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Short communication

Multiplex PCR tests sentinel the appearance of pandemic influenza viruses including H1N1 swine influenza

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Abstract

Background: Since the turn of the century seven new respiratory viruses have infected man and two of these have resulted in worldwide epidemics. Both SARS Coronavirus which quickly spread to 29 countries in February 2003 and H1N1 swine influenza that recently spread from Mexico to 30 countries in three weeks represent major pandemic threats for mankind. Diagnostic assays are required to detect novel influenza strains with pandemic potential.

Objective: In this report we evaluate the ability of a multiplex PCR test (xTAG RVP) to detect new, "non-seasonal" influenza viruses including the H1N1 swine influenza A/swine/California/04/2009.

Study design: Laboratory based study using retrospective and prospective specimens.

Results: This multiplex PCR test detected the present of non-seasonal (non-H1, non-H3) influenza in 20 of 20 patients infected with H1N1 swine flu virus. In addition to detecting the current swine flu the xTAG RVP test detected the H5N1 A/Vietnam/1203/2004 high pathogenicity avian influenza virus that circulated in South East Asia in 2003 as well as 17 out of 17 influenza A viruses representing 11 HA subtypes isolated from birds, swine and horses not yet seen in the human population.

Conclusion: Based on these results we believe that this molecular test can perform an important role as a sentinel test to detect novel non-seasonal influenza A viruses in patients presenting with influenza-like illness (ILI) and therefore act as an early warning system for the detection of future pandemic influenza threats.

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1. Background

The current outbreak of swine influenza that originated in Mexico in March 2009 has spread to 39 countries in one month causing 8,480 cases globally with 66 deaths as of May 17. The 2009 swine flu virus designated H1N1 A/swine/California/04/2009 is not zoonotic swine flu and is not transmitted from pigs to humans, but rather from person to person. In humans, H1N1 swine flu presents as an influenza-like illness (ILI) with symptoms similar to those of seasonal influenza viz. namely chills, fever, sore throat, muscle pains, severe headache, coughing, weakness and general discomfort. Since these symptoms are not specific to swine flu, early in the pandemic physicians were advised to consider swine influenza in the differential diagnosis of patients with acute febrile respiratory illness who had returned from Mexico or been in contact with persons with confirmed swine flu however, this epidemiological link will require modification. This new strain of H1N1 swine influenza appears to be a result of reassortment of two swine influenza viruses, one from North America and one from Europe with the North American virus itself the product of previous reassortments, carrying an avian PB2 gene for at least 10 years and a human PB1 gene since 1993. The hemagglutinin (HA) gene is similar to that of swine flu viruses present in United States pigs since 1999, while neuraminidase (NA) and matrix (M) genes resemble viruses present in European pigs. Viruses with this genetic makeup have not previously been found in humans or pigs, although there...
is no formal national surveillance system to identify what viruses are circulating in pigs in North America. The genetic makeup of this virus is germane to the design of molecular tests for diagnosis.

Multiplex PCR testing for the detection of respiratory viruses has seen major advances over the past decade resulting in the development of several commercially available tests. These tests can amplify one or more genes from a number of respiratory viruses and detect amplified products using microgene arrays. One test, the xTAG™ RVP test, was developed to detect 20 different virus types and subtypes in a single test using multiplex RT-PCR and detection with a microfluidic array on the Luminex 100 instrument. The xTAG™ RVP test was developed in 2005 immediately following the development of several commercially available tests. These tests can effectively detect the presence of non-seasonal (non-H1, non-H3) viruses and determine the H1 or H3 subtype. Since all seasonal influenza viruses in man over the past 20 years (prior to H5N1 in 2003 and swine influenza in 2009) have been either H1 or H3, the RVP test can detect the presence of non-seasonal influenza A virus by virtue of a positive matrix gene signal and negative H1 and H3 signals, a combination of results that flag a “new” influenza subtype and potential pandemic threat.

2. Objective

The objective of the study was to demonstrate that the use of multiplex PCR tests such as the xTAG™ RVP test that use a combination of matrix and hemagglutinin gene targets can detect novel non-seasonal strains of influenza.

3. Study design

The study was a laboratory based study using specimens from newly diagnosed H1N1 swine flu human cases and avian, swine and equine isolates of influenza A.

