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Original Article

A matrix gene–based multiplex real-time RT-PCR for detection and differentiation of 2009 pandemic H1N1 and other influenza A viruses in North America

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Background The emergence in humans of pandemic (H1N1) 2009 (pH1N1) with similarities to swine influenza viruses (SIVs) caused much concern for both human and animal health as potential for interspecies transmission was initially unknown.

Objectives The goal of this study was to develop a real-time RT-PCR test for the detection and differentiation of 2009 pH1N1 and endemic influenza A viruses in North America.

Methods Matrix (M) gene sequences from U.S. human pH1N1 cases and U.S. SIVs were aligned to determine a suitable region for an assay target. Primers were selected to amplify all influenza A. Two probes were designed to differentiate pH1N1 (EA matrix) from endemic (NA matrix) SIVs. The assay was validated using the first U.S. pH1N1 strain, 10 human pH1N1-positive specimens and nine U.S. SIV isolates, then evaluated on 165 specimens of swine and other animal origin submitted to the Iowa State University Veterinary Diagnostic Laboratory. Results were compared to other influenza A PCR assays. Sequences from additional pH1N1 strains and contemporary H1N1 SIVs were used to assess robustness of the selected primers and probes for the intended purpose.

Results The new assay's results from clinical specimens concurred with confirmatory PCR testing. The additional probe designed from sequence analysis improved detection of the NA matrix subtype when added to the reaction mixture.

Conclusion This assay detects and differentiates pH1N1 and US influenza A viruses in various sample matrices and species. Good bioinformatics support is critical when designing RT-PCR assays and monitoring their performance.

Keywords Influenza, matrix gene, novel H1N1, pH1N, real-time RT-PCR, swine.

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Introduction

Influenza is an acute respiratory disease caused by an RNA virus belonging to the *Orthomyxoviridae* family. Influenza viruses are classified into three types (A, B and C) based on matrix protein (M) or nucleoprotein (NP) and are further characterized by both hemagglutinin (H) and neuraminidase (N) proteins.[1] To date, 16 HA and 9 NA subtypes have been identified for influenza A.[2] Reassortment and mutations occur frequently, contributing to the variation and evolution of influenza viruses, and also influencing the potential of the virus to be the causative agent of epidemics and pandemics.[3] Following the emergence of a novel H1N1 influenza A virus strain, later named "the pandemic (H1N1) 2009" (pH1N1), in the human population, the initial concern in the general population was with regard to zoonotic potential and human-to-human transmission because of the high mortality rate associated with the virus in early cases. However, in the veterinary field, because this virus was quickly dubbed the "swine flu" because of its genetic similarity with influenza viruses of swine origin, the need at the veterinary diagnostic community quickly arose to be able to rapidly and accurately detect this organism and differentiate it from endemic influenza A viruses in non-human species.

A number of assays have been developed for the detection of pH1N1. Many of these[4–9] specifically detect the virus but not other influenza A viruses, which may increase the time and expense of diagnosing endemic influenza. Others target either the N[10] or H[11–15] genes, which tend to have a high rate of genetic mutation and may lead to false negative results if mismatches occur between the viral

sequence and the primer or probe sequence. Others are SYBR Green assays[16,17], which may lack the specificity of a probe-based assay as SYBR Green can intercalate with any double-stranded DNA product (including primer–dimers or non-specific products that may be amplified in the reaction) and cause fluorescence[18], whereas a probe-based assay requires complementarity of both primers as well as a probe. The lack of specificity can usually be overcome by performing a melt curve analysis[19] but this adds additional time to the assay. In some instances, the differentiation of primer–dimers and specific product is not possible and an additional confirmatory test must be performed.[20] The assay described herein is a probe-based assay, detecting pH1N1 as well as endemic, North American swine influenza viruses (SIVs) in a differential manner. The M gene is known to be genetically conserved among all influenza A viruses[21], so this was chosen as the target for a real-time RT-PCR assay capable of detecting all influenza A and differentiating pH1N1 from endemic influenza A virus directly from clinical specimens of various species origin.

