Chapter 34

Type A Influenza Virus Detection from Horses by Real-Time RT-PCR and Insulated Isothermal RT-PCR

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Abstract

Equine influenza (EI) is a highly contagious disease of horses caused by the equine influenza virus (EIV) H3N8 subtype. EI is the most important respiratory virus infection of horses and can disrupt major equestrian events and cause significant economic losses to the equine industry worldwide. Influenza H3N8 virus spreads rapidly in susceptible horses and can result in very high morbidity within 24–48 h after exposure to the virus. Therefore, rapid and accurate diagnosis of EI is critical for implementation of prevention and control measures to avoid the spread of EIV and to reduce the economic impact of the disease. The probe-based real-time reverse transcriptase polymerase chain reaction (rRT-PCR) assays targeting various EIV genes are reported to be highly sensitive and specific compared to the Directigen Flu A® test and virus isolation in embryonated hens’ eggs. Recently, a TaqMan® probe-based insulated isothermal RT-PCR (iiRT-PCR) assay for the detection of EIV H3N8 subtype has been described. These molecular based diagnostic assays provide a fast and reliable means of EIV detection and disease surveillance.

Key words Equine influenza, Equine influenza virus, Real-time RT-PCR, Insulated isothermal RT-PCR

1 Introduction

Equine influenza (EI) is an acute, highly contagious viral respiratory disease of equids (horses, donkeys, mules, and zebras) caused by infection with type A influenza virus [1]. Currently, equine H3N8 influenza virus continues to be the most important equine respiratory pathogen of horses in many countries around the world. Equine influenza is considered endemic in the USA, the UK, and many other European countries [2]. New Zealand and Iceland are the only countries that have remained continuously free of equine influenza. In 2005, interspecies transmission of H3N8 EI virus (EIV) from horse to dog was reported for the first time [3].

EIV possesses a segmented (eight segments), single-stranded RNA genome of negative sense. The first strain of EIV isolated in 1956 was of H7N7 configuration and designated influenza virus A/equine/Prague/1956 [4, 5]. The last confirmed outbreak
caused by an H7N7 subtype in horses was recorded in 1979 [4, 6]. Thus the H7N7 subtype is thought to be extinct or possibly still circulating at a very low level in nature [7–10]. A second EIV subtype, H3N8 was first isolated in 1963 and designated as influenza virus A/equine/Miami/1963 [6, 11]. This subtype has been associated with all confirmed outbreaks of equine influenza since 1980. Extensive antigenic drift has been detected in this virus over the years [12–18]. This led to categorization of H3N8 EIV isolates from around the world into two lineages: American and Eurasian [18–20].

In the past decade, nucleic acid amplification-based assays (standard reverse transcription PCR [RT-PCR] or real-time RT-PCR [RT-PCR]) were developed and evaluated by various groups [21–30]. However, such assays were not widely used for the routine diagnosis of this disease. This changed following the introduction of EI into Australia in 2007, when an rRT-PCR developed to detect the avian influenza virus matrix gene was used as the molecular diagnostic method of choice for EI [29–31]. In 2009, we developed a new panel of rRT-PCR assays capable of detecting a wide range of EIV strains comprising both subtypes of EIV [32]. The approach taken was to develop new rRT-PCR assays using a TaqMan® minor groove binding (MGB™) probe targeting the NP, M, H3, and H7 HA genes of the virus (see Table 1). The assays were developed using the subtype prototype strains of EI virus and then evaluated using archived strains of EIV and clinical specimens. These assays provided a fast and reliable means of virus detection and disease surveillance, and it would appear, the additional advantage of being able to identify antigenic shift between the two subtypes of EIV. We routinely use the NP-specific rRT-PCR assay for the detection of EIV clinical samples in our laboratory. Thus, the protocol for this assay is outlined in this chapter.

Recently, GeneReach (GeneReach USA, Lexington, MA, USA) has developed a TaqMan® probe-based insulated isothermal RT-PCR (iiRT-PCR) assay for the detection of EIV H3N8 subtype, and this assay has a very high specificity and equal or higher sensitivity as compared to the NP-specific rRT-PCR [32, 33]. The assay is based on iiRT-PCR for qualitative detection of influenza A virus subtype H3N8 in clinical samples. Fluorogenic probe hydrolysis chemistry is used to generate a fluorescent signal when a specific RNA sequence of influenza A virus subtype H3N8 is amplified. This assay is specially designed to be used on a compatible iiPCR instrument, POCKIT™ Nucleic Acid Analyzer (GeneReach USA, Lexington, MA) [34, 35]. The POCKIT Xpress™ Portable PCR Platform for on-site testing is suitable to be used in veterinary clinics, racetracks, breeding facilities, and diagnostic laboratories.
Table 1
Primer and probes for the detection of H3N8 and H7N7 EIV subtypes using RT-PCR assays [36]

