# Comparison of a singleplex real-time RT-PCR assay and multiplex respiratory viral panel assay for detection of influenza ''A'' in respiratory specimens

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**Background** Evaluation of different molecular tests for the detection of pandemic (H1N1) 2009 virus is important before the next wave of the pandemic.

**Objectives** To compare a hydrolysis probe-based real-time RT-PCR assay recommended by the CDC to the xTAG® respiratory viral panel (RVP) (Luminex® Molecular Diagnostics) for the detection of influenza A.

**Methods** Eleven thousand eight hundred and ninety-eight respiratory specimens were tested by the real-time RT-PCR and RVP assays for the detection of influenza A. The distribution of seasonal H1, H3 and pandemic H1N1 subtypes in these specimens was compared.

**Results** The RVP assay was generally unable to identify influenza A–positive samples with a low viral load, whereas the real-time RT-PCR assay detected most of these samples resulting in a subset of specimens that could not be confirmed as either seasonal or pandemic influenza A subtypes.

**Conclusions** When the prevalence of influenza A is high, the CDC recommended real-time RT-PCR has significant advantages as a frontline assay, namely higher sensitivity and shorter time to reporting a result. Anticipated scenarios would be during the peaks of the pandemic and episodes of seasonal influenza. Furthermore, the better sensitivity of the RT-PCR makes it the preferred assay to detect influenza in patients with severe respiratory disease tested late in their clinical course. If pandemic (H1N1) 2009 virus is not the dominant virus and there is a high proportion of other respiratory viruses circulating, laboratories will be faced with the decision to use the RVP assay for the detection of pandemic (H1N1) 2009 virus.

**Keywords** Influenza, pandemic (H1N1) 2009 virus, real-time RT-PCR, respiratory viral panel, swine.

## Background

Nucleic acid tests using a variety of amplification and detection technologies are available to diagnostic laboratories for the screening of pandemic (H1N1) 2009 virus.[1–8] Earlier studies have discussed the effectiveness of the xTAG® respiratory virus panel (RVP) (Luminex Molecular Diagnostics, Inc., Toronto, ON, Canada) and real-time reverse transcriptase-polymerase chain reaction (rtRT-PCR) assays using probes as tools for the diagnosis of the pandemic strain.[3,9] The RVP assay utilizes a suspension microarray for the detection of a panel of respiratory viruses.[10] This kit is approved by the United States Food and Drug Administration (USFDA) and facilitates the detection of an array of viruses in a cost-effective manner with a reasonable turn-around-time.[11,12] In addition to the RVP, an rtRT-PCR assay for influenza A developed by the Centres for Disease Control (CDC) using a hydrolysis probe was used in this study.[13] Probe-based assays provide rapid and sensitive results and are widely used for viral diagnosis; however, these assays are largely used in a singleplex format. Depending on resources, some laboratories may be using either one or both methods in routine diagnostics. It is thus important to understand and compare test characteristics for diagnosis and typing of influenza A subtypes.

## Objective

To compare and contrast the ability of the RVP and rtRT-PCR assays for the detection of pandemic (H1N1) 2009 virus during the first wave of the 2009 influenza pandemic.

