My favourite assay

Detection of novel influenza A(H1N1) virus by real-time RT-PCR

David M. Whileya, a, b, *, Seweryn Bialasiewicz a, b, Cheryl Bletchly c, Cassandra E. Faux a, b, Bruce Harrowerd, Allan R. Gould e, Stephen B. Lamberta, Michael D. Nissen a, b, c, Theo P. Sloots a, b, c

a Queensland Paediatric Infectious Diseases Laboratory, Sir Albert Sakzewski Virus Research Centre, Queensland Children’s Medical Research Institute, Children’s Health Service District, Queensland, Australia
b Clinical Medical Virology Centre, University of Queensland, Queensland, Australia
c Microbiology Division, Pathology Queensland Central, Royal Brisbane and Women’s Hospital Campus, Queensland, Australia
d Public Health Virology, Forensic and Scientific Services, Coopers Plains, Brisbane, Queensland, Australia
e Therapeutics and Molecular Mapping Unit, Sir Albert Sakzewski Virus Research Centre, Queensland Children’s Medical Research Institute, Children’s Health Service District, Queensland, Australia

ARTICLE INFO

Article history:
Received 20 May 2009
Accepted 22 May 2009

Keywords:
Influenza H1N1 Real-time PCR Detection

ABSTRACT

Accurate and rapid diagnosis of novel influenza A(H1N1) infection is critical for minimising further spread through timely implementation of antiviral treatment and other public health based measures. In this study we developed two TaqMan-based reverse transcription PCR (RT-PCR) methods for the detection of novel influenza A(H1N1) virus targeting the haemagglutinin and neuraminidase genes. The assays were validated using 152 clinical respiratory samples, including 61 Influenza A positive samples, collected in Queensland, Australia during the years 2008 to 2009 and a further 12 seasonal H1N1 and H3N2 influenza isolates collected from years 2000 to 2002. A wildtype swine H1N1 isolate was also tested. RNA from an influenza A(H1N1) virus isolate (Auckland, 2009) was used as a positive control. Overall, the results showed that the RT-PCR methods were suitable for sensitive and specific detection of novel influenza A(H1N1) RNA in human samples.

1. Introduction

The current outbreak of novel influenza A(H1N1) virus continues to expand globally. During outbreaks of emerging infectious diseases, accurate and rapid diagnosis is critical for minimising further spread through timely implementation of appropriate vaccines and antiviral treatment and prophylaxis where available, and other public health based, non-pharmaceutical measures. Real-time polymerase chain reaction (PCR) is more rapid and sensitive than traditional techniques including virus isolation by cell culture. A limitation of PCR methods is that false-negative results may occur due to sequence variation in primer and probe targets and is particularly relevant for the detection of emerging viruses. However the use of multiple targets can reduce such limitations, and may serve as a means of confirming positive results. For these reasons we have developed and validated two TaqMan-based reverse transcription PCR (RT-PCR) methods for the detection of novel influenza A(H1N1) virus.

2. Specimens

A total of 152 clinical respiratory samples (127 nasopharyngeal aspirates, 16 bronchial specimens and 9 swabs) collected in Queensland, Australia during the years 2008–2009 were retrospectively tested. These comprised 91 samples providing negative results and 61 samples providing positive results in a previously described Influenza A TaqMan assay targeting the Influenza A matrix gene. Two Influenza typing data were available for 29 of the 61 Influenza A positive samples and included seasonal H1N1 (n = 6) and H3N2 (n = 23).

Twelve clinical isolates collected from years 2000 to 2002 were also tested to further investigate assay specificity; seasonal H1N1 (n = 4) and H3N2 (n = 8) as well as a wildtype swine H1N1 isolate (A/SW/RAHABURI/00), provided by P. Selleck, Australian Animal Health Laboratory, Geelong, Victoria.

3. Selection of primers and probes

The primers and TaqMan probes used in the RT-PCR methods were designed using influenza A sequences available on the Genbank database (accessed 29 April 2009) and Primer Express 2.0 software (Applied Biosystems Pty Ltd., Australia). Primer Express 2.0 software was used to identify potential primer and probe
sequences, which were then subject to Genbank Blast searches to ascertain sequence specificity and conservation. Two TaqMan assays were designed, H1-PCR and N1-PCR, targeting the novel influenza A(H1N1) virus haemagglutinin and neuraminidase genes, respectively.

