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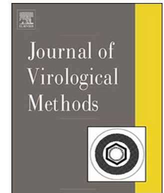

Differentiation of minute virus of mice and mouse parvovirus by high resolution melting curve analysis

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ABSTRACT

Murine parvovirus is one of the most prevalent infectious pathogens in mouse colonies. A specific primer pair targeting the *VP2* gene of minute virus of mice (MVM) and mouse parvovirus (MPV) was utilized for high resolution melting (HRM) analysis. The resulting melting curves could distinguish these two virus strains and there was no detectable amplification of the other mouse pathogens which included rat parvovirus (KRV), ectromelia virus (ECT), mouse adenovirus (MAD), mouse cytomegalovirus (MCMV), polyoma virus (Poly), *Helicobactor hepaticus* (*H. hepaticus) and Salmonella typhimurium (S. typhimurium)*. The detection limit of the standard was 10 copies/μL. This study showed that the PCR-HRM assay could be an alternative useful method with high specificity and sensitivity for differentiating murine parvovirus strains MVM and MPV.

1. Introduction

Minute virus of mice (MVM) and mouse parvovirus (MPV) are two murine parvovirus strains commonly found in experimental mice colonies (Jacoby and Lindsey, 1998).The first MVM strain was identified in 1966 and testing for the two serotypes is still required to screen rodent colonies (Joh et al., 2013). MPV is identified from cloned T cells and has a predilection for spleen, peripheral and mesenteric lymph nodes, and is serologically distinct from MVM (Ball-Goodrich and Johnson, 1994; Besselsen et al., 2006). MPV is grown well in murine cytotoxic T cells (CTLL-2). MVM prefers to grow in baby hamster kidney cells (BHK-21) (Besselsen et al., 1996). Antibody detection is the routine diagnostic assay for parvovirus infections in rodents because infections are often subclinical with no distinct pathology (Jacoby et al., 1996). However, serological assays cannot be used to screen immune-deficient mice, cell lines and biomaterials.

The polymerase chain reaction (PCR) is a common procedure used to test for the presence of viral nucleic acids and is independent of matrix type. Double PCR assay for detection of MVM and MPV infections in laboratory mice has been established (Wang et al., 2013) and real time PCR for MVM and MPV respectively (Boschetti et al., 2003; Redig and Besselsen, 2001; Zhan et al., 2002). DNA melting curve analysis using fluorescent detection is a successful new technology used to detect genetic variations in a PCR amplicon without sequencing (Erali and Wittwer, 2010). High resolution melting curve (HRM) analysis is a cost-effective alternative used in genotyping and other areas of genetic analysis (Ana Marek et al., 2010; Wittwer, 2009). PCR with HRM analysis has been effectively applied to detect and discriminate among animal pathogens. High-resolution melting analysis (HRMA) incorporating the fluorescent dye Eva Green has been used for differentiation nine bovine mastitis pathogens by detecting 16S rRNA gene (Ajitkumar et al., 2012). There are also PCR-HRM analysis for human brucellosis (Gopaul et al., 2014; Mohamed Zahidi et al., 2015) and five enterohepatic *Helicobacters* utilizing fluorescent dye LC Green (Wu et al., 2016). Differentiation of *Mycoplasma gallisepticum* strains based on vlhA gene utilizing PCR-HRM analysis has also been reported (Ghorashi et al., 2010). Analysis of PCR combined HRM is also applied in zoonotic virus such as bovine herpesvirus (Marin et al., 2016) and infectious bronchitis viruses (Hewson et al., 2010). More recently, HRM method has been reported as a diagnostic tool to identify *Pasteurellaceae* species for mice colonies but its application for the differentiation of murine parvovirus strains has not yet been reported (Miller et al., 2015).

The aim of this study was to establish a cost-effective closed-tube test method to differentiate MVM and MPV using PCR-HRM analysis.

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2. Materials and methods

2.1. Reference virus and samples

MVM reference strain (ATCC VR-1346) was obtained from the American Type Culture Collection (ATCC, Manassas, VA, USA) and cultivated in C6 cells according to ATCC standard conditions. A reference strain of MPV was stored in laboratory that confirmed by sequencing the VP2 gene and purified in CTLL-2 cells. Tissue and feces samples used in this study were previously submitted to our laboratory in Guangdong Laboratory Animals Monitoring Institute for routine animal health monitoring from different commercial laboratory animal providers in Guangzhou, China. These samples were stored at −80 °C upon the arrival to our laboratory. Samples which were mixture of liver and spleen were homogenized in phosphate-buffered saline (PBS) and centrifuged at 4000*g* for 5 min to clarify the supernatant. Total DNA was extracted from supernatant using viral DNA extraction kit (Tiangen, Beijing, China).The feces samples were resuspended with PBS, and then total DNA was extracted by feces DNA extraction kit (Tiangen, Beijing, China) according to the manufacturer's instructions.

2.2. Primers

The VP2 gene of MVM and MPV genomes were used as targeted genes as previously described (Bauer and Riley, 2006; Joh et al., 2013). A pair of degenerate primer M516F (GACCTYACWGCTTSCATGAT) and M516R (GTGWGGYTGACAAAATCCWACTT) amplified a 516 bp product were designed using Primer Premier 5.0 software. The primers located the VP2 gene ranged from 3304 to 3819 on the whole genome (GenBank accession no. M12032).