## Study design

### Samples tested for seasonal influenza and pandemic (H1N1) 2009 virus

The respiratory virus testing data were derived from the Data Integration for Alberta Laboratories (DIAL) application[14], a web-based, user-specific secure platform that has automatic data extraction and interpretation processes for respiratory virus testing data at the Provincial Laboratory for Public Health (ProvLab). A total of 19 159 samples were submitted to the ProvLab between April 24 and August 31, 2009, for respiratory virus testing. A subset of 11 898 samples (62·1%) were included in this analysis. The criterion for selection was that the samples be tested both by the xTAG® RVP assay (Luminex Molecular Diagnostics, Inc.) and by an rtRT-PCR assay developed by the CDC targeting the (M) gene (referred to as InfA in the original publication)[13], we will use the term CDC-M in this report. The RVP assay detects influenza A viruses and can identify seasonal H1 and H3 subtypes specifically. [10,15] According to the assay recommendation by the manufacturer, samples are reported as positive when the median fluorescence intensity (MFI) value is greater than 300, equivocal when the MFI is between 150–300 and negative when the MFI is less than 150. The CDC-M assay only detects influenza A viruses without providing additional subtyping information. Specimens that gave a positive result for influenza A but were not subtyped by the RVP assay were subjected to rtRT-PCR assays for typing seasonal H1 and H3 subtypes as described previously.[13] All influenza A–positive samples for which a valid subtype by RVP and the real-time subtyping methods were not obtained were subjected to conventional RT-PCR (cRT-PCR) assays for amplification and sequencing as described in the section for confirmation of pandemic (H1N1) 2009 virus from April 24 to June 5, 2009. An in-house-developed and validated rtRT-PCR assay targeting the hemagglutinin (HA) gene referred to as the HA assay was used for typing of pandemic (H1N1) 2009 virus[14] after June 5, 2009. Nucleic acid was extracted from respiratory samples using the easyMAG® automated extractor (bioMérieux, Durham, NC, USA) as previously published.[16]

### Confirmation of pandemic (H1N1) 2009 virus

The HA assay was designed to target the 5′ end of the HA gene using a hydrolysis probe for typing of pandemic (H1N1) 2009 virus. Amplification and detection were performed as described.[16] Conventional RT-PCR was performed for partial amplification and sequencing of the HA and M genes. Amplification and sequencing were performed as previously described.[2,16] Sequences with >99% nucleotide identity to database submissions were confirmed as pandemic (H1N1) 2009 virus.

### Data analysis

A true positive was defined as a specimen positive for influenza A by two or more independent assays, and a true negative was defined as a specimen positive for influenza A by only one assay or negative by all assays. The crossing threshold (Ct) values of the CDC-M assay for different subgroups were compared using the Kruskal–Wallis test. McNemar analysis was used to compare the performance of the CDC-M and RVP assays. Statistical analysis was performed using PASW Statistics 17·0.

## Results

### Specimen types tested by the RVP and CDC-M assays

A total of 11 898 specimens were included in this study, majority of the specimens tested (84·7%, $n = 10\,080$) were from the upper respiratory tract including nasal or nasopharyngeal swabs/aspirate and throat swabs. The lower respiratory tract specimens included bronchial and tracheal samples, pleural fluid (5·7%, $n = 676$) and lung tissue (0·2%, $n = 20$). There were 1121 (9·4%) swabs and fluid aspirates where the specimen source was not specified and one blood sample was included.

