European Network on Myalgic Encephalomyelitis/Chronic Fatigue Syndrome
COST Action - CA15111

Deliverable 5

Guidelines for usage of infection-associated biomarkers in ME/CFS diagnosis for use in biomedical and clinical research

Introduction

Infection by various viruses, including the Epstein-Barr virus (EBV), the human herpes virus (HHV)-6 and the human parvovirus B19 are known as triggers of ME/CFS. In a small subset of patients, there is evidence of chronic active infection (persistent infection in active stage) by serology and direct detection of the viral genomic sequences in cell-free blood plasma by PCR. While EBV persistently infects B cells and HHV-6 and HHV-7 preferentially CD4+ T cells detection of low level of viral genomic sequences in DNA isolated from the whole blood or PBMCs is not a mark for active infection. The only reliable way to detect chronic active infection (persistent infection in active stage) is by showing either presence of viral genomic sequences in DNA isolated from the cell-free blood serum/plasma, viral load in serum/plasma or by detection of viral RNA. In studies from our group evidence for an active HHV-6, HHV-7 or B19 infection was found in a subset of patients (Chapenko et al, 2012, Loebel et al., 2017).
Here we provide protocols to assess viral DNA and RNA.

Protocols for detection of virus (EBV, HHV-6, HHV-7 and B19) infection associated biomarkers

DNA isolation

To extract DNA from 0.5 ml of peripheral blood, 1 ml of lysis buffer [1M Tris HCl (pH 7.6), 2M $MgCl_2$, 4M NaCl] was added, samples mixed and centrifuged at 10000 rpm for 3 min to precipitate the cells. Supernatant was removed, 1 ml of 3X distilled water was added and centrifuged as before. Then supernatant was repeatedly removed and for cell lysis 80 µl proteinase K buffer [NaCl – 5.85 g, 0.5M EDTA], 20 µl 20% sodium dodecyl sulphate (SDS) for

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leukocyte membrane disruption, as well as 15 µl proteinase K and 3X distilled water up to 0.5 ml was added. The sample was mixed and ~ 4 hours in 55°C temperature incubated.

DNA was extracted from peripheral blood by phenol-chloroform extraction method. To previously prepared sample, 0.5 ml phenol was added, for 6 min mixed and centrifuged at 10000 rpm for 10 min. Then 250 µl of phenol and 250 µl of chloroform were added to supernatant, mixed and centrifuged as previously. 0.5 ml of chloroform was added to supernatant, mixed and centrifuged again. To precipitate DNA 1 ml 96% ice-cold ethanol was added to the sample, centrifuged at 14000 rpm for 15 min in +4°C temperature and the supernatant removed. Then DNA was washed with 1 ml 70% ice-cold ethanol, centrifuged as before and the supernatant removed. DNA pellet was air-dried, dissolved in 3X distilled water and stored overnight at +4°C, and for a longer period at −80°C temperature.

Blood plasma samples were treated with Deoxyribonuclease I (DNase I), RNase-free reagent to remove possible impurities of cell DNA. DNA from 200 µl cell-free blood plasma was extracted using QIAamp DNA Blood Kit, (Qiagen GmbH, Germany) according to manufacturer's instruction and stored overnight at +4ºC, and for a longer period at −80°C temperature.

RNA isolation

RNA from frozen PBMCs was extracted with 500 µl Tri Reagent (Applied Biosystems, USA) for cell lysis. 200 µl of chloroform was added, mixed for 15 s, incubated for 15 min at room temperature and centrifuged at 12000 g for 15 min at +4°C temperature. The supernatant was transferred to a new tube, 250 µl isopropanol added, mixed, incubated for 10 min at room temperature and centrifuged at 12000 g for 8 min at +4°C temperature. RNA pellet was washed with 1 ml 75% ethanol, centrifuged at 7500 g for 5 min at +4°C temperature. Ethanol was removed and RNA air-dried for 3 – 5 min, dissolved in DEPC-treated water and stored at −80°C temperature.

The presence of RNA was analysed electrophoretically in 1% agarose gel with 10x NorthernMax-Gly Gel Prep/Running Buffer and visualized using UVP BioSpectrum MultiSpectral Imaging System (United Kingdom).

Nucleic acid quantity analysis

Concentration of extracted DNA and RNA was measured spectrophotometrically with "NanoDrop" spectrophotometer. According to manufacturer's instruction nucleic acid concentration was determined at 260 nm wavelength with program ND1000. The obtained concentration units were ng/µl – nanograms per microliter. Nucleic acid purity was assessed by ratio 260/280 that is around 1.8 for pure DNA and ~ 2 for pure RNA.

