Chapter 8

Quantification of the Middle East Respiratory Syndrome-Coronavirus RNA in Tissues by Quantitative Real-Time RT-PCR

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Abstract

Since the emergence of the Middle East respiratory syndrome-coronavirus (MERS-CoV) in 2012, more than 2280 confirmed human infections and 800 associated deaths had been reported to the World Health Organization. MERS-CoV is a single-stranded RNA virus that belongs to the *Coronaviridae* family. MERS-CoV infection leads to a variety of clinical outcomes in humans ranging from asymptomatic and mild infection to severe acute lung injury and multi-organ failure and death. To study the pathogenesis of MERS-CoV infection and development of medical countermeasures (MCMs) for MERS, a number of genetically modified mouse models have been developed, including various versions of transgenic mice expressing the human DPP4 viral receptor. Tracking and quantifying viral infection, among others, in permissive hosts is a key endpoint for studying MERS pathogenesis and evaluating the efficacy of selected MCMs developed for MERS. In addition to quantifying infectious progeny virus which requires high-containment biosafety level (BSL)-3 laboratory, here we outlined an established real-time quantitative RT-PCR (RT-qPCR)-based procedure to unequivocally quantify MERS-CoV-specific RNAs within the lungs of infected human DPP4 (hDPP4, transgenic (hDPP4 Tg) mice under a standard BSL-2 laboratory.

Key words MERS-CoV, RT-qPCR, Animal models

1 Introduction

Middle East respiratory syndrome-coronavirus (MERS-CoV) is an emerging coronavirus that was first identified in Saudi Arabia in 2012 [1]. Since its emergence, MERS-CoV has infected more than 2280 individuals with over 800 deaths in 27 countries around the world, with the majority of the infections occurring in Saudi Arabia. MERS-CoV is classified as a lineage C betacoronavirus (Beta-CoV). Beta-CoV is one of four genera of the coronaviruses of the subfamily *Coronavirinae* in the family *Coronaviridae* [2]. In addition to MERS-CoV, the Beta-CoV genus also contains the SARS-CoV (lineage B), and other human coronaviruses such as OC43 and

HKU1 (lineage A) [2]. Coronaviruses are enveloped RNA viruses with a large positive-sense, single-stranded genome that ranges in size from 28 to 32 kbp and characterized by having crown-like projections on the virus particles [3]. The genome of the MERS-CoV is about 30.1 kbp long and contains 11 open reading frames (ORFs). Like other coronaviruses, the first two-thirds of the MERS-CoV genome contains two overlapping ORFs, ORF1a and ORF1b, that encode the viral replicase-transcriptase complex, the nonstructural proteins 1–16 (nsp1–16). The remaining one-third of the genome comprises ORFs that encodes the structural proteins, which include the spike (S), envelope (E), membrane (M), and nucleocapsid (N) proteins. In addition to these structural and nonstructural proteins shared by other coronaviruses, the MERS-CoV genome also contains several ORFs coding for accessory proteins, namely ORF3, ORF4a, and ORF4b [4].

Because of its high mortality rate and its potential to spread worldwide, it is important to study MERS-CoV pathogenesis in animal models. Mice are the most common and accessible laboratory animal species used for biomedical research in general. However, due to the disparity of two amino acids in the viral receptor—dipeptidyl peptidase 4 (DPP4)—from the human sequence that are critical for efficient binding to the receptor binding domain (RBD) of MERS-CoV, wild-type mice are not naturally permissive to MERS-CoV [5, 6]. We have recently developed a transgenic (Tg) mouse model, expressing the human DPP4, that is highly permissive to MERS-CoV infection and disease [7, 8]. The quantification of MERS-CoV RNA in specimens is important for studying MERS pathogenesis, evaluating the efficacy of selected MCMs and diagnostics. In this protocol, we outlined a stepwise RT-qPCR-based protocol to unequivocal quantify MERS-CoV-specific RNAs within the lungs of infected hDPP4 Tg mice.

