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Quantitative PCR technique for detecting lymphocytic choriomeningitis virus in vivo

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Abstract

Quantitative PCR (QPCR, or real time PCR (rtPCR)) has emerged as a powerful virologic technique for measuring viral replication and viral loads in humans and animal models. We have developed a QPCR assay to accurately quantify lymphocytic choriomeningitis virus (LCMV) in infected mice. We first validated this assay using plasmid DNA and LCMV viral stocks. We then demonstrated that the LCMV QPCR assay can detect LCMV in serum and tissues of chronically infected mice (LCMV-clone 13), with greater sensitivity than conventional plaque assay. Subsequently, we demonstrated that the QPCR assay can detect LCMV in tissues of CD40L$^{-/-}$ mice during a low grade chronic infection with LCMV Armstrong. Finally, we improved the assay further such that it was approximate 1000-fold more sensitive than plaque assay for detection of the presence of LCMV in tissue.

Keywords: RNA virus; Chronic infection; Arenavirus; RT-PCR; rtPCR

1. Introduction

Quantitative PCR (QPCR, or real time PCR (rtPCR)) has emerged as a powerful virologic technique for measuring viral replication and viral loads in humans and animal models, in serum and in tissue samples, and both for culturable viruses and non-culturable viruses. The most well known virologic applications of this technique are the measurement of HCV and HIV viral loads in the serum of infected human patients, but QPCR has now been applied to many viral species.

We have developed a QPCR assay to accurately quantify lymphocytic choriomeningitis virus (LCMV) in infected mice. There were several impetuses for developing such an assay. (1) It is known that that the sensitivity of LCMV plaque assays is imperfect, as a bioassay (mouse intracranial injection) can detect infectious virus that is undetectable by plaque assay, and there are reports of immunodeficient mouse strains that appear to clear an acute LCMV$_{arm}$ infection by plaque assay, but viral recrudescence is observed at later time points (Bachmann et al., 2004; Thomsen et al., 1996). (2) It is presumed that isolation of LCMV from infected tissues is inefficient due to the labile nature of the viral particles, whereas total RNA extraction is generally highly efficient. (3) Some LCMV strains plaque much more poorly than others due to their minimal cytotoxicity to Vero cells. (4) The current LCMV plaque assay technique takes 5 days, whereas a QPCR assay can be done in less than 1 day. Therefore, given the prevalence of LCMV use in viral immunology and viral pathogenesis research (Buchmeier and Zajac, 1999; Wherry and Ahmed, 2004), a better technique for quantifying LCMV would be highly valuable. Herein we describe our efforts to develop a sensitive and robust QPCR assay for LCMV.

2. Methods

2.1. Mice

C57BL/6J (B6) mice and CD40L$^{-/-}$ B6 mice were purchased from the Jackson Laboratory. Five-week-old mice were used for LCMV$_{cl13}$ experiments. Six to seventeen weeks old mice were used for LCMV$_{arm}$ experiments. Animals were bred and maintained in an accredited facility at the La Jolla Insti-

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tute for Allergy and Immunology (San Diego, CA), and the studies reported in this study conform to the principles outlined by the Animal Welfare Act and the National Institutes of Health guidelines for the care and use of animals in biomedical research.

2.2. Viruses

Plaque-purified clones of the Armstrong strain of LCMV ($LCMV_{arm}$) were propagated in BHK-21 cells (ATCC, Manassas, VA) (Ahmed et al., 1984), and tested for biological activity *in vitro* and *in vivo* (Crotty et al., 2006; Graham et al., 2006; McCausland et al., 2007). A second passage stock of plaque-purified subclone SC3 ($LCMV_{arm\text{-}sc3}$), was used for all $LCMV_{arm}$ experiments. Both genome segments of $LCMV_{arm\text{-}sc3}$ were fully sequenced (excluding the short loop domain), and amino acid differences from the original 1988 $LCMV_{arm}$ sequence (Salvato et al., 1988, 1989; Salvato and Shimomaye, 1989) were as follows: L^{S108T}, L^{S311R}, L^{T1513K}, L^{L1676V}, GP^{D176N}, GP^{R177A}, GP^{A313E}. For acute infections, mice received 1×10^5 PFU $LCMV_{arm}$ in a volume of 0.5 ml (suspended in RPMI-1640) by two intraperitoneal inoculations (i.p.) of 250 μl each.

