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Multiplex real-time PCR for the detection and quantification of latent and persistent viral genomes in cellular or plasma blood fractions

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ABSTRACT

In common with latent viruses such as herpesviruses, parvovirus B19, HBV and GBV-C are contained successfully by the immune response and persist in the host. When immune control breaks down, reactivation of both latent and persistent viruses occurs. Two multiplex assays were developed (B19, HBV, HHV-8), (EBV, CMV, VZV) for blood screening, and tested on blood donor samples from Ghana to determine baseline prevalence of viraemia in immunocompetent persons.

Single-virus real-time quantitative PCR (qPCR) assays were optimised for viral load determination of positive initial screening. The qPCR method utilised was absolute quantification with external standards. Multiplex and single-virus qPCR assays had similar sensitivity, except for the B19 assay in which sensitivity was 100-fold lower. Assays were optimised for reproducibility and repeatability, with R^2 of 0.9 being obtained for most assays. With the exception of B19 and CMV, assays had 100% detection limit ranging between 10^1 and 10^2 copies, IU or arbitrary units under single-virus and multiplex assay conditions.

The prevalence of viraemia was 1.6% HBV (0.8% DNA+/HBsAg−, 0.8% DNA+/HBsAg+), 0.8% parvovirus B19, and 3.3% GBV-C viraemia in the plasma fraction. The prevalence of four herpesviruses was 1.0% HHV-8, 0.85% CMV, and 8.3% EBV, and no detectable VZV viraemia.

1. Introduction

An increasing proportion of recipients of blood and blood components are immunocompromised either naturally or through chemotherapy in a variety of situations, from cancer to organ transplantation. Clinicians tend to attribute acute viral infections in transfused patients to transfusion despite unprecedented blood safety, but reactivation of common viruses has become an underestimated emerging issue (Allain and Compston, 2007). This is better known in the case of herpesviruses (EBV, CMV, HHV-8, VZV) that are able to maintain lifelong latency in a host until the balance of immune control is tipped by immunodeficiency, leading to reactivation and clinical manifestations. In addition, recent evidence indicated that, in common with the latent group, many common viruses once believed to be contained successfully, persist in the host in sanctuaries such as the liver (HAV, HBV), the bone marrow or the synovial tissue (B19) (Eis-Hubinger et al., 2001) and may leak into the circulatory system (Heegaard et al., 2002). This viral persistence is well controlled by immunocompetent persons, but in immunodeficient individuals these viruses may also reactivate becoming biologically detectable and clinically apparent (Allain and Compston, 2007; Besson et al., 2006; Chalandon et al., 2006; Cook et al., 2006; Savage and Waxman, 1997). It is possible that the reactivation profile of common latent and recovered persistent viral genome may reflect the overall immune status of the host. Reactivation has not been investigated in terms of overall profile of multiple common viruses.

In order to decrease the time and expense of traditional screening processes, multiplex PCR assays were used to enable simultaneous screening and individual virus identification. The testing strategy used was initial screening by triplex assays followed by confirmation and viral load quantification with single-virus assays. Assays were assessed for repeatability, reproducibility and sensitivity.

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The objective of the present study was to systematically screen and quantify a panel of common viruses that are able to either reactivate or cause a fluctuating persistent infection, with detectable viraemia becoming apparent as the immune defense wanes. The panel of viruses screened included latent (CMV, EBV, HHV-8, VZV) and persistent (HBV, parvovirus B19 and GBV-C) viruses in individuals assumed to be immunocompetent. GBV-C was selected to be included in the screening panel as a representative of flaviviruses with moderate background prevalence in sub-Saharan Africa (Sathar et al., 2000) and potential importance in the HIV infected and immunodeficient population (Lefrere et al., 1999). This study therefore focused on determining the background frequency and level of viraemia of each of the seven viruses in blood donors recruited at the Komfo Anokye Teaching Hospital in Kumasi, Ghana.

2. Materials and methods

2.1. Sample preparation

2.1.1. Blood donor screening

A total of 126 matched 1.5-ml EDTA cellular fraction (whole blood minus plasma) and plasma samples were screened with triplex 1 (B19/HBV/HHV-8), triplex 2 (EBV/CMV/VZV) and the single GBV-C real-time qPCR assays. The volunteer blood donors were recruited from Komfo Anoyke Teaching hospital, Kumasi, Ghana. Donors were informed of the study and signed an informed consent form according to the study protocol approved by the Addenbrooke's Hospital, Cambridge, UK and the University of Science and Technology School of Medical Sciences, Kumasi, Ghana ethics committees. Blood donor selection was carried out according to standard criteria as described by Owusu-Ofori et al. (2005).