### Table 1

| Patient | Specimen | Source | Matrix | H1 | H3 | Result |
|---------|----------|--------|--------|----|----|--------|
| 1       | NP       | Toronto | 8214   | 173| 50 | Flu A No subtype |
| 2       | NP       | Toronto | 5544   | 60 | 42 | Flu A No subtype |
| 3       | NP       | Toronto | 6691   | 105| 34 | Flu A No subtype |
| 4       | NP       | Toronto | 7754   | 115| 44 | Flu A No subtype |
| 5       | NP       | Toronto | 8335   | 54 | 20 | Flu A No subtype |
| 6       | NP       | Toronto | 510    | 23 | 66 | Flu A No subtype |
| 7       | NP       | Toronto | 2157   | 49 | 39 | Flu A No subtype |
| 8       | NP       | Toronto | 8425   | 34 | 30 | Flu A No subtype |
| 9       | NP       | Toronto | 9104   | 118| 2  | Flu A No subtype |
| 10      | NP       | Halifax | 9231   | 83 | 48 | Flu A No subtype |
| 11      | NP       | Halifax | 8986   | 82 | 54 | Flu A No subtype |
| 12      | NP       | Halifax | 552    | 78 | 55 | Flu A No subtype |
| 13      | NP       | Hamilton| 7510   | 60 | 55 | Flu A No subtype |
| 14      | NP       | Hamilton| 6503   | 75 | 67 | Flu A No subtype |
| 15      | NP       | Hamilton| 6019   | 35 | 32 | Flu A No subtype |
| 16      | NP       | Hamilton| 7975   | 81 | 50 | Flu A No subtype |
| 17      | NP       | Vancouver| 1556  | 33 | 24 | Flu A No subtype |
| 18      | NP       | Vancouver| 6273  | 48 | 26 | Flu A No subtype |
| 19      | NP       | Vancouver| 3767  | 36 | 37 | Flu A No subtype |
| 20      | NP       | Vancouver| 1919  | 21 | 12 | Flu A No subtype |

A: NP specimens were collected from patients who recently returned from Mexico or who were in contact with travelers to Mexico and presented with ILI in Toronto, Halifax, Hamilton, and Vancouver. The xTAG™ RVP was performed according to the manufacturer’s instructions and the cutoff for positivity was 300 MFI.

4. Results

The introduction of H1N1 swine flu this year provided a real life challenge for the RVP assay. The RVP test results for 20 confirmed cases of H1N1 swine influenza in four Canadian cities in three provinces (Nova Scotia, Ontario and British Columbia) are shown in Table 1. All 20 individuals who presented with ILI, had either recently returned from Mexico or had an epidemiologic link to travelers to Mexico, and were confirmed as positive for H1N1 A/Swine/California/04/2009 by either the National Microbiology Laboratory in Winnipeg, the Ontario Agency for Health Promotion in Toronto, or the BCCDC in Vancouver using a combination of real time matrix gene PCR, real time PCR targeting the A/Swine/California/04/2009 HA gene or by sequencing the HA gene. Matrix gene signals for all 20 patients were positive (mean signal