2   Materials

2.1 General Materials

1. Titrated MERS-CoV (EMC-2012 strain).
2. Vero E6 cells (ATCC® CRL-1586).
3. Minimal Essential Media (MEM): Minimal Essential Media (MEM) supplemented with 2% heat inactivated FCS, 1% L-glutamine, and 1% penicillin/streptomycin, referred to as M-2.
4. 2.0 mL screw cap tubes.
5. Polypropylene microcentrifuge tubes.
6. RNAlater RNA stabilization solution.
7. Tissue Homogenizer.

Table 1
Primers and probes for RT-qPCR

Primers and probe for MERS-CoV upE (*see* **Note 1**)	
Primer forward (5′-3′)	GCAACGCGC GAT TCAGTT
Primer reverse (5′-3′)	GCCTCTACACGGGACCCATA
Probe (5′-3′)	FAM/CTCTTCACATAATCGCCCCGAGCTCG/TAMRA
Primers and probe for endogenous control (mouse β-actin)	
Primer forward (5′-3′)	CTGGATGGCTACGTACATGG
Primer reverse (5′-3′)	ACCTTCACAATGAGCTGCG
Probe (5′-3′)	FAM/TCTGGGTCATCTTTTCACGGTTGGC/TAMRA

2.2   RNA Extraction

1. TRIzol® reagent.
2. Stainless steel beads, 5 mm.
3. Chloroform.
4. Isopropanol.
5. Ethanol, 75%.
6. Refrigerated centrifuge and rotor capable of atleast $12,000 \times g$ speed.
7. RNA storage solution.
8. Method to quantify RNA concentration in specimens (SpectraMax i3 Multi-mode Microplate reader from Molecular Device or other machines).

2.3   Viral RNA Quantification

1. TaqMan® primers/probes set for upE gene of MERS-CoV and endogenous host control (Table 1).
2. Superscript III One-Step RT-PCR kit.
3. Optical 96-well PCR plate or PCR tubes.
4. RT-qPCR machine.

3   Method

Biosafety: MERS-CoV is a biosafety level 3 (BSL3) pathogen. Thus, all work involves infectious MERS-CoV should be handled in a BSL3 facility following the institutional guidelines and regulations such as wearing the proper PPE and having the proper training to work at BSL3 and animal BSL3 laboratories. Since RNA is very unstable, make sure to use RNAse-free tubes and reagents. Always make sure to clean all the pipettes and bench to using any preferred reagent to remove RNAse.

3.1 Infection (Intranasal Route)

1. Prepare a virus inoculum in M-2 media as a concentration of 1×10^3 $TCID_{50}$ in 50 µl (equals to 100 LD_{50} in our Tg mouse model) [8]. Keep the virus on ice. Mock-infected mice should be included as a control to calculate the relative expression of MERS-CoV RNA compared to control.

2. Anesthetize mice using isoflurane vaporizer in an induction chamber (other approved methods like injectable anesthesia can also be used).

3. Once the mice are completely asleep, hold vertically and slowly deliver the virus inoculum (50 µl) into their nostrils. Make sure the solution gets entirely into the nose and not swallowed through the mouth.

4. Place the mice back in the cage until the desired time point to measure virus titer in the lung.

3.2 Tissue Collection and Homogenization

All procedures of RNA tissue homogenization and extraction should be done in BSL3 laboratory.

1. At the desired time point, euthanize the mice using CO_2 or other approved methods. In our Tg mice, we detect the highest viral titer in the lung at day 2 and 3 post infection (*see* **Note 2**).

2. Using standard necropsy technique, collect a piece of lung tissues (one-quarter of a lung) immediately into a tube containing RNAlater solution. The volume of the RNAlater solution should be at least ten times the size of the tissue.

3. Store the tissue at 4 °C until homogenization.

4. After at least 24 h, weight tissues and transfer into a 2.0 mL screw cap tube containing 1 mL TRIzol reagent and two stainless steel beads. The sample size should not exceed 10% of the volume of TRIzol reagent used for homogenization.

