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Bulgarian Participation in European Network on Myalgic Encephalomyelitis/Chronic Fatigue Syndrome: Human Herpesvirus Infection Associated Biomarkers of ME/CFS – Optimization of PCR-Based Detection

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Abstract

Infections of Epstein-Barr virus (EBV), cytomegalovirus (CMV), human herpesvirus-6 and -7 (HHV-6, HHV-7) are suspected as etiological agents of Myalgic Encephalomyelitis/Chronic Fatigue Syndrome (ME/CFS). The aim of this study was to select and optimize PCR-based systems for detection of EBV, CMV and HHV-6 in biological samples from ME/CFS patients and controls.

Whole blood and plasma samples obtained from 5 individuals, and DNA samples containing the entire viral genome of EBV, CMV and HHV-6 were used in this study. Conventional and nested PCR systems were assessed for their efficiency in virus DNA amplification. For PCR optimization modifications in reaction volume, concentration of primers and $MgCl_2$, and in annealing temperatures were assessed. Sensitivity of reactions was estimated by serial dilutions of viral DNA. PCR systems were tested with HSV, VZV, EBV, CMV and HHV-6 for cross-reactivity.

EBV, CMV and HHV6 DNA were not detected in blood and serum samples by conventional PCR and the first round of nested PCR. EBV, CMV and HHV-6 positive samples were only detected in the second round of nested PCR in blood samples (two samples were EBV-positive, CMV and HHV-6 were detected in one sample each). The PCR conditions for each virus in both steps of amplification were optimized. The sensitivity of nested PCR was determined to be not less than 200 copies/mL of sample. No cross-reactivity was detected with other herpes viruses. The optimized nested PCR assays are highly sensitive and specific and allow detection of EBV, CMV and HHV-6 in clinical specimens to study the potential role of these viruses in ME/CFS.

Keywords: Encephalomyelitis/Chronic Fatigue Syndrome, biomarkers, EBV, CMV, HHV-6, PCR

Резюме

Съществува хипотеза, че инфекциите с Епщайн-Бар вирус (EBV), цитомегаловирус (CMV), човешки херпесен вирус-6 и -7 (HHV-6, HHV-7) са етиологичен фактор за развитието на миалгичен енцефаломиелит /синдром на хроничната умора (ME/CFS). Целта на настоящото изследване е да селектира и оптимизира PCR системи, подходящи за доказване на EBV, CMV и HHV-6 в биологични проби от пациенти с ME/CFS и контроли.

Цяла кръв и плазма от 5 пациенти, както и ДНК проби, съдържащи целия геном на EBV, CMV и HHV-6 бяха използвани в това изследване. Конвенционални и нестед PCR системи бяха изследвани за определяне на тяхната ефективност при амплификация на вирусна ДНК. За оптимизация на PCR системите бяха тествани модификации в обема на реакциите, концентрацята на праймерите и $MgCl_2$ и в температурата на анилинг. Чувствителността на реакциите определяхме чрез изследване на серийни разреждания на вирусна ДНК. PCR системите бяха тествани с HSV, VZV, EBV, CMV и HHV-6 за кръстосана реактивност.

Не установихме EBV, CMV и HHV-6 ДНК в кръвни и серумни проби с конвенционален PCR

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и при първата реакция на нестед PCR. EBV, CMV и HHV-6 бяха открити само при втората реакция на нестед PCR в кръвни проби (две проби бяха EBV-позитивни, CMV и HHV-6 бяха открити в по една проба). PCR протоколите за всеки вирус в двата етапа на амплификация бяха оптимизирани. Установихме, че чувствителността на нестед PCR е не по-малка от 200 копия /mL от пробата. Не открихме кръстосана реактивност с други херпесни вируси. Оптимизираните нестед PCR системи са с висока чувствителност и специфичност и позволяват откриване на EBV, CMV и HHV-6 в клинични проби с цел изследване на потенциалната роля на тези вируси в ME/CFS.

Introduction

Myalgic Encephalomyelitis/Chronic Fatigue Syndrome (ME/CFS) is a serious, chronic, multisystem disease characterized by severe fatigue and other disabling symptoms (Christley *et al*., 2012). ME/CFS has a considerable social and economic impact and a prevalence rate between 0.2% and 2.6% (Reid *et al.*, 2000; Afari and Buchwald, 2003). There are no data concerning ME/CFS in Bulgaria.

