



Contents lists available at ScienceDirect

Journal of Virological Methods

journal homepage: www.elsevier.com/locate/jviromet



Protocol

Rapid and sequential quantitation of salivary gland-associated mouse cytomegalovirus in oral lavage

Yosuke Kamimura, Lewis L. Lanier*

Department of Microbiology and Immunology and the Cancer Research Institute, University of California, San Francisco, CA, USA

ABSTRACT

Article history:

Received 15 January 2014
Received in revised form 14 March 2014
Accepted 18 March 2014
Available online xxx

Keywords:

Cytomegalovirus
Protocols
PCR

Cytomegalovirus (CMV) establishes a persistent infection in the salivary glands and transmits to other hosts. Mouse cytomegalovirus (MCMV) is a well-characterized model for studying the mechanisms of host responses against CMV. The viral load in salivary glands has been measured traditionally because it has been considered to reflect the consequence of anti-virus responses by T cells and natural killer (NK) cells. However, the standard plaque assay is cumbersome and it is impossible to monitor sequentially the viral load in same host. Hence, the goal of this study was to develop a real-time quantitative PCR (qPCR)-based procedure to measure the viral load in oral lavage. This report demonstrates that the viral load in oral lavage correlates well with viral titers in the salivary glands. This method allows sequential quantitation of viral loads without sacrificing mice and provides a technique that will facilitate kinetic studies of anti-viral immunity mediated by the innate and adaptive immune systems.

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1. Introduction

Cytomegalovirus (CMV) is a double-stranded DNA virus and a member of β -herpesvirus family, which is widely disseminated in mammalian hosts. Mouse CMV (MCMV) shares many features in common with human CMV (HCMV) in terms of disease progression, dissemination, tropism, latency, and recurrence following immunosuppression, thus providing a useful model of the human disease. MCMV initially replicates in visceral organs, including

spleen, liver, kidney, and lung. Control of MCMV is mediated initially by Natural Killer (NK) cells, followed by the subsequent development of B and T cell immunity. In the mouse model of CMV infection, NK cells are critical to the early control of infection (Scalzo et al., 1992). In C57BL/6 mice, NK cells expressing the activating Ly49H receptor are responsible for control of MCMV replication (Brown et al., 2001; Daniels et al., 2001; Lee et al., 2001). The Ly49H receptor recognizes the m157 glycoprotein encoded by MCMV, which is displayed on the surface of infected cells within hours after infection (Arase et al., 2002; Smith et al., 2002). NK cells successfully limit the replication of MCMV during the first few days of infection, and together with T cells and B cells, completely eliminate virus from the blood, spleen, and liver within two weeks.

* Corresponding author. Tel.: +1 4155140829; fax: +1 4155028424.
E-mail address: lewis.lanier@ucsf.edu (L.L. Lanier).

However, MCMV accumulates in salivary glands and persists for weeks or months after primary infection (Henson and Strano, 1972; Jonjić et al., 1989; Andrews et al., 2010). The viral load in salivary glands is correlated inversely with the anti-MCMV responses by T cells and NK cells (Bukowski et al., 1984; Jonjić et al., 1989; Lucin et al., 1992; Orr et al., 2009, 2010). Therefore, it is important to know the viral load in salivary glands to evaluate the immunity against MCMV. Typically, the viral load of MCMV is quantified by plaque assays, in which salivary gland homogenates are dispensed onto a monolayer of mouse embryonic fibroblasts (MEFs) or a MCMV-permissive cell line, followed by visual counting of plaques after 7–10 days. However, this technique is time consuming and requires euthanizing the mice at each time point evaluated, thus not allowing the kinetic analysis of viral load in the same animal. In the mouse oral cavity, the orifice of submandibular duct (also known as Wharton's duct) is located sublingually on both sides of the tongue and is connected to the submandibular glands (Kuriki et al., 2011). The goal of the study was to test whether MCMV is detectable and quantifiable in oral lavage fluid collected from the mouse sublingual cavity where MCMV is excreted from the submandibular duct along with saliva secretion.

2. Material and methods

2.1. Mice

C57BL/6 mice and BALB/c mice were purchased from the National Cancer Institute (Frederick, MD) and bred at UCSF. Mice were maintained and used in accordance with guidelines of the Institutional Animal Care and Use Committee.

2.2. MCMV infection

Mice 8 weeks of age were infected by i.p. injection with 10^4 plaque-forming units (pfu) of Smith strain MCMV. The virus stock was prepared by homogenizing salivary glands harvested from BALB/c mice 6 weeks of age, which had been infected with 10^4 pfu of MCMV at 3 weeks of age.