5. Under the biosafety cabinet, homogenize tissue using automated tissue homogenizer ($2\times$ for 60 s at 25 strokes/s).

6. Spin down for 1 min at $5000 \times g$ to pellet tissue debris and collect supernatant that contains total RNA into fresh screw cap tubes.

7. Incubate at room temperature for 2–5 min after homogenization. You can proceed with total RNA isolation immediately or store the homogenized sample at -80 °C.

3.3 Total RNA Extraction

1. Add 0.2 mL chloroform to the tube for each 1 mL TRIzol reagent used for homogenization.

2. Shake vigorously by hand for 15–30 s and incubate at room temperature for 5 min.

3. Centrifuge at $12{,}000 \times g$ for 20 min at 4 °C.

4. Carefully remove the upper aqueous phase, which contains the total RNA, and place into a fresh microcentrifuge tube.

5. Add 0.5 mL of isopropanol to the tube containing total RNA and incubate at room temperature for 10 min (or at $-20\ ^{\circ}$C for 1 h).

6. Centrifuge at $12,000 \times g$ for 10 min at $4\ ^{\circ}$C.

7. Remove the supernatant leaving only the RNA that appears as a gel-like pellet on the side of the tube.

8. Wash the pellet with at least 1 mL of 75% ethanol per 1 mL TRIzol used.

9. Centrifuge at $12,000 \times g$ for 5 min at $4\ ^{\circ}$C and discard the supernatant.

10. Repeat the wash one more time (**steps 8** and **9**) to ensure complete removal of guanidine salt present in sample.

11. Remove supernatant and dry the pellet at room temperature for no more than 10 min (do not let the pellet dry completely).

12. Suspend the pellet in 150–200 μL of RNA storage solution.

13. Measure the concentration of RNA in a SpectraMax i3 Multimode Microplate reader or equivalent.

14. The expected total RNA yield from 1 mg lung tissue is 5–10 μg.

15. Keep RNA at $-80\ ^{\circ}$C until RT-qPCR analysis or on ice if proceeding immediately.

3.4 Quantitative Real-Time RT-PCR (RT-qPCR) and Calculation of Relative Copy Number of MERS-CoV RNA

RT-qPCR to quantify MERS-CoV RNA is performed in triplicate using the Superscript III One-Step RT-PCR kit (Invitrogen) with MERS-CoV-specific primers and probes (Table 1):

1. Set up a 25 μL one-step RT-qPCR reaction in an optical 96-well plate or on PCR tubes on ice: SuperScript III RT/Platinum Taq mix (1 μL), 2× reaction (12 μL), forward primer 10 μM (1 μL), reverse primer 10 μM (1 μL), fluorogenic probe 10 μM (1 μL), 1 μg of the total RNA ($\leq$5 μL), and up to 25 μL of RNAse/DNase-free water (*see* **Notes 3** and **4**).

2. Seal or cap the PCR reaction tube/plate and gently mix.

3. Centrifuge the plate or the tubes for 1 min at $500 \times g$ to settle down any droplets on the inner sides of the wells.

4. Place the PCR tubes/plate in a preheated real-time PCR machine and run RT-qPCR reaction using the conditions recommended by the manufacturer as shown in Table 2.

5. Calculate the relative copy number of MERS-CoV RNA normalized to the endogenous control (mouse β-actin) using the standard threshold cycle ($\Delta\Delta$Ct) as follows:

ΔCt 1 (MERS-CoV infected) = Ct MERS-COV upE $-$ Ct β-actin.

Table 2
RT-qPCR program conditions

Step	Temperature (°C)	Time	Cycles
Reverse transcription	50	30 min	1
Initial denaturing	95	2 min	1
Denaturation	95	15 s	40
Annealing/extension	60	30 s	

ΔCt 2 (Control (Mock-infected)) = Ct MERS-COV upE − Ct β-actin.

$\Delta\Delta$Ct = ΔCt 1 − ΔCt 2.