So far, the etiology and pathogenesis of ME/CFS are unknown and there are no validated disease biomarkers, in part due to the limited numbers of patients included in the ME/CFS studies and to the heterogeneity of the illness. Many factors, such as immunological, infection related, metabolic or neurological are associated with the pathogenesis of ME/CFS (Bansal *et al.*, 2012). Viral infection has been reported as a trigger of ME/CFS and the hypothesis is based on the sudden onset of "flu-like" symptoms in the majority of cases. Infection with certain viruses, especially persistent infections with Epstein-Barr virus (EBV), cytomegalovirus (CMV), human herpesviruses 6 and 7 (HHV-6, HHV-7), have been suspected to be associated with ME/CFS (Ablashi *et al.* 2000; Komaroff, 2006; Cameron *et al.*, 2010; Bansal *et al.*, 2012; Chapenko *et al.*, 2012). It was suggested that these infections may cause chronic activation of the immune system with abnormal regulation of cytokine production leading to ME/CFS (Bansal *et al*., 2012). However, the role of viruses in ME/CFS remains controversial and so far, no particular virus has been implicated as the causative agent of the disease.

EUROMENE is a European network of researchers and clinicians on ME/CFS supported by the European COST program within Horizon 2020 (http://www.cost.eu/ COST_Actions/ca/CA15111). The aims of EUROMENE are to foster strategies for collaboration and harmonization of diagnosis and research on ME/CFS. As a partner of EUROMENE network and member of the Working group on biomarkers, particularly Infection-associated ME/CFS biomarkers, we are involved in characterization of EBV, CMV and HHV-6 and 7 infections in ME/CFS patients in Bulgaria. One of the methods, most commonly used for detection of these viruses in different biological samples is PCR. Therefore, as a first step, we have selected and optimized PCR-based systems for detection of EBV, CMV and HHV-6 in biological samples obtained from patients with ME/CFS and controls. The results of our work are reported here.

Material and Methods

Whole blood and plasma samples obtained from 5 individuals, and DNA samples containing the entire viral genome of EBV (Genekam, Duisburg, Germany), CMV (Genekam, Duisburg, Germany) and HHV-6 (kindly provided by Prof. M. Murovska, August Kirchenstein Institute of Microbiology and Virology, Riga Stradins University, Latvia) were used in this study.

DNA extraction from the whole blood and serum samples was performed by PureLink® Genomic DNA Mini Kit (Invitrogen™) and PureLink® Viral RNA/DNA Kit (Invitrogen™), respectively, and 5 µl was used in each PCR. Conventional and nested PCR systems were assessed for efficiency of amplification of EBV, CMV and HHV-6 genomic sequences. The primers were selected according to the data available in the literature. The EBV specific primers were directed at EBNA-1 region. The following sets of primers were used: set 1 for conventional PCR (5'-ccaccagcagcaccagca-3' and 5'-cagggccaccatggtggc -3') (Yap *et al.*, 2007) and set 2 - for nested PCR (outer primers 5'-aaggaggtggtttggaaag-3' and 5'-agacaatggactcccttagc-3'; inner primers 5'-gctagaacgtatttgctgcagaacg-3' and 5'-atccgaaacaactgtctgactggca-3') (Cinque *et al.*, 1993). PCR for CMV detection was directed to the gH region. For conventional PCR the primer set was 5'-tggacctggccaaacgagccc-3' and 5'-tggacgaggctgcccatgagg-3' (Allen *et al.*, 1995). Nested PCR was performed using the following set of primers: outer primers - 5'-tggacctggccaaacgagccc-3' and 5'-tggacgaggctgcccatgagg-3' and inner primers 5'-tcaccgacatcaccagcctcg-3' and 5'-cttggcgcgcgaaggctgaaag-3'. The primers used for the conventional HHV-6 PCR were 5'-cccatttac-

gatttcctgcaccacctctgc-3‘ and 5‘-ttcagggaccgttatgtcattgagcatgtcg-3‘(Torelli *et al.*, 1991). Nested PCR for HHV-6 detection was directed to a highly conserved region encoding the major capsid protein with the outer primers -5’-gcgttttcagtgtgtagttcggcag-3‘ and 5‘-tggccgcattcgtacagatacggagg-3‘, and inner primers 5‘-gctagaacgtatttgctgcagaacg-3‘ and 5‘-atccgaaacaactgtctgactggca-3‘ (Secchiero *et al.*, 1995; Bandobashi *et al.*, 1997).

In all nested PCR systems a sample of the first round product was used as template for the second round. DNA samples containing the entire viral genome of EBV, CMV and HHV-6 were used as positive controls and distilled water as negative control.

