Development of a Novel Multiplex PCR (M-PCR) for Combined Influenza A Virus Subtyping and Oseltamivir Resistnance (H275Y) Genotyping

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INTRODUCTION

The identification of influenza A virus subtypes in clinical specimens is becoming increasingly important for clinical laboratories since seasonal H1N1, H3N2 and pandemic H1N1 influenza A viruses currently have characteristic antiviral resistance patterns and subtyping is often used as a surrogate for antiviral resistance testing. Both seasonal and pandemic influenza A continue to mutate and resistance to either amantadine or oseltamivir will continue to vary with subtype over time.

OBJECTIVE

To develop a novel M-PCR for the identification of both influenza A subtype and oseltamivir resistance genotype (N1 gene, H275Y) in a combined assay using Luminex xMAP™ technology.

METHODS

Specimens – 54 nasopharyngeal swab specimens (FLOQSwabs™; Copan, Spa Brescia Italy) confirmed to be influenza A positive by either direct fluorescent antibody (DFA) staining or real time PCR, submitted to the Regional Virology Laboratory at St. Joseph's Healthcare (Hamilton Ontario) during the 2007 to 2009 respiratory seasons were characterized for both its subtype and oseltamivir resistance status in the newly developed M-PCR bead assay.

Nucleic Acid Extraction – Total nucleic acid was extracted from 200µl of NPS specimen using the bioMérieux easyMAG™ automated extractor and eluted in 60µl of buffer.

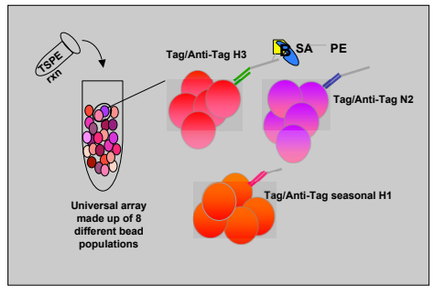
M-PCR- M-PCR was performed using 5 degenerate primer pairs designed to amplify both the hemagglutinin (HA) and neuraminidase (NA) genes of known seasonal H3N2, seasonal H1N1 and the most recent pandemic H1N1 influenza A strains found in all geographic regions. The final reaction volume of 25µl contained 1.2 x OneStep RT-PCR Buffer (Qiagen, Mississauga ON) with 3mM magnesium chloride, 0.4mM dNTP, primers at a final concentration of 0.4µM to 1µM, 2µl of OneStep RT-PCR Enzyme Mix (Qiagen, Mississauga ON) and 5µl total nucleic acid. Amplification was performed using the MJ Research thermocycler, PTC-200 under the following conditions: 50^0C x 30 mins, 95^0C x 15 mins, 40 cycles of: 95^0C x 30 secs, 52^0C x 30 secs, 72^0C x 30 secs and a final extension at 72^0C for 5 mins.

Luminex xMAP Detection System– For micro-fluidic bead interrogation, PCR amplicons were enyzmatically treated with 20 U exonuclease (USB, Cleveland OH) and 2.5 U of shrimp alkaline phosphatase (USB, Cleveland OH) for 30 minutes at 37^0C to remove excess primers and dNTP. An aliquot (5 ul) of the treated PCR product was subjected to a target specific primer extension (TSPE) multiplex reaction with 8 target specific primers (TSP) ranging in concentration from 0.025µM to 0.075µM, 1.6x PCR buffer (TaKaRa Bio, Madison WI), 5µM of d(A, T, G)TP (Invitrogen, Burlington ON), 5µM biotin dCTP (Invitrogen, Burlington ON) and 2U DNA polymerase (TaKaRa Bio, Madison WI). There was one TSP for each H1 (seasonal strain), H1 (pandemic strain), H3 and N2. For the detection of the H275Y mutation, 4 TSP were required. For seasonal H1 and pandemic H1 strains, there was each a N1 oseltamivir sensitive primer and a N1 oseltamivir resistant primer. The multiplex TSPE reaction was performed on the MJ Research PTC-200 thermocycler under the following conditions : 96^0C x 2 mins and 35 cycles of: 95^0C x 30 secs, 50^0C x 30 secs, 72^0C x 45 secs, during which amplified products generated in the initial M-PCR were hybridized to type specific primers containing a unique tag sequence. The DNA polymerase extended, generating complements with biotin dCTP incorporated into the product. The biotinylated products (5µl) were captured onto 20µl bead mix consisting of 8 populations of xTAG™ microspheres (Luminex, Austin TX) by hybridization for 30 minutes at 45° C. Captured products are then detected using 100µl of the fluorescent reporter molecule, streptavidin-phycoerythrin (SAPE) diluted 1:135 in wash buffer. Each biotin-labeled TSPE product hybridized only to a specific microsphere containing a complementary anti-tag oligonucleotide sequence. Each set of colored beads represents a specific influenza A subtype or presence/absence of the H275Y mutation by virtue of the bead/anti-tag/tagged primer association. After 20 minute incubation with the SAPE at room temperature, the beads were analyzed on the Luminex 100 IS instrument containing two lasers: one laser identifies the colour coded bead, and the other identifies the presence of the target specific nucleic acid sequence. Fluorescent signals were expressed as MFI readings.