Complementary DNA synthesis

Complementary DNA (cDNA) was synthesized with reverse transcription (RT) using commercially available RevertAid First Strand cDNA Synthesis Kit (Thermo Scientific, USA). In a sterile tube 0.1 ng – 5 µg of total RNA, 1 µl oligo $(dT)_{18}$ primer and nuclease free water up to 12 µl was mixed, centrifuged, incubated at 65°C temperature for 5 min and chilled on ice. Following reagents were added to the sample:

- 4 µl 5× Reaction Buffer;
- 1 µl RiboLock RNase Inhibitor (20 u/µl);
- 2 µl 10 mM dNTP Mix;
- 1 µl RevertAid M-MuLV Reverse Transcriptase (200 u/µl).

The sample was mixed, centrifuged and incubated at 42°C for 60 min. Synthesis reaction was terminated at 70°C for 5 min. Aliquots were stored at −70°C temperature.

DNA and cDNA quality analysis

To assure the quality of cDNA and DNA from peripheral blood and exclude possible contamination of plasma DNA by cellular DNA, PCR was carried out to detect β-globin gene sequence according to Vandamme et al., 1995 with following primers: GS268 5'-ACACAACTGTGTTCACTAGC-3' and GS269 5'-TGGTCTCCTTAAACCTGTCTTG-3'.

Peripheral blood DNA and cDNA were considered as qualitative if 200 bp products were acquired by PCR. Negative β-globin PCR result in plasma DNA samples indicated on pure DNA (without cell DNA contamination), though obtaining 200 bp product from plasma DNA was considered invalid for virus-specific genome sequence analysis and repeated DNA extraction was carried out.

Components for 25 µl PCR amplification mix for one sample: 2.5 µl 10× Taq PCR buffer +KCl-$MgCl_2$; 2 µl 25 mM $MgCl_2$; 0.5 µl 10 mM dNTP; 0.1 µl 100 µM GS268 primer; 0.1 µl 100 µM GS269 primer; 0.1 µl 5U/µl Taq polymerase; 17.7 µl molecular biology grade water; 2 µl DNA sample (300 ng/reaction).

Amplification conditions: Initial denaturation – 3 min, 95°C; Amplification (40 cycles: DNA denaturation – 30 s, 95°C; Primer hybridisation – 30 s, 55°C; DNA synthesis – 45 s), 72°C; Final synthesis – 7 min, 72°C.

Virus genomic sequence detection by nested PCR

nPCR was used to detect virus or provirus genomic sequences in patients and apparently healthy individuals' DNA.

Cycle 1 components for 50 µl PCR amplification mix for one sample with corresponding primers to detect virus or provirus gene sequences were:

5 µl 10× Taq PCR buffer +KCl-$MgCl_2$;

3 µl 25 mM $MgCl_2$;

1 µl 10 mM dNTP;

0.2 µl 100 µM corresponding forward primer;

0.2 µl 100 µM corresponding reverse primer;

0.2 µl 5U/µl Taq polymerase;

DNA sample (To detect HHV-6: 1 µg; HHV-7: 1 µg; EBV: 1 µg; B19: 1 µg per reaction);

Add molecular biology grade water up to 50 µl.

Cycle 2 components for 25 µl PCR amplification mix for one sample:

2.5 µl 10× Taq PCR buffer +KCl-$MgCl_2$;

1.5 µl 25 mM $MgCl_2$;

0.5 µl 10 mM dNTP;

0.1 µl 100 µM corresponding forward primer;

0.1 µl 100 µM corresponding reverse primer;

0.1 µl 5U/µl Taq polymerase;

15.2 µl molecular biology grade water;

5 µl of cycle 1 amplification product.

HHV-6 genome sequence detection using nPCR

nPCR was used to amplify specific virus DNA sequence in DNA isolated from peripheral blood (a marker of persistent infection) and cell-free blood plasma (a marker of an active infection).

The detection of HHV-6 genomic sequence was performed in accordance with Secchiero et al., 1995. Used primers were complementary to the gene that encodes main capsid proteins for both HHV-6A and HHV-6B. HHV-6 genomic DNA (Advanced Biotechnologies Inc, Columbia, MD, USA) was used as a positive control. Sensitivity of HHV-6-specific primers was three copies per reaction (Tomsone et al., 2001; Kozireva et al., 2008).

Presence of HHV-6 U3 gene sequence was detected with following primers:

Cycle 1: HV1 forward – 5'-GCGTTTTCAGTGTGTAGTTCGGCAG-3'

HV2 reverse – 5'-TGGCCGCATTCGTACAGATACGGAGG-3'

Cycle 2: HV3 forward – 5'-GCTAGAACGTATTTGCTGCAGAACG-3'

HV4 reverse – 5'-ATCCGAAACAACTGTCTGACTGGCA-3'

Amplification conditions for both cycles: Initial denaturation – 3 min, 95°C; Amplification (30 cycles: Denaturation – 1 min, 94°C; Primer hybridisation – 1 min, 57°C; Synthesis – 1 min, 72°C); Final synthesis – 7 min, 72°C.