### Table 2

| Source | Subtype | Strain               | Matrix | H1 | H3 | Result       |
|--------|---------|----------------------|--------|----|----|--------------|
| Human  | H1N1    | A/New Caledonia/20/99| 8,345  | 8,716| 64 | 32           |
| Human  | H3N2    | A/Brisbane/10/2007  | 4,443  | 88  | 1,566| 20           |
| Human  | H3N2    | VR4788               | 9,849  | 72  | 3,255| 48           |
| Human  | Flu B   | B/Yamagata/16/88    | 27     | 42  | 35  | 22           |
| Avian  | H2N2    | G01-30214            | 6,982  | 49  | 62  | 38           |
| Avian  | H4N6    | A/DK/Czech/56       | 9,490  | 57  | 108 | 46           |
| Avian  | H5N1    | A/Mallard/Vietnam/133/2004 | 1,739 | 47  | 31  | 787          |
| Avian  | H5N1    | A/Vietnam/1203/2004 | 1,544  | 34  | 20  | 17           |
| Avian  | H6N5    | A/Swine/Canada/82   | 9,722  | 62  | 70  | 68           |
| Avian  | H7N3    | A/Chicken/British Columbia/04 | 1,972 | 37  | 26  | 9            |
| Horse  | H7N7    | A/Prague/56         | 10,333 | 37  | 45  | 38           |
| Horse  | H7N7    | A/Equine/Cambridge/1/63 | 3,056 | 25  | 28  | 16           |
| Avian  | H8N4    | A/Turkey/Canada/68  | 9,697  | 98  | 58  | 48           |
| Avian  | H9N2    | A/Turkey/Wisconsin/66| 2,104  | 36  | 33  | 24           |
| Avian  | H10N8   | A/Ukraine/Italy/65  | 9,815  | 49  | 53  | 52           |
| Avian  | H14N5   | A/Mallard/263/82    | 9,901  | 54  | 62  | 41           |
| Avian  | H15N8   | A/Duck/Aus/314/83   | 9,948  | 72  | 57  | 17           |
| Swine  | untyped | OVC-07-10901        | 8,577  | 60  | 34  | 34           |
| Swine  | untyped | OVC-06-28600        | 8,148  | 51  | 49  | 42           |
| Swine  | untyped | OVC-04-23866        | 6,607  | 102 | 41  | 46           |
| Swine  | untyped | OVC-06-58285        | 10,865 | 57  | 43  | 41           |

$^a$ The xTAG™ RVP was performed according to the manufacturer’s instructions and the cutoff for positivity was 300 MFI. VR4788 is an H3N2 virus isolated from a patient returning from Mexico who was negative for H1N1 swine flu using two swine flu HA gene real time PCR assays. G01-30214 is an H2N2 swine isolate determined by sequencing the HA gene. The influenza B/Yamagata/16/88 virus tested negative for the influenza A matrix gene but was positive for influenza B gene in the RVP test.
was 6,970 MFI) while the H1 and H3 signals were negative indicating the presence of a non-seasonal, non-H1, non-H3 influenza A virus.

To further validate the ability of the RVP test to identify non-H1, non-H3 influenza viruses we tested 17 influenza A viruses representing 11 of the 16 HA subtypes and four untyped isolates. The influenza subtypes tested included both high pathogenicity and low pathogenicity avian influenza viruses isolated from four bird species (turkey, quail, mallard, chicken) in five countries (Canada, Italy, England, U.S.A., and Vietnam), four swine and two equine viruses. The results show that all 17 viruses had a positive matrix signal (mean, 7,692) and negative signals for both H1 and H3 genes (Table 2). For comparison, one H1 and two H3 seasonal influenza specimens gave readings of 8,716, 1,566, and 3,255 for the H1 and H3 targets, respectively. The RVP test also correctly flagged two H5N1 isolates as H5 subtypes (using unmasked software). These results indicate that the RVP test can distinguish between seasonal H1, H3 influenza and non-H1, non-H3, non-seasonal avian or animal influenza.

5. Discussion

The emergence of H1N1 swine influenza has provided a real life challenge for the RVP test to detect new pandemic strains. The xTAG™ RVP successfully flagged 20 out of 20 swine flu patients as having non-seasonal influenza while at the same time correctly identified seasonal influenza (8 H1N1 and 14 H3N2), 3 parainfluenza type 3, 6 rhino/enterovirus, 2 coronavirus 229E, and 1 metapneumovirus infections in Hamilton during the month of April. By running the test daily as a sentinel test, we have been able to provide public health authorities with a probable swine flu result with a 24 h turn around time faster than that provided by our Public Health Laboratories. While laboratories rush to build H1N1 swine flu specific assays, currently available molecular tests such as the xTAG™ RVP assay provide a solution for detecting swine flu cases in the absence of specific H1N1 swine flu tests. We believe that the RVP test if implemented in diagnostic algorithms can play an important role as a sentinel test to detect novel non-seasonal influenza A viruses in patients presenting with IIL and therefore act as an early warning system for the detection of future pandemic influenza threats.

Conflict of interest

All authors have agreed to the content of the manuscript and its submission to the journal. JBM is an inventor on a patent relating to the xTAG™ RVP test. All authors declare that the content of the manuscript is original and has not been published or submitted for publication to another journal.

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