, Veglia F, Gobbi A, Lusso P and Malnati M: Real-time quantitative PCR for human herpesvirus 6 DNA. J Clin Microbiol *38(11)*: 4042-4048, 2000.
21 Zerr DM, Huang ML, Corey L, Erickson M, Parker HL and Frenkel LM: Sensitive method for detection of human herpesviruses 6 and 7 in saliva collected in field studies. J Clin Microbiol *38(5)*: 1981-1983, 2000.
22 Lo YMD, Chan LYS, Lo KW, Leung SF, Zhang J, Chan ATC, Lee JCK, Hjelm NM, Johnson PJ and Huang DP: Quantitative analysis of cell-free Epstein-Barr virus DNA in plasma of patients with nasopharyngeal carcinoma. Cancer Res *59(6)*: 1188-1191, 1999.
23 Gruber F, Falkner FG, Dorner F and Hämmerle T: Quantitation of viral DNA by real-time PCR applying duplex amplification, internal standardization, and two-color fluorescence detection. Appl Environ Microbiol *67(6)*: 2837-2839, 2001.
24 Sashihara J, Tanaka-Taya K, Tanaka S, Amo K, Miyagawa H, Hosoi G, Taniguchi T, Fukui T, Kasuga N, Aono T, Sako M, Hara J, Yamanishi K and Okada S: High incidence of human herpesvirus 6 infection with a high viral load in cord blood stem cell transplant recipients. Blood *100(6)*: 2005-2011, 2002.
25 Boeckh M and Nichols WG: Immunosuppressive effects of beta-herpesviruses. Herpes *10(1)*: 12-16, 2003.
26 Clark DA: HHV-6 and HHV-7: emerging pathogens in transplant patients. Int J Hematol *76(S2)*: 246-252, 2002.
27 Dolcetti R: B lymphocytes and Epstein-Barr virus: the lesson of post-transplant lymphoproliferative disorders. Autoimmun Rev *7(2)*: 96-101, 2007.
28 Kerr JR and Tyrrell DA: Cytokines in parvovirus B19 infection as an aid to understanding chronic fatigue syndrome. Curr Pain Headache Rep *7(5)*: 333-341, 2003.
29 Kerr JR: Pathogenesis of parvovirus B19 infection: host gene variability, and possible means and effects of virus persistence. J Vet Med B Infect Dis Vet Public Health *52(7-8)*: 335-339, 2005.
30 Kerr JR and Mattey DL: Preexisting psychological stress predicts acute and chronic fatigue and arthritis following symptomatic parvovirus B19 infection. Clin Infect Dis *46(9)*: e83-87, 2008.

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