2.3. Plaque assay

Salivary glands were collected from euthanized mice and snap-frozen on dry ice in 1 mL of DMEM with 2% (vol/vol) BSA and 5 mM HEPES. Samples were stored at -80°C until use, then were thawed and homogenized, plated on a monolayer of C57BL/6 MEFs in DMEM without FCS, and incubated for 2–8 h at 37°C . DMEM with 10% (vol/vol) FCS and 0.75% (wt/vol) carboxymethyl cellulose was

added and samples were incubated for 7–10 days. MEF monolayers were fixed with 4% (wt/vol) paraformaldehyde in PBS and plaques were visualized by staining with 0.1% (wt/vol) crystal violet.

2.4. Sample collection for qPCR

For oral lavage collection, the mouse sublingual cavity was washed by inserting the tip of a P20 Pipetman (Ranin, Oakland, CA) with 20 μL of sterile saline under anesthesia. After pipetting 10 times, 5–10 μL of lavage fluid was collected and 1 μL was used for qPCR analysis. To measure virus copy number in peripheral blood, 10 μL of peripheral blood was processed with a GenElute™ Blood Genomic DNA kit (Sigma-Aldrich, St. Louis, MO), eluted with 20 μL of sterile nuclease-free water, and 1 μL was used for qPCR. To measure virus copy number in salivary glands, homogenates of salivary glands were prepared as described above and 10 μL of homogenate was processed as described for peripheral blood.

2.5. MCMV quantitation by qPCR

SYBR green-based real-time qPCR was performed to measure viral load as previously described with a slight modification (Wheat et al., 2003). Primers used: MCMV-IE1 Forward: 5'-AGCCACCAACATTGACCACGCAC-3' and MCMV-IE1 Reverse: 5'-GCCCAACCAGGACACACAAC-3'. To establish a standard curve for quantitation of viral load a full-length MCMV-IE1 gene was amplified using primers; IE1-full Forward: 5'-TGTCGCCAACAAAGATCCTCG-3' and IE1-full Reverse: 5'-CCCTGCCTGCTGTTCTT-3' and the gene was cloned into the TOPO-PCR2.1 vector (Invitrogen™, Thermo Fisher Scientific, Waltham, MA).

The reaction mixture contained 1 μL of sample, primers (0.5 μM), and 10 μL of SYBR GreenERTM qPCR mix (Invitrogen™, Thermo Fisher Scientific, Waltham, MA), and was adjusted to 20 μL with nuclease-free water. The PCR was performed using a Chromo4 Real-Time PCR system (Bio-Rad, Hercules, CA) as follows: initial incubation at 95°C for 10 min, subsequent 40 cycles consisting of 95°C for 15 s and 60°C for 1 min.

2.6. Statistical analysis

Results were analyzed by using the Pearson test.

3. Results

Roy and colleagues initially reported MCMV quantitation by SYBR green-based qPCR (Wheat et al., 2003); however,

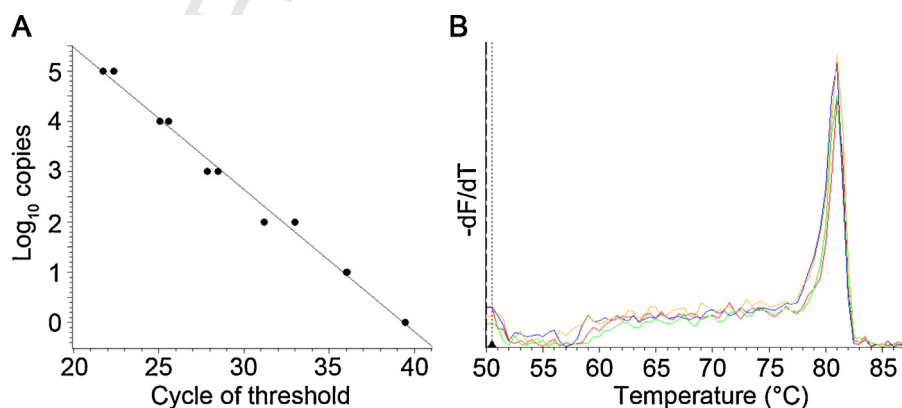


Fig. 1. Sensitivity and specificity of qPCR MCMV viral load assay. (A) Serially diluted MCMV-IE1 plasmids were amplified. Copy number of IE-1 amplicons was plotted against cycle of threshold. (B) Template DNA was prepared from MCMV-infected salivary glands ($n=4$). The melting curve was graphed by plotting $-dF/dT$ against temperature ($^\circ\text{C}$). Note that peak (T_m) for IE-1 amplicons was 81°C .

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