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A new quantitative PCR for human parvovirus B19 genotypes

M. Toppinen[a,*], P. Norja[a], L.-M. Aaltonen[b], S. Wessberg[c], L. Hedman[a,d], M. Söderlund-Venermo[a], K. Hedman[a,d]

[a] University of Helsinki, Department of Virology, Haartmaninkatu 3, 00290 Helsinki, Finland
[b] Helsinki University Hospital, Department of Otorhinolaryngology-Head and Neck Surgery, Haartmaninkatu 4, 00290 Helsinki, Finland
[c] Finnish Red Cross Blood Service, Kivihaantie 7, 00310 Helsinki, Finland
[d] Helsinki University Hospital Laboratory Division, Haartmaninkatu 3, 00290 Helsinki, Finland

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Parvovirus B19 (B19V) is a minute ssDNA virus associated with a wide range of diseases from childhood erythema to fetal death. After primary infection, the viral genomes persist lifelong in solid tissues of most types. Quantification of the viral DNA is important in the timing of primary infection, assessment of tissue persistence and screening of blood donor plasma. In this study, we present a new PCR assay for detection and quantification as well as for differentiation of all three B19V genotypes.

A new B19V qPCR was designed to target a 154-bp region of the NS1 area. Serum, plasma and solid tissue samples were suitable for testing in the assay. The *WHO International Reference Panel for Parvovirus B19 Genotypes* was utilized to validate the assay for detection of different genotypes of B19V in clinical material. Each panel member yielded, by the new qPCR, a quantity similar to the one reported by National Institute for Biological Standards and Control (NIBSC). The qPCR was specific for B19V and amplified and quantified all three genotypes with detection sensitivities of ≤10 copies/reaction. The differentiation of B19V genotypes was performed by Sanger sequencing of the amplified products.

1. Introduction

Human parvovirus B19 (B19V) was discovered in 1975 (Cossart et al., 1975). The virus has an ssDNA genome of 5.6 kb (Cotmore and Tattersall, 1984; Deiss et al., 1990) and belongs to the family *Parvoviridae*, genus *Erythroparvovirus* (Cotmore et al., 2014). The genome codes for five proteins: two structural (VP1 and VP2) and three non-structural (NS1, 11 kDa and 7.5 kDa) (Ozawa et al., 1987; Ozawa and Young, 1987). All five are encoded from a single promoter (p6) (Doerig et al., 1987). Palindromic hairpin structures are located at both termini of which the 3′ end hairpin acts as a DNA synthesis primer (Astell and Blundell, 1989; Deiss et al., 1990).

In 1999 and 2002 two new B19V genotypes were found (Nguyen et al., 1999, 2002; Hokynar et al., 2002). Sequences identical to the prototype virus are called genotype 1. Illnesses presently occurring in western countries are mostly due to this genotype. Genotype 2 circulated in Northern Europe decades ago and disappeared from circulation by the 1970s (Norja et al., 2006; Manning et al., 2007; Kuethe et al., 2009). Genotype 3, encountered primarily in Ghana and Brazil as well as in India, has two subtypes: V9-like (genotype 3a) and D91.1-like (genotype 3b) (Servant et al., 2002; Candotti et al., 2004; Sanabani et al., 2006; Freitas et al., 2008; Jain et al., 2015). To date, only a few genotype 3 positive individuals have been reported in western countries aside from the discovery of this genotype in France (Nguyen et al., 1999; Servant et al., 2002; Rinckel et al., 2009). The three genotypes differ by 10% in the genomic coding region and 20% in the promoter region but are serologically indistinguishable (Hokynar et al., 2002; Blümel et al., 2005; Ekman et al., 2007).