The relative expression of MERS-CoV upE RNA in the sample compared to control = 2- $\Delta\Delta$Ct (*see* **Note 5**).

3.5 Standard Curve to Quantify MERS-CoV RNA as TCID$_{50}$Eq/Gram of Tissue

To determine the amount of the viral RNA load as TCID$_{50}$ eq/gram of tissue, a standard curve need to be generated.

1. Collect lung tissues from uninfected hDPP4 Tg mice and place them in a vial containing 1 mL RNAlater solution before extracting total RNAs as described in Subheading 3.2. This sample will be spiked with viral inoculum to serve as a positive control for standard curve preparation.

2. Weigh and homogenize the collected lung tissue as described.

3. To make the positive control standards, divide the homogenized sample into five equal aliquots, each containing 1 mL of Trizol reagent (*see* **Note 6**).

4. Spike the aliquots with different dilutions of MERS-CoV starting from 10^{5} TCID$_{50}$/mL until 10^{1} TCID$_{50}$/mL (*see* **Note 6**).

5. Isolate spiked RNA from these samples as described in Subheading 3.3.

6. Use 1 μg of spiked RNA from each standard to perform one-step real-time RT-qPCR in triplicate using Superscript III One-Step RT-PCR kit as described in Subheading 3.4.

7. Determine the mean Ct value of each standard dilution, which corresponds to the viral titers.

8. Construct a 5-point standard curve by plotting the obtained mean Ct values against the titers of MERS-CoV used for spiking as TCID$_{50}$/mL (Fig. 1).

9. Calculate the TCID$_{50}$ eq/gram of tissue of your sample using the established standard curve.
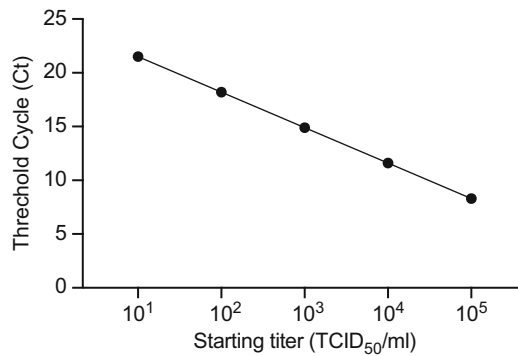
Fig. 1 Standard curve of the tenfold dilution (10^5 to 10^1) of MERS-CoV by real-time RT-qPCR: Lung tissue were isolated from uninfected hDPP4 Tg mouse, and homogenized. For RNA isolation, homogenized lung tissues sample were divided into five equal parts and spiked with different dilutions of $TCID_{50}$/mL of stock MERS-CoV in the range from 10^5 $TCID_{50}$/mL to 10^1 $TCID_{50}$/mL. Standard curve was generated by RT-qPCR technique using real-time PCR machine by loading uniform amount of RNA in triplicate. Total of 5 standards were constructed by plotting mean Ct values over titers of infectious MERS-CoV in the samples

4 Notes

1. Probes with different fluorescent dyes can be used based on the used instrument and the manufacturer's instructions.

2. We focus here on lung tissues because the lung is the target organ for MERS-CoV infection. However, viral RNA can be detected in other organs such as heart, brain, and spleen in low titers [8]. This protocol can be followed to detect viral RNA in these tissues as well.

3. In the protocol, we use one-step RT-qPCR without the need for separate reaction for cDNA synthesis, which is quick and simple. However, creating a cDNA first can be done and used for qPCR, especially if there is a need to stock cDNA to quantify other targets.

4. For multiple samples, prepare a master mix and add an appropriate volume to the plate wells, followed by adding the RNA template. Master mix preparation is crucial to reduce pipetting errors.

5. Alternatively, the viral RNA titer can be represented as TCID50 eq/gram of tissue as detailed in Subheading 3.5.

6. More aliquots could be prepared to make higher range standard curve by spiking with higher concentrations of MERS-CoV.

Acknowledgment

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