First, the amplification mixtures and the cycling protocols for each set of primers were followed as described in the literature (Torelli *et al.*, 1991; Cinque *et al.*, 1993; Allen *et al.*, 1995; Secchiero *et al.*, 1995; Bandobashi *et al.*, 1997; Yap *et al.*, 2007) without any modifications. Further, the optimal amplification conditions were determined experimentally. For PCR optimization modifications were made in reaction total volume (25µl and 50 µl), concentration of primers (0.005µM, 0.01µM, 0.1µM, 0.2µM, 0.5µM), concentration of $MgCl_2$ (1.5mM, 2mM, 3mM, 4mM, 5mM) and annealing

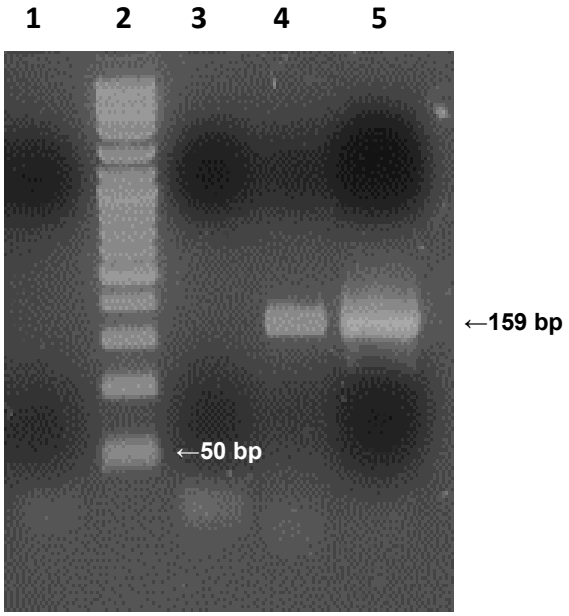

Fig. 2. Detection of CMV by nested PCR: amplification of 159 bp fragment in CMV positive sample (line 4), negative (line 1) and positive (line 5) controls, line 3 - CMV negative sample, line 2 - MW marker, 50 bp DNA ladder.

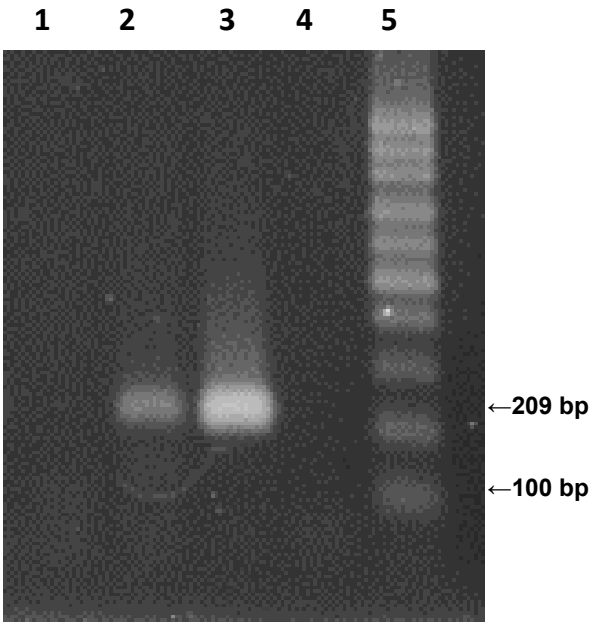

Fig. 1. Detection of EBV by nested PCR (second PCR): amplification of 209 bp fragment in EBV positive sample (line 2), negative (line 4) and positive (line 3) controls, line 1 - EBV negative sample, line 5 - MW marker, 100 bp DNA ladder.