### Samples with concordant results by the RVP and CDC-M assays

Of the 11 898 samples tested by the CDC-M and RVP assays, 11 576 (97·3%) samples tested negative and 220 (1·8%) tested positive for influenza A by both assays. The Ct values by the CDC-M assay for 220 positive samples ranged from 13·5 to 39·3 with a median value of 26·2. Of the 220 samples, 19 (8·6%) were typed as seasonal H1, 47 (21·4%) as seasonal H3 and 154 (70·0%) samples could not be subtyped by the RVP assay (Figure 1). Of the 154 samples that could not be typed by the RVP assay, the rtRT-PCR typing assays successfully subtyped 14 samples (9·1%) as seasonal H3 of which eight samples had an equivocal result for influenza A subtype H3 by RVP. Majority of the remaining samples (89·6%, $n = 138$) were subtyped as pandemic (H1N1) 2009 virus as shown in Figure 1. One sample was not subtyped using the rtRT-PCR typing assays because another sample from the same patient had been typed and reported as pandemic (H1N1) 2009. Another sample that did not provide a subtype by the rtRT-PCR assays was serotyped at the National Microbiology Laboratory (NML, Winnipeg, Manitoba, Canada) as pandemic (H1N1) 2009 virus.
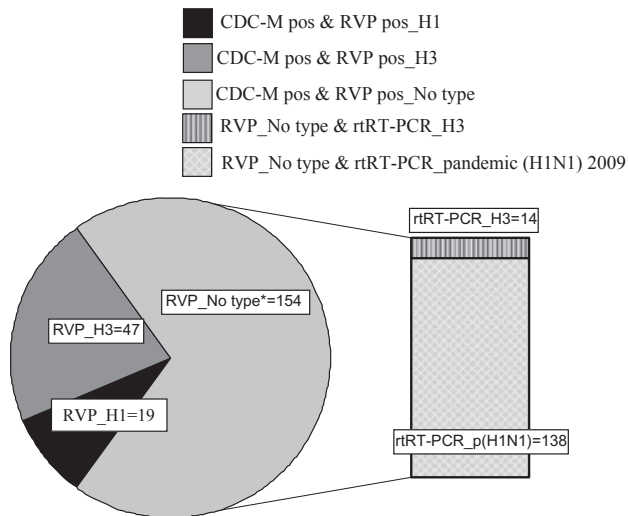
Figure 1. Typing of samples positive for influenza A by respiratory viral panel (RVP) and CDC-M assays. The pie chart shows a distribution of influenza A subtypes as detected by the RVP assay, subtyping of samples that could not be typed by the RVP assay is provided by the bar graph. *One sample was not typed; another sample from the same patient was typed as pandemic (H1N1) 2009 virus. One sample was subtyped by sero-typing.

**Table 1.** Typing of samples discordant for influenza A by the CDC-M and RVP assays

| Influenza A subtype | CDC-M pos & RVP neg | CDC-M pos & RVP equivocal | CDC-M neg & RVP pos | CDC-M neg & RVP equivocal |
|---|---|---|---|---|
| H1 | 1 | 0 | 0 | 0 |
| H3 | 6 | 5 | 0 | 0 |
| Pandemic (H1N1) 2009 | 8 | 43 | 2 | 2 |
| NoType | 17 | 4 | 0 | 0 |
| Total | 32 | 52 | 2 | 2 |

Distribution of influenza A subtypes as detected by the rtRT-PCR subtyping assays in specimens that were discordant for Influenza A by the CDC-M and RVP assays.
RVP, respiratory viral panel.

## Samples with discordant results by the RVP and CDC-M assays

Eighty-four (0·7%) samples tested positive for influenza A by the CDC-M assay and equivocal ($n = 52$, 0·4%) or negative ($n = 32$, 0·3%) by the RVP assay. Of these 84 samples, 63 were successfully subtyped, which included 48 of the 52 (92·3%) equivocal RVP positives, and 15 of the 32 (46·9%) RVP-negative samples (Table 1). A total of 62 samples were subtyped by rtRT-PCR assays and included one seasonal H1, 11 seasonal H3 and 50 pandemic (H1N1) 2009 virus subtypes; one sample was subtyped as pandemic (H1N1) 2009 virus at the NML. The median Ct values of the 48 equivocal RVP, 15 RVP-negative samples that were successfully subtyped and the 19 negative samples (only positive by CDC-M) that could not be subtyped were 35·6 (29·7–38·2), 35·8 (30·8–38·0) and 36·5 (31·6–39·0), respectively ($P < 0·05$, Kruskal–Wallis test).

Sixteen (0·1%) samples tested negative for influenza A by the CDC-M assay and equivocal by the RVP assay. Only two of these 16 samples (12·5%) were successfully subtyped as pandemic (H1N1) 2009. Two samples tested negative by the CDC-M assay and positive by the RVP assay and both were subtyped as pandemic (H1N1) 2009.