Uniplex PCR- For the confirmation of seasonal H1N1 or H3N2, positive samples were tested by in-house uniplex PCR assays targeting the hemagglutinin gene H1 and H3 and the neuraminidase gene for N1 and N2. The final reaction volume of 25µl contained 1x OneStep RT-PCR Buffer (Qiagen, Mississauga ON) with 2.5mM magnesium chloride, 0.4mM dNTP, 0.6µM primers, 2µl of OneStep RT-PCR Enzyme Mix (Qiagen, Mississauga ON) and 5µl total nucleic acid. Amplification was performed using the MJ Research PTC-200 thermocycler under the following conditions: 50^0C x 30 mins, 95^0C x 15 mins, 40 cycles of: 95^0C x 30 secs, 52^0C x 30 secs, 72^0C x 30 secs and a final extension at 72^0C for 5 mins. Products were visualized on a 2% (w/v) agarose gel with ethidium bromide staining under UV detection.

Pandemic H1N1 Probe LightCycler Assay- All pandemic H1N1 strains were confirmed with an in-house LightCycler PCR assay with specific primers targeting the matrix gene. The final reaction volume of 20µl contained 1 x QuantiTect Probe RT-PCR Master Mix (Qiagen, Mississauga ON) with 4mM magnesium chloride, 1µM primers, 0.2µM FAM-Iowa Black FQ probe, 0.2µl of QuantiTect RT Mix (Qiagen, Mississauga ON) and 5µl total nucleic acid. Amplification was performed using LightCycler 2.0 instrument (Roche, Laval QB) under the following conditions: 50^0C x 20 mins, 95^0C x 15 mins, 45 cycles of: 95^0C x 0 secs, 60^0C x 1 min with data requisition.

Sequencing of the H275Y Region- For confirmation of the oseltamivir sensitive/resistant status, the 234bp N1 PCR product encompassing the H275Y region of all H1N1 strains, was sequenced by the Institute for Molecular Biology and Biotechnology (MOBIX) at McMaster University (Hamilton, ON) using the ABI sequencer (Applied Biosystems, Foster City CA).



RESULTS

The combined M-PCR subtyping and genotyping assay correctly subtyped all 54 influenza A positive samples, including 13/13 seasonal H3N2, 17/17 seasonal H1N1 and 24/24 pandemic H1N1 for both HA and NA gene targets. Table 1 shows typical MFI readings for 11 of the 54 samples.

Table 1: Typical MFI readings for the M-PCR assay for influenza A subtyping and H275Y resistance genotyping

Sample	H1 Pandemic	H1 Seasonal	H3 Seasonal	Pandemic N1 Resistant	Pandemic N1 Sensitive	Seasonal N1 Resistant	Seasonal N1 Sensitive	N2 Seasonal
VR1939	47	3837	13	59	19	1129	99	8
90129	25	4515	26	45	20	1104	93	18
70592	22	4024	17	46	22	48	2784	19
72587	58	5352	38	40	64	59	5735	33
VR1456	56	26	875	49	35	36	20	691
70139	30	30	825	71	32	45	12	589
VR14196	4078	32	23	85	2295	36	34	24
VR13124	8072	103	20	179	6401	40	24	23
90524	5874	23	29	3829	333	31	17	15
VR10016	6117	265	8	3677	1462	21	16	10
VR14488	3770	459	6	3727	3169	13	6	0

MFI cutoffs were established as follows: pandemic H1, 500; seasonal H1, 500; seasonal H3, 200; seasonal N1 (sensitive and resistant), 300; pandemic N1 (sensitive and resistant), 500; and seasonal N2, 100.

The average signal to noise ratio for each bead ranged from 20:1 to 97:1, enabling easy interpretation of results. Table 2 shows the average positive and negative MFI readings for each respective bead.