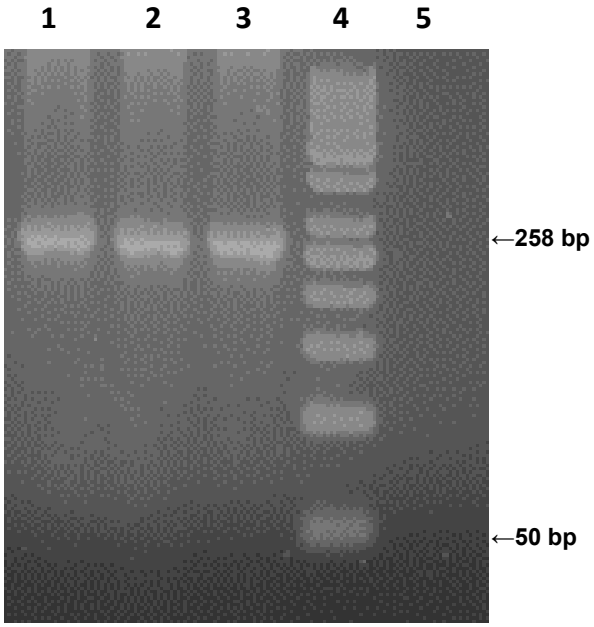

Fig. 3. Detection of HHV-6 by nested PCR: amplification of 258 bp fragment in HHV-6 positive samples (lines 1 and 2), negative (line 5) and positive (line 3) controls, line 4 - MW marker, 50 bp DNA ladder.

temperature (45, 50, 55, 56, 57, 58, 60, and 65°C).

Sensitivity of reactions was estimated by testing serial10-fold dilutions (starting with 1µg) of DNA samples containing the entire viral genome. Each PCR system was tested with HSV, VZV, EBV, CMV and HHV-6 for potential cross-reactivity.

Amplifications were performed on a DNA Engine Opticon 2 system (MJ Research) Thermal Cycler. 10 µl of each PCR product was analyzed by electrophoresis on 2% agarose gel.

Results and Discussion

Initially, we tested all PCR systems following the amplification mixtures and cycling protocols as described in the literature without any modifications (Torelli *et al.*, 1991; Cinque *et al.*, 1993; Allen *et al.*, 1995; Secchiero *et al.*, 1995; Bandobashi *et al.*, 1997; Yap *et al.*, 2007). As expected, nested PCR systems were more sensitive. We were not able to detect EBV, CMV and HHV-6 DNA in blood and serum samples by conventional PCR and the first round of nested PCR. EBV, CMV and HHV-6 positive samples were only detected through nested PCR. Two of the blood samples we were EBV positive – we observed amplicons of expected length of 209 bp in the second round of nested PCR (Fig. 1).

CMV and HHV-6 were detected in one blood sample each – the expected 159 bp (CMV) and 258 bp (HHV-6) amplicons were generated in the second round of nested PCR (Fig. 2 and Fig. 3). All 5 serum samples were negative for EBV, CMV and HHV-6 by all PCR systems and their modifications. These results indicated that the two-step amplification procedure increased the sensitivity of reactions.

Due to non-specific amplification products, we additionally optimized the PCR tests. We evaluated several parameters that are known to influence the outcome of the reaction. We made modifications in reaction volume, primers and $MgCl_2$ concentration and in cycling protocols. After optimization, the reaction total volume was fixed to 25µl for EBV and CMV PCR and 50µl for HHV-6 PCR. The optimal final concentration of primers according to our studies was 0.01µM and 3mM of $MgCl_2$. We tested several annealing temperatures – the best results were observed at an annealing temperature of 55°C (EBV/firs PCR), 60°C (EBV/second PCR), 56°C (CMV/first and second PCR), 57°C (HHV-6/ first and second PCR). The optimized cycling profiles were as follows: initial denaturation of 5 min at 94°C and final extension - 5 min at 72°C for all PCR; EBV - first round of amplification consisted of 40 cycles (30 sec at 94°C, 30 sec at 55°C and 45 sec at 72°C) and the second round – of 40 cycles (30 sec at 94°C, 30 sec at 60°C and 45 sec at 72°C); CMV first round – 40 cycles (30 sec at 94°C, 30 sec at 56°C, 45 sec at 72°C), CMV second round – 30 cycles (30 sec at 94°C, 30 sec at 56°C, 1 min at 72°C); HHV-6 first and second round - 40 cycles (1 min at 94°C, 1 min at 57°C, 1 min at 72°C).

By serial dilutions of viral DNA we determined that the sensitivity of nested PCR was not less than 200 copies/mL of sample. We also assessed our optimized PCR systems for specificity. We did not observe cross-reactivity after we tested each PCR system with HSV, VZV, EBV, CMV and HHV-6.

Although our studies showed that due to their higher sensitivity nested PCRs are appropriate systems for detection of EBV, CMV and HHV-6 in clinical samples, there is a risk of false-positive results because of cross-contamination among samples during preparation of the second round of PCR. Therefore, in order to minimize the risk, it is obligatory to apply special measures to avoid contamination.

Conclusions

The selected and optimized nested PCR assays are highly sensitive and specific and allow detection of EBV, CMV and HHV-6 in clinical specimens to study the potential role of these viruses in ME/CFS.

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