Plaque-purified LCMV-clone 13 ($LCMV_{cl13}$) clones were propagated in BHK-21 cells and tested for biological activity *in vitro* and *in vivo* (Crotty et al., 2006). A second passage stock of subclone SC9 ($LCMV_{cl13\text{-}sc9}$) was used for all $LCMV_{cl13}$ experiments shown. To establish chronic infections, 5-week-old mice received 2×10^6 PFU $LCMV_{cl13}$ by intravenous inoculation (i.v.) via retroorbital injection (0.2 ml). Both genome segments of $LCMV_{cl13\text{-}sc9}$ were fully sequenced (excluding the short loop domain) and mutations GP^{F260L} and L^{K1079Q} were confirmed (Matloubian et al., 1990, 1993). Additional changes in $LCMV_{cl13\text{-}sc9}$ from the original 1989 sequences were L^{S108T} L^{T1513K}, L^{H1665N}, L^{V177I}, GP^{R177A}, and GP^{A313E}. Note that L^{S108T}, L^{T1513K}, GP^{R177A}, and GP^{A313E} were also present in $LCMV_{arm\text{-}sc3}$.

2.3. Cell, tissue, and serum preparation

Organ samples were surgically removed and frozen at −80 °C, then weighed and homogenized using an Omni PCR Tissue Homogenizer (Omni). Normal tissue sample sizes were: spleen, 10 mg; liver, 50 mg; kidney, 50 mg; brain, 50 mg; lymph node, 5 mg. Tissue samples for plaque assays were homogenized in 1 ml of phosphate buffered saline (PBS, pH 7.4). The homogenizer was washed multiple times between each tissue sample. Tissue samples were prepared differently for QPCR (see below).

Single cell suspensions of spleen were prepared by standard gentle mechanical disruption through a 70 μm nylon mesh screen (BD Falcon) using a 3 ml syringe plunger (BD Biosciences), followed by removal of red blood cells with ACK Lysis Solution (Biosource).

Bone marrow cells were obtained by surgical removal of both femurs followed by flushing the shaft of each femur using a syringe containing 5 ml cold DMEM + 10% FCS.

2.4. LCMV plaque assay

Plaque forming units (PFU) of viral stocks, and serum and tissue samples from infected mice, were determined by 5 day plaque assay on VeroE6 cells (to titer $LCMV_{arm}$ virus stock) or Vero cells (to titer $LCMV_{cl13}$ stock, or serum and tissue samples from chronically infected mice) (Ahmed et al., 1984). Cells were plated the previous day such that they would be 75–90% confluent at time of infection. Monolayers were infected with virus for 60 min at 37 °C, and then covered with a 0.5% agarose (Sigma) and complete 1× Medium 199 (Life Technologies) semisolid overlay. Cells were incubated for 4.5 days at 37 °C, 5–8% CO_2. Then an overlay of 0.02% neutral red in 0.5% agarose and 1× complete Medium 199 was added for overnight staining, followed by plaque enumeration on a lightbox.

2.5. LCMV viral load quantitative PCR (QPCR)

2.5.1. Samples

To obtain serum samples, whole blood was collected from metafane ($LCMV_{cl13}$ infected mice) or isofluorane (healthy mice) sedated animals into 1.5 ml tubes by capillary tube rupture of the retroorbital sinus. Heparinized capillary tubes were used (Sigma). Blood samples were then centrifuged for 20 min at 12,000 × *g* and 4 °C to separate the serum. Tissue samples were obtained using sterilized surgical tools, and tools were wiped with 70% ethanol between each mouse to prevent viral cross-contamination, and samples from negative control (uninfected) mice were always obtained first. Normal tissue sample sizes were: spleen, 10 mg; liver, 50 mg; kidney, 50 mg; brain, 50 mg; lymph node, 5 mg. Tissue samples were immediately snap frozen on dry ice at the time of harvest. All serum and tissue samples were frozen at −80 °C until time for RNA extraction.

2.5.2. RNA isolation

RNA was isolated using the RNAqueous mini spin column based system (Ambion, Austin, TX), which allows for rapid RNA isolation for both RNA sparse and RNA rich samples (serum and tissue samples, respectively). For serum samples, RNA was isolated from 50 μl serum. For tissue samples, RNA was isolated from 5 to 50 mg tissue homogenized in the presence of lysis buffer (∼5 mm bore Tissuemiser, Fisher Scientific). Extreme care must be taken to avoid cross-contamination, particularly at this stage. Therefore, tissue homogenization was done in a laminar flow hood, and the homogenizer was washed once with PBS, followed by once with 10% bleach, and again with PBS. Note: standard washing of the homogenizer with ethanol and PBS between samples is insufficient and results in RNA cross-contamination. RNA was eluted from RNAaqueous spin columns in a volume of 20 μl and purified RNA was frozen at −80 °C until use.

2.5.3. Primers

Original NP primers (NP1 set, no longer used) = NP1-R, AAGCTGAAGGCCAAGATCAT; NP1-F, GAGGCTTTCT-