<table>
<thead>
<tr>
<th>rRT-PCR assay name</th>
<th>Primer or probe*</th>
<th>Sequence 5'-3'</th>
<th>Nucleotide location (nt)</th>
<th>GenBank accession number</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>H3N8</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>EqFlu NP</td>
<td>EqFlu NP F</td>
<td>GAAGGGCGGCTGATTTCAGA</td>
<td>(157–175)</td>
<td>DQ124184</td>
</tr>
<tr>
<td></td>
<td>EqFlu NP R</td>
<td>TTCGTCGAATGGCCGAAAGTAC</td>
<td>(199–219)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>EqFlu NP Pr</td>
<td>CAGCATACAATAATAGAAAGGA</td>
<td>(177–196)</td>
<td></td>
</tr>
<tr>
<td>EqFlu M</td>
<td>EqFlu M F</td>
<td>ACCGGAGGTCAACGTAGTCTCA</td>
<td>(38–57)</td>
<td>DQ124188</td>
</tr>
<tr>
<td></td>
<td>EqFlu M R</td>
<td>CGCGAYCTCGGCCCTTCCAGT</td>
<td>(84–100)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>EqFlu M Pr</td>
<td>CTCTCTATCGTACCATCAGG</td>
<td>(59–78)</td>
<td></td>
</tr>
<tr>
<td>EqFlu HA3</td>
<td>EqFlu HA3 F</td>
<td>TCACTATGGACAGGTTCACTCA</td>
<td>(448–469)</td>
<td>L39914</td>
</tr>
<tr>
<td></td>
<td>EqFlu HA3 R</td>
<td>GGCCTATACCCCTTTTTTGA</td>
<td>(485–506)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>EqFlu HA3 Pr</td>
<td>AACCGGGAGGATGGAGGC</td>
<td>(471–487)</td>
<td></td>
</tr>
<tr>
<td>EqFlu HA3-Mia</td>
<td>EqFlu HA3-Mia F</td>
<td>GCAGTGCCTTACGCAATTGC</td>
<td>(346–365)</td>
<td>M29257</td>
</tr>
<tr>
<td></td>
<td>EqFlu HA3-Mia R</td>
<td>AGAGCCCGGAGCGATGCA</td>
<td>(389–406)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>EqFlu HA3-Mia Pr</td>
<td>CCATATGACGTCCTGACT</td>
<td>(369–387)</td>
<td></td>
</tr>
<tr>
<td><strong>H7N7</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>EqFlu NP-Pra</td>
<td>EqFlu NP-Pra F</td>
<td>GGCCTTCAAGGCCACCA</td>
<td>(48–65)</td>
<td>M63748</td>
</tr>
<tr>
<td></td>
<td>EqFlu NP-Pra R</td>
<td>TCTGCGGTCTTCACGCTTGT</td>
<td>(87–106)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>EqFlu NP-Pra Pr</td>
<td>GCACCTTATGAACAAATG</td>
<td>(67–84)</td>
<td></td>
</tr>
<tr>
<td>EqFlu M-Pra</td>
<td>EqFlu M-Pra F</td>
<td>CGCGCAGAGACTTGGAGATTG</td>
<td>(97–116)</td>
<td>CY005801</td>
</tr>
<tr>
<td></td>
<td>EqFlu M-Pra R</td>
<td>CATTCATAGGAGGCTCAAGATCT</td>
<td>(136–159)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>EqFlu M-Pra Pr</td>
<td>TTTGCAAGGAAAATAA</td>
<td>(119–134)</td>
<td></td>
</tr>
<tr>
<td>EqFlu HA7-Pra</td>
<td>EqFlu HA7-Pra F</td>
<td>CAATGGGAGAGACTACGC</td>
<td>(441–462)</td>
<td>X62552</td>
</tr>
<tr>
<td></td>
<td>EqFlu HA7-Pra R</td>
<td>AGAAGGCCATTTTCTCACTCT</td>
<td>(483–506)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>EqFlu HA7-Pra Pr</td>
<td>AAGGTCAAGATCTTC</td>
<td>(465–480)</td>
<td></td>
</tr>
<tr>
<td>EqFlu HA7</td>
<td>EqFlu HA7 F</td>
<td>TCCCTCTGTGACGTCAGAGA</td>
<td>(59–84)</td>
<td>X62556</td>
</tr>
<tr>
<td></td>
<td>EqFlu HA7 R</td>
<td>GGGGTTGCTTACTTGGTTC</td>
<td>(106–130)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>EqFlu HA7 Pr</td>
<td>CCTAGGACGGTACAGCT</td>
<td>(87–103)</td>
<td></td>
</tr>
</tbody>
</table>