The most common B19V manifestations are the childhood rash *erythema infectiosum*, also known as fifth or slapped cheek disease, and arthralgias (Anderson et al., 1984). Most B19V infections are mild or asymptomatic yet in immunosuppressed individuals and pregnant women B19V can cause chronic anemias and fetal death, respectively (Brown et al., 1984; Knott et al., 1984; Kurtzman et al., 1987). B19V transmission occurs via the respiratory route, blood products or vertically (Anderson et al., 1985; Siegl and Cassinotti, 1998; Schmidt et al., 2001; Enders et al., 2006). Whereas in early primary infection, the viral DNA load in blood can be very high (up to 1.00E+14 viral copies/ml), the DNAemia can remain detectable for months despite a specific antibody response (Musiani et al., 1995; Enders et al., 2006).

* Corresponding author. Tel.: +358 44 511 51 37.
E-mail address: mari.toppinen@helsinki.fi (M. Toppinen).

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Post-infection, B19V DNA persists in tissues lifelong, detectable in synovia, skin, tonsillar tissue, bone marrow, liver, heart, kidney, brain, muscle, thyroid and testicular tissue of both seropositive symptomatic and asymptomatic persons (Kurtzman et al., 1987; Söderlund et al., 1997; Gray et al., 1998; Tanawattanacharoen et al., 2000; Chevrel et al., 2000; Norja et al., 2006; Manning et al., 2007; Mori et al., 2007; Schenk et al., 2009; Bock et al., 2010; Adamson et al., 2014).

Traditional diagnosis of B19V infection relies on antibody detection. However, knowledge of the viral DNA load in blood assists in timing of the infection, especially in immunosuppressed individuals and pregnant women; therefore quantitative PCR, aside of serology, is increasingly recommended for B19V diagnosis (Liefeldt et al., 2005; Enders et al., 2006; Maple et al., 2014).

B19V is a concern in plasma product safety due to its resistance to most viral inactivation methods (Willkommen et al., 1999; Soucie et al., 2012). The US Food and Drug Administration (FDA) regulated in 2004 that B19V DNA levels in plasma pools for manufacturing plasma-derived products must not exceed 1.00E+04 International Units/ml (IU/ml). Thus, quantitative PCR (qPCR) amplifying all B19V genotypes is a mandatory security method.

While several qualitative PCRs for detection of B19V DNA have been introduced in the literature, far fewer quantitative PCRs have been published (Baylis et al., 2004; Candotti et al., 2004; Blümel et al., 2005; Lindblom et al., 2005; Liefeldt et al., 2005; Bonvicini et al., 2013; Maple et al., 2014). All detect and amplify the three B19V genotypes but only the modified *artus*® Parvo B19 LC PCR kit (Qiagen, Germany) can distinguish them, albeit genotype 3b at a 3-log reduced sensitivity (Hokynar et al., 2004). Liefeldt et al. (2005) published a B19V qPCR suitable for solid tissue samples. While that assay employs genotype-specific probes it cannot be used in multiplex format for genotype differentiation.

A new quantitative Pan-B19V PCR was developed for clinical and scientific purposes. This method was designed to amplify and quantify all three B19V genotypes equally, both in body fluids and solid tissues as well as to genotype by Sanger sequencing the amplified products.

2. Materials and methods

2.1. Plasmids

B19V genotype 1 and 2 plasmids have been previously cloned in our laboratory and the B19V genotype 3a and 3b plasmids were kind gifts from Professor Antoine Garbarg-Chenon. The four B19V genotype plasmids served as PCR positive controls. The concentrations of the plasmids were measured spectrophotometrically and copy numbers calculated according to the concentrations and weights of the plasmids. Ten-fold dilution series of the genotype 1 plasmid was used as quantification standard.

Serial dilutions of a plasmid containing the partial human RNase P gene, served as standards for determination of cell count via the single copy gene. The plasmid was a kind gift from Doctor Janet Butel (McNees et al., 2005).

All the plasmid dilution series were stored at −70 °C in 20–100 μl aliquots.