4. Protocol

Respiratory samples and controls were extracted using the Corbett X-tractor Gene (Corbett Robotics, Australia) according to manufacturer’s instructions. The H1-PCR and N1-PCR assays were performed using the Qiagen One-Step RT-PCR Kit (Qiagen, Australia) comprising 0.8 μM of forward and reverse primers (H1-F and H1-R for the H1-PCR; N1-F and N1-R for the N1-PCR; Table 1) and 0.2 μM of TaqMan probe (H1-TM for the H1-PCR; N1-TM for the N1-PCR; Table 1) in a total reaction volume of 25 μL, including 5 μL of nucleic acid extract. Amplification and detection were performed on Rotorgene 3000 and 6000 instruments (Qiagen, Australia) with the following conditions: initial holds at 50 °C for 20 min and 95 °C for 15 min followed by 45 cycles at 95 °C for 15 s and 60 °C for 1 min.

RNA from an influenza A(H1N1) virus isolate (Auckland, 2009), provided by the Australian World Health Organization Collaborating Centre for Reference and Research on Influenza (Melbourne, Australia), was used as a positive control. Appropriate negative controls were included in each test run.

5. Performance

All 152 clinical samples (2008/2009) and the 12 seasonal H1N1 and H3N2 isolates (2000–2002) provided negative results in both the H1-PCR and N1-PCR assays. This indicates the assays are specific and do not cross-react with seasonal H1 and H3 influenza A strains affecting humans. The influenza A(H1N1) virus (Auckland, 2009) control RNA produced positive results with the same cycle threshold (Ct) values (23 cycles) in both assays whereas the wild-type swine H1N1 isolate was positive in the N1-PCR but provided negative results in the H1-PCR assay. This shows that these assays may potentially cross-react with wild-type influenza A strains infecting swine, but this need not be of concern when testing influenza A infecting humans. Furthermore, it is unlikely that a wild-type swine H1N1 would produce positive results in both methods.

During the course of these validations a nose swab from a subject returning to Australia from the USA with suspected influenza A(H1N1) infection was submitted for diagnostic testing. The specimen provided positive results in both assays with similar Ct values (36 cycles in the H1-PCR and 35 cycles in the N1 PCR). This result was subsequently confirmed by the Australian WHO Collaborating Centre for Reference and Research on Influenza, Melbourne, to be the first confirmed case of novel influenza A(H1N1) identified in Australia.3

Overall, these results suggest these assays may be used successfully for the detection of novel influenza A(H1N1) RNA in human samples. Our findings will be validated in other settings as the current outbreak evolves.

Conflict of interest

The authors have no conflicting interests.

References


### Table 1

Oligonucleotides used in this study.

<table>
<thead>
<tr>
<th>Name</th>
<th>Sequence</th>
<th>Target</th>
<th>Position</th>
<th>Product size</th>
</tr>
</thead>
<tbody>
<tr>
<td>H1-F</td>
<td>GGTGGAGATATCCCCAAGACA</td>
<td>Haemagglutinin</td>
<td>389–411</td>
<td>75 bp</td>
</tr>
<tr>
<td>H1-R</td>
<td>GAGGACATGCGCTGATACA</td>
<td>Haemagglutinin</td>
<td>463–444</td>
<td>9 bp</td>
</tr>
<tr>
<td>H1-TM</td>
<td>FAM-TCATGGCCCAATCATGACTCGAACA-BHQ</td>
<td>Haemagglutinin</td>
<td>415–439</td>
<td>9 bp</td>
</tr>
<tr>
<td>N1-F</td>
<td>CAGAGGGCGACCCAAAGAGA</td>
<td>Neuraminidase</td>
<td>1281–1300</td>
<td>93 bp</td>
</tr>
<tr>
<td>N1-R</td>
<td>GGCCAAGACCAACCCACA</td>
<td>Neuraminidase</td>
<td>1373–1356</td>
<td>9 bp</td>
</tr>
<tr>
<td>N1-TM</td>
<td>FAM-CACAATCTGGACTAGCGGGAGCAGCAT-BHQ</td>
<td>Neuraminidase</td>
<td>1302–1328</td>
<td>9 bp</td>
</tr>
</tbody>
</table>

* Genbank accession number GQ117040.
* Genbank accession number GQ117036.