Table 2: Signal to noise ratios for each bead in the M-PCR assay

Bead	Average Positive MFI Reading	Average Negative MFI Reading	Signal to Noise Ratio
H1 (seasonal)	4659.3	95	49:1
H1 (pandemic)	4650.6	48.1	97:1
H3 (seasonal)	801.6	20.9	38:1
N1 (seasonal, H275)	4259.8	47.4	90:1
N1 (seasonal, H275Y)	1116.7	39.5	28:1
N1 (pandemic, H275)	3825.3	53.5	72:1
N1 (pandemic, H275Y)	2673.6	91.4	29:1
N2 (seasonal)	391	20	20:1

Of the 17 seasonal H1N1 specimens tested, the M-PCR bead assay found 15 to be oseltamivir resistant with the H275Y mutation and 2 to be oseltamivir sensitive. Among the 24 pandemic H1N1 specimens, 3 were H275Y positive (resistant), 12 were H275 (sensitive) and 9 showed a mix of H275 and H275Y genotypes. All M-PCR results were in agreement with sequencing data. For the viruses with mixed H275 and H275Y genotypes the MFI readings for the pandemic N1 sensitive and resistant beads were variable. Table 3 shows the MFI readings from a mixing experiment that was performed to determine the effect of different ratios of viral genotypes for the sensitive and resistant beads.

Table 3: Detection of both sensitive and resistant H275Y genotypes in a single sample achieved by combining different ratios of oseltamivir sensitive and resistant virus

	H1 (pandemic)	H1 (seasonal)	H3	N1 (pandemic)-resistant	N1 (pandemic)-sensitive	N1 (seasonal)-resistant	N1 (seasonal)-sensitive	N2
100% Sensitive	6907	339	46	198	7673	77	37	52
75% Sensitive, 25% Resistant	6621	485	30	2370	6755	28	30	32
50% Sensitive, 50% Resistant	2978	85	11	1478	2515	44	29	25
25% Sensitive, 75% Resistant	5909	460	35	3762	4805	63	16	34
100% Resistant	6616	35	33	4440	370	36	39	39

Serial specimens collected from 2 patients infected with pandemic H1N1 and undergoing oseltamivir treatment were tested in the M-PCR bead assay. Results are summarized in Table 4. Briefly, patient 1 was oseltamivir sensitive on day 1 with a MFI reading on the N1 sensitive bead of 4,918. By day 15, the patient showed a mixed H275 and H275Y genotype. A week later the patient was completely oseltamivir resistant. A similar pattern was seen with patient 2.

Table 4: Detection of oseltamivir sensitive and resistant virus in serially collected specimens from 2 patients infected with pandemic H1N1

	Collection Date	H1 (pandemic)	N1 (pandemic) -resistant	N1 (pandemic)-sensitive	N1 (seasonal)-resistant	N1 (seasonal)-sensitive	Oseltamivir Resistance Status
Patient 1	09/08/2009	6564	117	4918	39	22	sensitive
Patient 1	24/08/2009	6117	3677	14623	22	16	sensitive/resistant
Patient 1	01/09/2009	5874	3830	333	31	17	resistant
Patient 2	04/11/2009	7240	162	6615	46	17	sensitive
Patient 2	11/11/2009	4557	239	3979	34	20	sensitive
Patient 2	13/11/2009	4953	3990	1422	7	0	sensitive/resistant
Patient 2	14/11/2009	3770	3728	3169	13	6	sensitive/resistant
Patient 2	14/11/2009	855	1949	977	6	14	sensitive/resistant
Patient 2	16/11/2009	neg	neg	neg	neg	neg	
Patient 2	22/11/2009	2430	2297	3398	29	2	sensitive/resistant
Patient 2	22/11/2009	4660	299	5378	32	4	sensitive
Patient 2	22/11/2009	4370	1383	4411	25	17	sensitive/resistant
Patient 2	24/11/2009	2426	768	2518	28	0	sensitive/resistant
Patient 2	25/11/2009	neg	neg	neg	neg	neg	

SUMMARY

1. We developed a M-PCR bead assay for the combined subtyping and oseltamivir resistance genotyping (H275Y) of both seasonal and pandemic H1N1 influenza A virus.
2. The M-PCR bead assay correctly subtyped 54 influenza A specimens, including 17/17 seasonal H1N1, 13/13 seasonal H3N2 and 24/24 pandemic H1N1, for both the HA and NA gene targets.
3. The H275Y mutation was correctly identified in 15 seasonal H1N1 viruses. Two seasonal H1N1 specimens showed to be oseltamivir sensitive.
4. Of the 24 pandemic H1N1 specimens tested by the M-PCR bead assay, 3 were H275Y positive (resistant), 12 were H275 (sensitive) and 9 showed a mixed H275 and H275Y mixed genotype. All M-PCR assay results were in agreement with sequencing data.
5. The newly developed M-PCR bead assay can detect the presence of the H275Y mutation earlier than sequencing. This combined subtyping and oseltamivir resistance test should provide useful information to clinicians and facilitate appropriate patient management.