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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.

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.[1] 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.[2] 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.[3] 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

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is no formal national surveillance system to identify what viruses are circulating in pigs in North America. The genetic makeup of this virus is germaine 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.[4] 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.[5] The xTAG™ RVP test was developed in 2005 immediately following SARS and H5N1 influenza and was designed to detect and type the three influenza A subtypes circulating at that time *viz.* H1, H3 and H5. Understanding how the xTAG™ RVP test identifies influenza A is important to its detection of new "non-seasonal" influenza viruses. The test amplifies a conserved part of the matrix gene found in all influenza A viruses and specific regions of the H1 or H3 genes.[6] The test therefore simultaneously detects influenza A and determines 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 effectively detect the presence of non-seasonal (non-H1, non-H3) 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
xTAG™ RVP results for matrix, H1 and H3 hemagglutinin targets for 20 confirmed cases of H1N1 swine influenza[a].

Patient[a]	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	7764	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	7	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 Prevention and 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
xTAG™ RVP results for the matrix and hemagglutinin gene targets for 17 influenza A isolates from human, avian, equine and swine[a].

Source	Subtype	Strain	Matrix	H1	H3	H5
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/Czeck/56	9,490	57	108	46
Avian	H5N1	A/Mallard/Vietnam/133/2004	1,739	47	31	787
Human	H5N1	A/Vietnam/1203/2004	1,544	34	30	624
Avian	H6N5	A//Shearwater/Aus/72	9,722	62	70	68
Avian	H7N3	A/Chicken/British Columbia/04	1,972	37	26	9
Horse	H7N7	A/Prague/56	10,333	71	45	38
Horse	H7N7	A/Equine/Cambridge/1/63	3,006	25	28	16
Avian	H8N4	A/Turkey/Ontario/68	9,697	98	58	48
Avian	H9N2	A/Turkey/Wisconsin/66	2,104	36	33	24
Avian	H10N8	A/Quail/Italy/65	9,815	49	53	52
Avian	H14N5	A/Mallard/263/82	9,901	54	62	41
Avian	H15N8	A/Duck/Aus 341/83	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 patent 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^{TM} 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^{TM} 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 ILI 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^{TM} 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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