*F forward primer, R reverse primer, Pr probe
*Reporter dye (FAM, 6-carboxyfluorescein) labeled nucleotide
*Non-fluorescent quencher dye (minor groove binding, MGB<sup>™</sup>) labeled nucleotide
*Reporter dye (VIC-) labeled nucleotide
*Reporter dye (NED-) labeled nucleotide
2 Materials

2.1 Materials for Real-Time RT-PCR

1. TaqMan® One-Step RT-PCR Master Mix Reagents Kit (Life Technologies, Grand Island, NY, USA) or equivalent.
2. Forward primer (EqFluNP: 5’-GAAGGGCGGCTGATT CAGA-3’).
3. Reverse primer (EqFluNPR: 5’-TTCGTCGAATGGCCGAAAG TAC-3’).
4. Probe (EqFluNPPr: 5’-6FAM-CAGCATAAATAGAAA GGA-MGBNFQ-3’).
5. Nuclease-free water.
6. Test RNA samples (see Note 1).
7. Positive control EIV H3N8 RNA.
8. Real-time RT-PCR instrument [e.g., ABI PRISM Applied Biosystems 7500 Fast Real-time PCR machine (Life Technologies, Grand Island, NY, USA)].
9. Reaction vials for the real-time PCR instrument [e.g., MicroAmp® Fast 8-Tube Strip, 0.1 ml and caps (Life Technologies, Grand Island, NY, USA), or MicroAmp® Fast Optical 96-well Reaction Plate, 0.1 ml and MicroAmp® Optical Adhesive Film (Life Technologies)].

2.2 Materials for iiRT-PCR

1. POCKIT™ Influenza H3N8 Detection Kit (GeneReach, Lexington, MA, USA) (see Note 2) (see Table 2). The kit consists of the following reagents for 48 tests and R-tube™, 48 tubes (see Note 3).
2. Nuclease-free water.
3. Test RNA samples (see Note 4).
4. POCKIT™ Nucleic Acid Analyzer (GeneReach, Lexington, MA, USA).
5. Microcentrifuge or cubee™ Mini-centrifuge (see Note 5).
6. Other materials: Single-channel and multichannel pipettes; RNase-free, aerosol-resistant tips (see Note 6); lab coat, gloves, and goggles (see Note 7); RNase decontamination solution (see Note 8).

3 Methods

3.1 Detection of EIV by Real-Time RT-PCR

1. The TaqMan® One-Step RT-PCR Master Mix Reagents Kit is used for the RT and PCR amplification of EIV RNA. Both RT and PCR reactions are done in a single tube, and the reaction proceeds without the addition of reagents between the RT and PCR steps.
Table 2
Components of the POCKIT™ influenza H3N8 detection kit (GeneReach, Lexington, MA, USA)

<table>
<thead>
<tr>
<th>Component</th>
<th>Contents or purpose</th>
<th>Amount</th>
</tr>
</thead>
<tbody>
<tr>
<td>Premix pack (see Note 13)</td>
<td>Influenza H3N8 premix (lyophilized pellet) containing dNTPs, primers, probe, and enzyme for amplification</td>
<td>Eight influenza H3N8 premix vials/bag (six bags/kit)</td>
</tr>
<tr>
<td>Premix buffer B (see Note 13)</td>
<td>Reaction buffer to redissolve the lyophilized pellet</td>
<td>Two vials (1.3 ml/vial)</td>
</tr>
<tr>
<td>Positive control (P) (see Note 14)</td>
<td>Dried plasmid containing influenza H3N8 partial sequence</td>
<td>One vial</td>
</tr>
<tr>
<td>Standard buffer</td>
<td>Reaction buffer to redissolve positive control standard</td>
<td>One vial (110 µl/vial)</td>
</tr>
</tbody>
</table>

Table 3
TaqMan® One-Step RT-PCR Master Mix (Life Technologies, Grand Island, NY, USA) for real-time RT-PCR amplification of equine influenza virus RNA

<table>
<thead>
<tr>
<th>Reagent</th>
<th>Stock concentration</th>
<th>Volume per Rxn (µl)</th>
<th>Final concentration</th>
</tr>
</thead>
<tbody>
<tr>
<td>TaqMan® Universal Master Mix</td>
<td>2x</td>
<td>12.5</td>
<td>1x</td>
</tr>
<tr>
<td>40× Multiscribe reverse transcriptase and RNase inhibitor mix</td>
<td>40x</td>
<td>0.625</td>
<td>1x</td>
</tr>
<tr>
<td>EqFluNPF primer</td>
<td>50 µM</td>
<td>0.45</td>
<td>900 nM</td>
</tr>
<tr>
<td>EqFluNPR primer</td>
<td>50 µM</td>
<td>0.45</td>
<td>900 nM</td>
</tr>
<tr>
<td>EqFluNPPr primer</td>
<td>10 µM</td>
<td>0.625</td>
<td>250 nM</td>
</tr>
<tr>
<td>Nuclease-free water</td>
<td></td>
<td>5.35</td>
<td></td>
</tr>
<tr>
<td>Master mix total volume (µl)</td>
<td></td>
<td>20</td>
<td></td>
</tr>
</tbody>
</table>