## Sensitivity and specificity analysis

Using the definitions of true positive and negative, the number of samples classified as true positive and negative for influenza A was 287 and 11 611, respectively. The sensi-

tivity and the specificity of the CDC-M assay were 98·6% (CI: 96·5–99·6%) and 99·8% (CI: 99·7–99·9%), respectively. Considering the samples that gave an equivocal result by the RVP assay as negative, the sensitivity and specificity of the assay were 77·4% (CI: 72·1–82·1%) and 100% (no false positive was detected), respectively. Considering the samples that gave an equivocal result by the RVP assay as positive, the sensitivity and specificity of the assay were 94·8% (CI: 91·5–97·0%) and 99·8% (99·8–99·9%), respectively. These results are shown in Table 2. When the samples with an equivocal result by the RVP assay were classified as negative or excluded from the comparison with CDC-M assay, there was a significant difference between the two assays ($P < 0·05$, McNemar analysis); however, when these samples were considered as positive, the $P$ value changed to 0·065 indicating no significant difference between the assays.

## Discussion

The CDC-M and RVP assays utilized here provide unique benefits for the detection of influenza A. In an acute care setting, the higher sensitivity of CDC-M assay and shorter time to a reportable result have a significant impact for the management and treatment of severe respiratory disease cases caused by influenza A and for epidemiologic classification. Furthermore, the CDC-M assay requires less hands-on-time, which is important for dealing with large volumes of samples, especially during a pandemic wave, when the dominant circulating virus is influenza A.

A dilemma caused by the CDC-M assay is the higher sensitivity when compared to the typing assays, thus generating a subset of specimens that could not be confirmed as either seasonal or pandemic influenza subtypes. From a laboratory perspective, effort to resolve all or some of these

Table 2. Two-by-two table used for the calculation of sensitivity and specificity of the CDC-M and RVP assays

|  | True positive* (*n* = 287) | True negative* (*n* = 11611) | Sensitivity (%CI) | Specificity (%CI) |
|---|---|---|---|---|
| CDC-M-Pos | 283 | 21 | 98·6% (96·5–99·6) | 99·8% (99·7–99·9) |
| CDC-M-Neg | 4 | 11590 | | |
| RVP-Pos** | 222 | 0 | 77·4% (72·1–82·1) | 100% |
| RVP-Neg** | 65 | 11611 | | |
| RVP-Pos*** | 272 | 18 | 94·8% (91·5–97·0) | 99·8% (99·8–99·9) |
| RVP-Neg*** | 15 | 11593 | | |

RVP, respiratory viral panel.
*A true positive was defined as a specimen positive for influenza A by two or more independent assays, and a true negative was defined as a specimen positive for influenza A by only one assay or negative by all assays.
**Classify equivocal RVP samples as negative for RVP.
***Classify equivocal RVP samples as positive for RVP.

samples has to be weighed considering patient management and the significance in surveillance, as the resources required are significant.

The primary advantage of the RVP assay is that it can simultaneously detect other respiratory agents that also present as an influenza-like illness. This information is valuable when agents such as parainfluenza virus and respiratory syncytial virus can have adverse outcomes in immunocompromised patients in an acute care setting and for establishing an infectious etiology in suspected viral respiratory outbreaks. Surveillance programs that rely on collecting data for the complete spectrum of co-circulating viral agents in the community will benefit from this assay.

The difference in assay sensitivity based on whether equivocal samples are considered positive or negative warrants attention. In our algorithm, we have re-tested equivocal samples by target-specific confirmatory assays and found in this study that the CDC-M assay was more sensitive when the RVP assay was used as a first screen. Thus, it is important to examine equivocal samples with other tests if possible during validation to conclude whether these samples should be considered as ''positive'' or ''negative''. The decision to use one or both assays in a stepwise algorithm will depend upon the local circumstances, as each has its own benefits.[3,9,10,16] This study provides data on the performance of two commonly used assays in a pandemic influenza setting and their relative merits as a first or second-line assay.

## Acknowledgements

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