HHV-7 genome sequence detection using nPCR

Detection of HHV-7 specific genomic sequence in DNA isolated from peripheral blood is a marker of a persistent infection, whereas in cell-free blood plasma – a marker of an active infection.

HHV-7 genome sequence detection was done using primers according to Berneman et al., 1992. Primers were complementary to U10 gene (Pfeiffer et al., 1995). HHV-7 genomic DNA (Advanced Biotechnologies Inc., Columbia, MD, USA) was used as a positive control. Sensitivity of HHV-7-specific primers was one copy per reaction (Tomsone et al., 2001; Kozireva et al., 2008).

Presence of HHV-7 U10 gene sequence was detected with following primers:

Cycle 1: HV7 forward – 5'-TATCCCAGCTGTTTTCATATAGTAAC-3'

HV8 reverse – 5'-GCCTTGCGGTAGCACTAGATTTTTTG-3'

Cycle 2: HV10 forward – 5'-CAGAAATGATAGACAGATGTTGG-3'

HV11 reverse – 5'-TAGATTTTTTGAAAAAGATTTAATAAC-3'

Amplification conditions for the first and second cycles were: Initial denaturation – 4 min, 94°C; Amplification (30 cycles: Denaturation – 1 min, 94°C; Primer hybridisation – 2 min, 60°C for cycle 1 and 2 min, 55°C for cycle 2; Synthesis – 2 min, 72°C); Final synthesis – 7 min, 72°C.

EBV genome sequence detection using nPCR

Detection of EBV genomic sequence in DNA isolated from peripheral blood is marker of persistent infection and cell free blood plasma is a marker of active viral infection.

The detection of EBV DNA was performed using primers according to Landgren et al., 1994. Sensitivity of EBV-specific primers was two copies per reaction (Landgren et al., 1994).

Presence of EBV specific gene sequence was detected with following primers:

Cycle 1: W1 forward – 5'- CTAGGGGAGACCGAAGTGAA -3'

W2 reverse – 5'- CTGAAGGTGAACCGCTTACCA -3'

Cycle 2: W3 forward – 5'- GGTATCGGGCCAGAGGTAAGT -3'

W4 reverse – 5'- GCTGGACGAGGACCCTTCTAC -3'

With the first primer set, amplification was performed by 30 incubation cycles and with the second primer set – by 40 incubation cycles of thermal denaturation at 92°C for 15 seconds, primer annealing at 66°C for 10 seconds, and primer extension at 72°C for 15 seconds. Each extension time was increased by 1 second on each subsequent cycle. Final synthesis – 7 min, 72°C.

B19V genome sequence detection using nested PCR

Considering virus genomic sequence detection in DNA isolated from whole peripheral blood but not in DNA from cell free blood plasma, the virus genomic sequence is located in peripheral blood cells. The presence of B19V genomic sequence only in DNA from peripheral

blood cells indicated a persistent infection, whereas B19V genomic sequence detected also in DNA from cell free blood plasma is a marker for an active infection.

Presence of human parvovirus B19V genomic sequence was determined according to Barah et al., 2001, using primers complementary to NS1 gene. Viremic serum DNA (kindly provided by Prof. K. Hedman, Department of Virology, Heartman Institute, University of Helsinki) was used as a positive control. Sensitivity of B19-specific primers was 1 – 10 copies per reaction (Barah et al., 2001).

Presence of B19V NS1 gene sequence was detected with following primers:

Cycle 1: NS1 F-out – 5'-AATACACTGTGGTTTTATGGGCCG-3'

NS1 R-out – 5'-CCATTGCTGGTTATAACCACAGGT-3'

Cycle 2: NS1 F-in – 5'-GAAAACTTTCCATTTAATGATGTAG-3'

NS1 R-in – 5'-CTAAAATGGCTTTTGCAGCTTCTAC-3'

Amplification conditions for both cycles were: Initial denaturation – 6 min, 95°C; Amplification (40 cycles: Denaturation – 30 s, 95°C; Primer hybridisation – 30 s, 55°C; Synthesis – 30 s, 72°C); Final synthesis – 7 min, 72°C.