2.2. Specimens

Test specimens comprised serum, plasma and tissue. Panel 1 consisted of 40 samples of serum acquired from the Helsinki University Hospital Laboratory Division (HUSLAB). This included (i) 20 B19V IgM-positive, (ii) 8 IgM-negative but IgG-positive and (iii) 12 IgM- and IgG-negative sera representing acute and past infection, as well as seronegative individuals, respectively.

Panel 2 consisted of minipools ($n = 208$) of which each included plasma from 16 individual blood donations. Minipools were tested for B19V and hepatitis A virus (HAV) DNA at the Finnish Red Cross Blood Service (Procleix Parvo/HAV Assay, Gen-Probe and Novartis Diagnostics), as part of their routine virus screening. All minipools had B19V DNA values below the Finnish Red Cross Blood Service cut-off. One minipool had B19V DNA at 7.36E+02 IU/ml.

Panel 3 was the WHO International Reference Panel for Parvovirus B19 Genotypes (National Institute for Biological Standards and Control) (Baylis et al., 2012). It contains one B19V DNA-negative, and three B19V DNA-positive (genotype 1, 2 and 3a like viruses) plasma samples and therefore served as a control for each genotype. The positive samples contained B19V DNA at ~1.00E+06 IU/ml plasma (Baylis et al., 2012).

Panel 4 consisted of 212 tonsillar tissue samples and sera collected from the tonsillectomy patients. The tonsillectomies had been done due to chronic tonsillitis or tonsillar hypertrophy. The median age was 21.5 years (range 1 month to 57 years). The tonsillar samples were analyzed with Pan-B19V and RNase P qPCRs (McNees et al., 2005; Sadeghi et al., 2014). B19V IgG and IgM EIAs were performed of the sera, as described previously (Kaikkonen et al., 2001; Maple et al., 2014). Serum samples from IgM-positive individuals were also analyzed for B19V DNA by the Pan-B19V qPCR.

Panel 5 consisted of cultured cells of A549 human lung carcinoma cell line, obtained from HUSLAB.

The Ethics Committee of the Hospital District of Helsinki and Uusimaa approved the study.

2.3. Nucleic acid extraction

DNA from serum and plasma samples (Panels 1–4) was extracted with the DNA Blood Mini Kit (Qiagen, Germany) according to the manufacturer's instructions for viral DNA, without addition of carrier DNA. DNA from tonsillar tissues (Panel 4) was purified with the MagNA Pure 96 DNA and Viral NA Small Volume Kit (Roche, Switzerland) from 25 to 50 mg of tissue. DNA of A549 cells (Panel 5) was purified with the DNA Blood Mini Kit (Qiagen) according to the manufacturer's instructions for cell cultures.

2.4. Primers and hydrolysis probe

A comprehensive analysis of published B19V sequences included alignment of 61 near-full-length sequences (acquired from GenBank) with ClustalW2, examination of secondary structures (RNA fold WebServer), and search of conserved genome areas among the genotypes (BioEdit). According to the sequence analysis a primer pair (forward primer 5′CCACTATGAAAACTGGGCAATA3′, reverse primer 5′GCTGCTTTCACTGAGTTCTTCA3′) and a probe (5′[6FAM]-AATGCAGATGCCCTCCACCCAG-[BHQ1]3′) were designed to amplify and quantify a 154-bp target area of the NS1 gene of B19V. No degenerative nucleotides were included. The primers and the probe were subjected to a BLAST search to reveal any unspecific binding to other genomes.

2.5. Quantitative PCR for B19V

After optimization, the qPCR reaction consisted of 1x Fermentas Maxima qPCR Master mix (Thermo Scientific, Lithuania) with 0.03 μM of ROX passive reference dye, 0.4 μM each of the forward and reverse primers, 0.15 μM probe, 5 μl template, and nuclease-free water to a final volume of 25 μl. After an initial denaturation at 95 °C for 10 min, the qPCR cycles were 95 °C for 15 s, 60 °C for 15 s and 62 °C for 30 s, altogether 45 cycles. The adaptive fluorescence baseline was automatically calculated by the MxPro – Mx3005P v.