2.1.2. Nucleic acid extraction

For the isolation of DNA or RNA standards and clinical samples, the commercially available high-pure viral nucleic acid kit (Roche Diagnostics, Lewes, England) was used according manufacturer's protocol modified by the addition of an 1-h lysis step at room temperature instead of the recommended 10 min.

2.1.3. Controls

A negative control (deionised water) was extracted alongside the viral nucleic acid samples to act as an indicator of potential cross-contamination between individual samples or within common extraction reagents.

2.2. Virus strains

For the standardisation of quantitative TaqMan qPCR, qRT-PCR and confirmatory nested PCR assays, NIBSC (National Institute of Biological Standards and Controls, Potters Bar, UK) calibrated DNA or RNA virus standards, in-house cloned plasmids or high-titre virus preparations from clinical samples were used in 10-fold serial dilutions for the production of the standard curves.

2.2.1. NIBSC standards

First international standard for parvovirus B19 DNA NAT assays 99/800, and WHO international standard for hepatitis B virus DNA for nucleic acid amplification technology (NAT) assays 97/746 were used.

2.2.2. Plasmid standards

Laboratory grown virus strains of EBV (in Raji Cells), HHV-8 (BC-3) were obtained (gift from Prof. Henri Agut, Hôpital Pitié/Salpétrière, Paris, France), and CMV isolated from a clinical sample. These samples were extracted using the Roche high-pure viral nucleic acid kit, amplified using the TaqMan assay primers only (Fig. 1). The amplicon was purified with the Qiagen QIAquick PCR purification protocol (Qiagen), and cloned using the Qiagen PCR cloning kit according to the manufacturers' instructions. Following transformation, the DH5α cells were sub-cultured overnight at 37 °C on LB ampicillin plates, individual colonies were subsequently picked onto a grid plate (LB ampicillin) and incubated under standard conditions for 5 h. Each colony (with a designated grid reference number) was screened directly in TaqMan PCR using Brilliant QPCR Core Reagent kit (Stratagene, La Jolla, CA, USA) to determine if the virus-specific amplicon had successfully been transformed. Successful transformants were sub-cultured in broth culture (LB broth + ampicillin) and incubated overnight at 37 °C. Plasmids for use in qPCR were isolated with the QIAprep spin mini kit (Qiagen) and resultant DNA was quantified by UV spectroscopy. Due to lack of availability of the VZV virus or VZV DNA template, an oligonucelotide encompassing the TaqMan assay target region was synthesised commercially (Thermo Electron Corporation, Germany) and cloned according to the standard procedure detailed above.

2.2.3. Clinical standards

A high titre clinical sample of GBV-C was serially diluted and used as a standard for quantification as described (Li et al., 2006). Due to the unknown initial viral load, a standard dilution was set-up and the dilution giving repeatable 100% limit of detection was designated as 10 AU (arbitrary unit).

2.3. PCR amplification

2.3.1. Single-virus real-time qPCR

All reactions were validated initially in single-virus PCR, each assay was optimised in terms of reagent concentrations and cycling conditions to provide the highest sensitivity, reproducibility, repeatability and the dynamic range for each assay was assessed to determine assays suitability for viral load quantification. The optimal concentrations of primers were evaluated by performing PCR's with the recommended conditions. However, if these concentrations gave suboptimal results, a primer concentration range of 50–900 nM was tested. Similarly, a $MgCl_2$ gradient was assessed at concentrations ranging between 2 and 8 mM. The real-time PCR (TaqMan) assays utilised for this study were those of previously published assays (Table 1) (Kimura et al., 1999; Visconti et al., 2004; Furuta et al., 2001; Candotti et al., 2004a,b; Weinberger et al., 2000; Pennington et al., 2002; Candotti and Allain, 2007; Li et al., 2006). All single-virus real-time PCR reactions were set up in a total volume of 50 μl containing 5 μl PCR buffer (10×), Rox reference dye, optimal reagent concentration, 10 μl of target DNA or cDNA; the total volume was adjusted to 50 μl with deionised water. The PCR reactions were standardised for standard cycling conditions with an initial denaturation step at 95 °C (10 min), followed by 40 cycles of denaturation 95 °C (1 min), and annealing at 60 °C (1 min) using Mx3000P and Mx4000 platforms (Stratagene). The qPCR method used for viral load quantification is that of non-competitive quantification using external standards.

2.3.2. Multiplex real-time PCR assays

Multiplex assays were optimised for initial screening purposes, in terms of the reagent concentrations required to allow similar detection sensitivity and dynamic range for each target virus.