2.3. Establishing the PCR-HRM assay

PCR was carried out using Premix ExTaq Kit (Takara Bio, Dalian, China) according to the manufacturer's recommendations. The PCR reactions were performed in a 20 μL reaction volume containing 0.5 μM each primer, 1 × Premix ExTaq, 1 μL of LC Green (Idaho Technology, USA), about 100 ng template DNA for fecal extraction or 500 ng template DNA for tissue extraction. Reaction tube performed in PCR amplification (Biometra, German) following PCR cycling conditions: 95 °C for 3 min; 35 cycles at 94 °C for 30 s, 55 °C for 30 s, and 72 °C for 35 s; and a final extension at 72 °C for 5 min. PCR products were analyzed directly using a Rotor-gene Q (Qiagen, Germany) fluorescence quantitative PCR instrument that performed HRM analysis cooperating with instrument software. PCR products were gel purified using the Tiangen gel extraction kit (Tiangen, Beijing, China) according to the manufacturer's instructions and sequenced.

The temperature of melting curve analysis ranged from 80 to 90 °C at intervals of 0.3 °C with a hold of 2 s at each step. Normalized and first derivative plots were computed using instrument software v.2.0.2.1.4

2.4. Specificity and sensitivity of the PCR-HRM assay

Control DNA samples from seven pathogens were used to evaluate the specificity of the PCR-HRM assay. These included rat parvovirus (KRV), ectromelia virus (ECT), mouse adenovirus (MAD), mouse cytomegalovirus (MCMV), polyoma virus (Poly), *Helicobactor hepaticus* (*H. hepaticus) and Salmonella typhimurium (S. typhimurium)*. Reference strains of MVM and MPV were used as positive controls. Amplified products were analyzed by electrophoresis in 2% agarose gels. To assess the sensitivity of the method compared with conventional PCR, two recombinant plasmids constructed by cloning target sequence into pMD-19T Vector (Takara Biotechnology, Dalian, China) were purified and measured by spectrophotometer (NanoDrop, USA). Then both recombinant plasmids were quantified and serially diluted from 1×10^8 to 1×10^1 copies/μL.

2.5. Sample detection and simulation of co-infection

12 tissue DNA samples(mixture of liver and spleen from BALB/c mice)extracted by tissue DNA extraction kit (Tiangen China) were used to validate our PCR-HRM assay, including ten MVM and two MPV positive samples. While the DNA of 24 feces samples of BALB/c mice were extracted by feces DNA extraction kit (Tiangen China).

In order to imitate co-infection, a series of plasmids constructed by insertion of MVM and MPV DNA with pMD-19T (1×10^5 copies/μL) were mixed with the different proportion. The proportion of plasmids of MVM and MPV amplicons ranged from 1:9, 1:7, 1:5, 1:3, 1:1, 3:1, 5:1, 7:1 and 9:1.

3. Results

3.1. Establishing the PCR-HRM assay

The purpose of this study was to verify the utility of PCR-HRM analysis for differentiating MVM from MPV. We targeted a 516 bp region of the *VP2* gene and used these amplicons for HRM analysis. Seven negative control DNA samples were used to verify specificity and none of them was detected (Fig. 1A and B).

However, the MVM and MPV positive control DNA samples generated different melting curves. The MVM strain generated two peaks in the first derivative plots during the temperature ramp from 80 to 90 °C. The MPV strain exhibited only one in this interval (Fig. 1A and B). The PCR products from HRM analysis were checked by agarose gel electrophoresis and single electrophoresis bands were obtained from each sample. Both positive control samples had sizes of about 500 bp indicating that a single product was amplified (Fig. 2).

These data indicated that HRM analysis could differentiate MVM and MPV according to the specific melting temperature and shape of melting curves. The negative control samples including five mouse virus and two bacteria samples showed no distinct fluorescence (Fig. 1A) and no visible bands from gel analysis (Fig. 2). The primer pair only amplified fragments of MVM and MPV for the expected size, indicating that the primer sets were suitable for HRM analysis.

3.2. Sensitivity evaluation of the PCR-HRM assay

Sensitivity evalution was tested by using the PCR primer pair and analyzing a series of gradient dilutions of recombinant plasmid DNA. When template concentrations were ranged from 1×10^8 to 1×10^2 copies/μL, the resulting amplicons gave the correct melting curves although the fluorescent signals decreased. Interestingly, the fluorescence values of template with high concentration (1×10^8 copies/μL) were decreased compared with the lower copies(1×10^7 copies/μL) (Fig. 1C and D). When the templates of high concentration were not selected, a template with concentration of 10 copies/μL also showed appropriate melting curves (Fig. 1E and F).

3.3. Differentiating MVM and MPV strains targeting tissue samples by PCR-HRM

When utilizing 12 tissue samples to verify the assay, ten samples generated two peaks with values of 84.58 ± 0.13 °C (T_m1) and 86.72 ± 0.13 °C (T_m2) while two samples generated single peaks with average T_m values of 85.53 ± 0.19 °C that was a value located between the temperature range of the two MVM peaks (Fig. 3AB).

As 24 fecal samples of unknown status were screened by PCR-HRM, two samples generated specific two melting peaks curves with average values of 84.56 ± 0.05 °C (T_m1) and 86.64 ± 0.07 °C (T_m2) which were similar to reference strain of MVM and one sample generated specific one peak melting curves with values of 85.44 ± 0 °C similar to reference strain of MPV (Fig. 3CD).

The total 12 positive samples generated two peaks with the values

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