HHV-6A and HHV-6B determination by nPCR and HindIII restriction

HHV-6A and HHV-6B according to Lyall and Cubie, 1995 were differentiated. Amplification components for both cycles were prepared as previously described for nPCR. Presence of HHV-6 large tegument protein gene sequence was detected with following primers:

Cycle 1: O1 – 5'-AGTCATCACGATCGGCGTGCTATC-3'

O2 – 5'-TATCTAGCGCAATCGCTATGTCG-3'

Cycle 2: I3 – 5'-TCGACTCTCACCCTACTGAACGAG-3'

I4 – 5'-TGACTAGAGAGCGACAAATTGGAG-3'

Amplification conditions for both cycles were: Initial denaturation – 5 min, 95°C; Amplification (30 cycles: Denaturation – 1 min, 94°C; Primer hybridisation – 1 min, 60°C; Synthesis – 1 min, 72°C); Final synthesis – 10 min, 72°C.

Amplification products were analysed electrophoretically in 1.7% agarose gel as described in the section below "Electrophoretic analysis" to determine 163 bp amplification product. Obtained nPCR amplification products were digested with HindIII restriction

endonuclease (Thermo Scientific, USA) which cleaves HHV-6B 163 bp amplification product into 66 bp and 97 bp fragments, whereas does not cleave HHV-6A.

Detection of virus gene expression using PCR

PCR was used to amplify virus specific DNA sequences in cDNA samples, which were obtained from RNA that was extracted from PBMCs. Each reaction mix contained corresponding primers to detect HHV-6 U89/90, HHV-7 U57 or B19V NS1 gene sequences. Amplification components were prepared as previously described for nPCR second cycle 25 µl adding 2 µl of cDNA sample.

HHV-6 U89/90 immediate-early gene expression was detected according to Van den Bosch et al., 2001 using primers complementary to both HHV-6A and HHV-6B:

C1bis – 5'-GTTCCTGTTTCATGGCA-3'

C2bis – 5'-TCCAGTAATGTGGAAGAAGG-3'

HHV-6 U89/90 amplification conditions: Initial denaturation – 10 min, 95°C; Amplification (40 cycles: Denaturation – 20 s, 95°C; Primer hybridisation – 45 s, 50°C; Synthesis – 30 s, 72°C); Final synthesis – 5 min, 72°C.

HHV-7 U57 gene expression was detected according to Ito et al., 2013 using following primers:

U57F – 5'-CGGAAGTCACTGGAGTAATGACAA-3'

U57R – 5'-CCAATCCTTCCGAAACCGAT-3'

HHV-7 U57 amplification conditions: Initial denaturation – 5 min, 95°C; Amplification (40 cycles: Denaturation – 30 s, 95°C; Primer hybridisation – 1 min, 60°C; Synthesis – 1 min, 72ºC); Final synthesis – 5 min, 72°C.

B19V NS1 gene expression was detected in accordance with Ito et al., 2013 with following primers:

B19RT_F – 5'-GGGTTTCAAGCACAAGYAGTAAAAGA-3'

B19RT_R – 5'-CGGYAAACTTCCTTGAAAATG-3'

B19V NS1 amplification conditions: Initial denaturation – 5 min, 95°C; Amplification (40 cycles: Denaturation – 30 s, 95°C; Primer hybridisation – 1 min, 51.7°C; Synthesis – 1 min, 72°C); Final synthesis – 1 min, 72°C.

Electrophoretic analysis

Electrophoretic analysis was done to separate and identify by PCR amplified DNA fragments. Agarose gel contained 1.7 or 2.5 grams of agarose powder and 100 ml of 1× TAE buffer. 6× Loading buffer with GelRed (fluorescent nucleic acid dye) was mixed with DNA at a volume ratio of 1:5 and transferred to gel. Marker pUC19 DNA/MspI (HpaII) 23 and GeneRuler 1 kb DNA Ladder was used to estimate amplification product size. Results were visualised using UVP BioSpectrum MultiSpectral Imaging System (United Kingdom).

β-globin PCR amplification products were analysed in 1.7% agarose gel determining expected amplification product size – 200 bp.

HHV-6, HHV-7, EBV and B19V nPCR amplification products were analysed in 1.7% agarose gel for detection of following size amplification products: HHV-6 – 258 bp; HHV-7 – 124 bp; EBV – 192 bp and B19V – 103 bp.

In addition, HHV-6 U89/90, HHV-7 U57 and B19V NS1 amplification products were analysed in 2.5% agarose gel to detect amplification products with size: U89/90 – 115 bp; U57 – 147 bp; B19V – 190 bp.

Viral load detection with real-time PCR

HHV-6, HHV-7 and B19V load was estimated in DNA extracted from peripheral blood using real-time PCR according to manufacturer's instructions. HHV-6 load was determined with HHV-6 Real-TM Quant (Sacace Biotechnologies, Italy) and B19V – with Parvovirus B19 Real-TM Quant kit (Sacace Biotechnologies, Italy). HHV-7 load was detected using Human Herpes Virus 7 genomes genesig kit (Primerdesign, United Kingdom) and Realquality RQ-HHV-7 (AB Analitica Advanced Biomedicine, Italy).

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