Methods

Primer and probe design

A suitable target region for a real-time RT-PCR assay was determined by using Clustal W alignment function of Lasergene® software (DNAstar, Inc., Madison, WI, USA) for the sequences of the matrix gene from six U.S. human pH1N1 cases (GenBank accession numbers: EPI176471, FJ966954, FJ966968, FJ966972, FJ966975, and FJ966983) and 16 U.S. SIVs (GenBank accession numbers: CY031252, CY031253, CY031254, CY031255, CY032930, CY035071, CY036800, CY036864, DQ145542, DQ889688, EU409951, EU409957, EU409962, EU409966, EU409970 and EU604695). Primer Express® software (Applied Biosystems, Carlsbad, CA, USA) was used for the primer and probe design as summarized in Table 1. Initially, one forward (M_F) and three reverse primers (M_R1, M_R1a and M_R1b) were selected to amplify all influenza A targets, and 2 MGB probes (M_EAPR and M_NAPR1) were incorporated to differentiate pH1N1 (Eurasian or EA) matrix gene from endemic SIV (North American or NA) matrix gene. Sequence information and additional test validation resulted in the incorporation of a second probe (M_NAPR2) into the assay to improve the detection of endemic influenza A in clinical specimens.

Real-time reverse transcriptase-polymerase chain reaction (rRT-PCR)

Extraction of RNA from isolates or clinical specimens was performed using the Applied Biosystems/Ambion MagMAX® Viral RNA Isolation kit and a KingFisher® semi-automated magnetic particle processing system (Thermo Scientific, Waltham, MA, USA) according to manufacturer's recommendations. Real-time RT-PCR was carried out with the Applied Biosystems/Ambion Path-ID® Multiplex one-step RT-PCR kit in a 20- μl reaction according to supplier's recommendations, with the addition of 10 U of MultiScribe® reverse transcriptase (Applied Biosystems) per reaction. The forward primer (M_F) was added at a final concentration of 0·375 μm. The three reverse primers (M_R1, M_R1a and M_R1b) were combined in equal molar concentrations and added at a final concentration of 0·375 μm. Each probe (M_EA and M_NA1) was added at a final concentration of 0·125 μm. When an additional probe (M_NA2) was incorporated into the assay, each of the NA probes was added at a final concentration of 0·0625 μm. Reactions were run on an Applied Biosystems 7500Fast instrument with the following cycling conditions in fast mode: 45°C for 10 minutes, followed by 95°C for 10 minutes, then 45 cycles of 97°C for 2 seconds and 60°C for 40 seconds. Fluorescence curves crossing the threshold at or before 40 cycles were considered positive for the respective genotype. A cycle threshold (Ct) between 40 and 45 was considered suspect and retested if appropriate. Criteria for retesting include the presence of clinical signs supporting a diagnosis of influenza, compatible lesions or a real-time fluorescence curve of atypical shape.

Table 1. Primers and probes used in assay

Oligo name	Sequence (5′ to 3′)	5′ label	Target
M_F	TCAGGCCCCCTCAAAGC	N/A	Influenza A
M_R1	CATTCCATGAGAGCCTCAAGATC	N/A	Influenza A
M_R1a	CACTCCATGAGAGCCTCAAGATC	N/A	Influenza A
M_R1b	CATTCCATGAGTGCCTCAAGATC	N/A	Influenza A
M_EAPr	CAGAGACTGGAAAGTGT	VIC	pH1N1 (Eurasian Matrix)
M_NAPR1	CAGAGACTYGAAGAYGT	FAM	North American Matrix
M_NAPR2	CAGAAACTYGAAGAYGT	FAM	North American Matrix

Validation and evaluation

The assay was initially validated with the first U.S. pH1N1 strain (A/California/04/2009)[22], 10 human specimens positive for pH1N1 using the CDC PCR[23] obtained from the University of Iowa State Hygienic Laboratory (UIHL) and two SIV isolates [A/Swine/Iowa/73 (H1N1) and A/Swine/TX/98 (H3N2), both supplied by the National Veterinary Services Laboratory, Ames, IA].

In addition, eight isolates that had previously been tested with the ISU-VDL general and subtyping assays[24] and further characterized by sequencing were tested with this assay and were also sent to the UIHL in a blind fashion where they were tested by the CDC PCR protocols. These included swine H1α clade (A/Swine/Iowa/1973 H1N1), swine H1β clade (A/Swine/IA/15231/2008 H1N1), swine H1γ clade (A/Swine/IA/13381/2008 H1N1; A/Swine/IA/14913/2008 H1N1;A/Swine/IA/31256/2006 H1N2), swine H1δ clade (A/Swine/IA/18755/2008 H1N2), cluster I H3N2 (A/Swine/IA/41305/1998) and cluster IV H3N2 (A/Swine/IA/18916/2008) viruses. Classifications of H1 clades and H3 clusters are described by Vincent *et al.*[25] and Olsen *et al.*[26], respectively. The CA04 isolate (A/California/04/2009) served as positive control for pH1N1. Phylogenetic relationship between these viruses and contemporary human influenza viruses including pH1N1 based on H gene is illustrated in Figure 1.