2. Prepare the master mix without RNA template as shown in Table 3.
3. Aliquot out 20 µl of master mix reaction into 96-well plate or reaction tubes (see Note 9).
4. Add 5 µl of sample RNA per well. One no-template amplification control (add 5 µl of nuclease-free water) is included per test plate. Similarly, one positive amplification control per test is included using 5 µl of EIV control RNA (see Note 10).
5. Seal the plate or tubes, and spin down the contents before placing the plate in the real-time PCR instrument.
6. The cycling conditions are as follows:
   (a) Probe dye setting: Reporter dye = FAM, quencher dye = MGB (non-fluorescent MGB dye).
(b) Thermal cycler conditions (Applied Biosystems 7500 Fast real-time PCR Machine, use standard mode): reverse transcription = 48 °C for 30 min, pre-denaturation = 95 °C for 10 min, then 40 cycles of denaturation = 95 °C for 15 s and 60 °C for 1 min.

7. Analysis of results: Results are analyzed using automatic threshold software function.

3.2 Detection of EIV by iRT-PCR

1. Label R-tube(s) with the sample identification number.
2. Prepare one premix for each sample by adding 50 µl of premix buffer B to each premix tube.
3. Add 5 µl test nucleic acid extract or positive control standard to each premix tube. Mix by pipetting up and down.
4. Transfer 50 µl premix/sample mixture into R-tube.
5. Seal the top of each R-tube with a cap. Make sure that R-tube is capped tightly. Spin tube briefly in cubee™ or in microcentrifuge to make sure that all solution is collected at the bottom of R-tube.
6. Turn on POCKIT™ Nucleic Acid Analyzer, which should complete self-testing within 5 min. Select "520 nm" (see Note 11).
7. When "System READY" is displayed, place the holder with R-tube(s) into the reaction chamber.
8. Tap the cap of each R-tube to make sure that the tube is positioned properly.
9. Close lid, and press "Run" to start reaction program.
10. Test results are shown on the monitor after reaction is completed.
11. Data interpretation: An example of results shown on the monitor is shown in Fig. 1 (see Note 12).

4 Notes

1. EIV RNA is extracted using MagMAX™-96 Viral RNA Isolation Kit as described in Chapter 33.
2. For more information on the POCKIT Xpress™ Portable PCR Platform (GeneReach USA, Lexington, MA, USA) see http://genereach-us.com.
3. Storage and stability of the POCKIT™ reagents:
   (a) The kit should be stored at 4 °C and is stable until the expiration date stated on the label.
   (b) Store premix vials in sealed premix pack to avoid hydration of lyophilized components.
Reconstituted positive control is stable for 6 months at 4 °C. Aliquot reconstituted positive control to avoid degradation of nucleic acid.

4. EIV RNA extracted using MagMAX™-96 Viral RNA Isolation Kit as described in Chapter 33 or PetNAD™ Nucleic Acid Co-prep Kit (GeneReach USA) is also recommended for nucleic acid extraction.

5. The cube™ Mini-centrifuge (cube™) is included in the POCKIT Xpress™ Portable PCR Platform for on-site testing.

6. Use RNase-free, aerosol-resistant tips during the RNA extraction procedures, and always use clean tips with stock reagent containers.

7. Gloves and protective gear: Wear laboratory gloves and other protective gear for protection from the reagents. Gloves will protect the RNA from the nucleases that are present on the skin.

8. Clean the bench or the hood and pipettes with an RNase decontamination solution.

9. Samples could also be tested by using primers and probes specific for HA and M genes of H3N8 or HA, M, and N genes of H7N7 (see Table 1) as described in Lu et al. [36]. The H3N8 subtype-specific primers and probes also could be used for the detection of canine influenza virus in clinical specimens [37].
10. EIV RNA copy number could be calculated using tenfold serial dilutions of in vitro-transcribed RNA (IVT RNA) as described in Lu et al. [36].

11. Please see the user manual of POCKIT™ for details.

12. Test freshly prepared RNA sample with EIV NP-specific TaqMan® real-time RT-PCR.

13. If the premix pellet is not found at the bottom of the tube, spin tube briefly to bring it down.

14. Before using for the first time, add 100 µl standard buffer to positive control. Store reconstituted positive control at 4 °C.

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References