The assay was then evaluated on specimens from 165 animals representing 32 cases submitted to the Iowa State University Veterinary Diagnostic Laboratory (ISU-VDL). These specimens were from a variety of species, including porcine, feline (domestic and lion), canine, primate (chimpanzee) and rodent (hamster). Samples tested included lung tissue, swabs (nasal, pharyngeal or tracheal) and bronchoalveolar lavage. All samples were also tested by ISU-VDL's in-house screening PCR, which targets influenza A nucleoprotein (NP) gene[27], ISU-VDL's H subtyping PCR that identifies and differentiates H1 and H3,[24] NAHLN SIV surveillance PCR[28] and/or sequencing for the confirmation of virus classification.

Matrix gene sequencing

Matrix gene sequences from two additional pH1N1-positive influenza cases and 122 contemporary H1N1 SIVs, all from clinical specimens submitted to the ISU-VDL, were used to further assess primer and probe suitability. Specimens with sequences in which a mismatch was observed between primer/probe sequence and viral sequence were tested with the assay. Sequence of samples not giving the expected result were evaluated and utilized to design an additional probe for incorporation into the assay.

For sequencing, extraction of RNA was performed using MagMAX® Viral RNA Isolation Kit (Applied Biosystems/Ambion) in conjunction with the KingFisher® magnetic particle processor according to manufacturer's recommendations. RT-PCR was performed using the SuperScriptIII® One-step RT-PCR System (Invitrogen Life Technologies, Carlsbad, CA, USA) with each primer being added at a level of 1·32 μm in the reaction mixture. Also, 32 U of RNaseOUT® RNase inhibitor (Invitrogen) was added to each reaction. Reverse transcription was performed at 50°C for 30 minutes, followed by denaturation at 94°C for 2 minutes. PCR amplification was executed for 35 cycles of 94°C for 20 seconds, 58°C for 30 seconds and 68°C for 7 minutes. Final extension was carried out at 68°C for 7 minutes. PCR product purification was performed using QIAquick® PCR Purification Kit (Qiagen, Valencia, CA, USA) or the

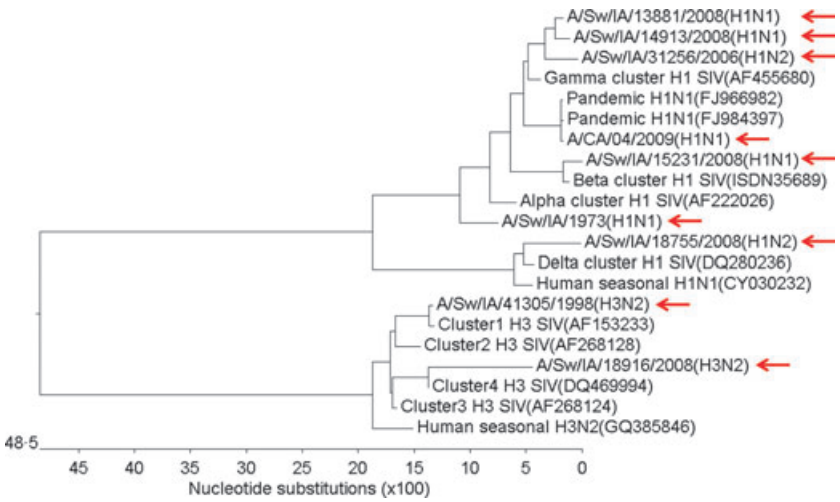

Figure 1. Phylogenetic relationship between representative swine influenza viruses and human influenza viruses based on hemagglutinin gene sequence. The viruses that were tested by various PCR protocols (ISU, CDC, NAHLN) are marked with arrow.

Table 2. Matrix gene sequencing primers

Primer name	Sequence (5′ to 3′)	Use	Source
M-1F	TAT TCG TCT CAG GGA GCA AAA GCA GGT AG	PCR full length and sequencing	Hoffman *et al.*[29]
M-1072R	ATA TCG TCT CGT ATT AGT AGA AAC AAG GTA GTT TTT	PCR full length and sequencing	Hoffman *et al.*[29]
M-404F	TGC ATG GGC CTC ATA TAC AAC AG	Sequencing	This paper
M-564R	ACC ATT CTG TTC TCG TGC CTG ATT	Sequencing	This paper

ChargeSwitch® PCR Clean-up kit (Invitrogen) in conjunction with the Kingfisher Magnetic Particle Processor, according to manufacturer's recommendations. Purified PCR product and sequencing primers were submitted to the Iowa State University DNA Sequencing and Synthesis Facility. Data were analyzed with Lasergene® software. Primers used for amplification and sequencing reactions are listed in Table 2.

Results

The 10 human pH1N1-positive specimens provided by UIHL were detected by only the VIC dye, as expected for the EA gene of the pH1N1 virus. The A/Swine/Iowa/73 (H1N1, α clade) and A/Swine/TX/98 (H3N2, cluster I) viruses were detected only by the FAM dye, confirming specificity of the assay for the North American matrix gene.

Classification of the eight SIV isolates sent to UIHL is summarized in Table 3. Although all eight isolates were endemic SIVs, five of the eight viruses were identified as positive for pH1N1 by CDC PCRs and the remaining three isolates as reassortant between pH1N1 and human seasonal influenza viruses. In contrast, the NAHLN SIV surveillance PCR did not identify any of the eight isolates as pH1N1 as shown in Table 3. These isolates all tested NA matrix gene positive (i.e., endemic influenza virus) by the assay described herein.

Specimens from 165 animals were tested by the differential M gene RT-PCR. All results (e.g., positive for influenza A, identification of virus genotype) were confirmed by ISU-VDL in-house RT-PCR assay (NP, H, N genes) or by the NAHLN surveillance PCR (M and N genes) and/or by sequencing for H and M genes. Results are summarized in Table 4. In summary, six samples were positive for endemic (NA matrix) influenza A (five porcine and one feline),

Table 3. Comparison of detection and subtyping influenza A real-time RT-PCR assays

		ISU SIV PCRs			ISU M Gene PCR		NAHLN SIV PCRs		CDC Influenza PCRs			CDC seasonal flu PCR	
		NP	HA subtyping		M diff								
Virus	Classification		H1	H3	NA-M	EA-M	M	N1 diff	M	swHA	swNP	huH1	huH3
SIV	α H1*	+	+	–	+	–	+	–	+	+	+	–	–
SIV	β H1**	+	+	–	+	–	+	–	+	+	+	–	–
SIV	γ H1***	+	+	–	+	–	+	–	+	+	+	–	–
SIV	δ H1†	+	+	–	+	–	+	–	+	–	+	+	–
SIV	H3N2††	+	–	+	+	–	+	–	+	–	+	–	+
Human	2009 pandemic H1N1†††	+	+	–	–	+	+	+	+	+	+	–	–

SIV, swine influenza virus.
*A/Swine/Iowa/1973 (H1N1).
**A/Swine/Iowa/15231/2008.
***A/Swine/Iowa/13381/2008 (H1N1); A/Swine/Iowa/14913/2008 (H1N1); A/Swine/Iowa/31256/2006 (H1N2).
†A/Swine/Iowa/18755/2008 (H1N2).
††A/Swine/Iowa/41305/1998 (cluster I); A/Swine/Iowa/18916/2008 (cluster IV).
†††A/California/04/2009.

Table 4. Number of clinical samples tested by matrix differential rRT-PCR assay

Species	NA positive	EA positive	Negative
Porcine	5	2	125
Feline (cat, lion)	1	2	24
Canine	0	1	2
Primate (Chimpanzee)	0	0	1
Rodent (Hamster)	0	0	2

five samples were positive for pandemic (EA matrix) influenza A (two porcine, two feline and one canine) and the remaining 154 samples were negative for influenza A virus.

Sequencing was performed on 122 clinical porcine samples or virus isolates from samples that had previously tested as H1N1 by the ISU-VDL assay and on two pH1N1 samples. Analysis revealed total homology between the EA matrix primer/probe sequences and that from the pH1N1 matrix sequences. However, minor mismatches in the probe target region were found in six of the endemic H1N1 matrix sequences from clinical specimens. These samples, all confirmed by sequencing as NA genotype, were tested with the RT-PCR assay described here. Three of these samples tested as NA matrix positive by the assay but the remaining three samples were either inconclusive or negative for influenza A by the assay. The matrix sequence derived from the latter three specimens (CAGA**A**ACTTGAAGATGT) had a single mismatch to the NA genotype probe (CAGA**G**ACTYGAAGAYGT). A probe reflecting this mismatch (M_NAPR2) was synthesized and incorporated into the PCR mixture. All six samples tested as NA matrix positive with the updated reagent mix.

Discussion

The newly developed real-time PCR assay targeting the M gene detects and differentiates pH1N1 and US influenza A viruses in various sample matrices and species. It was used to detect the first-reported human-to-feline reverse zoonotic transmission of pH1N1 virus[28] and has also been used to detect this virus in canine and porcine specimens. (For the USDA's list of pH1N1 influenza presumptive and confirmed results in non-human species, see: http://www.usda.gov/documents/FINAL_RESULTS_2009_PANDEMIC_H1N1_INFLUENZA_CHT.pdf). The assay is rapid (approximately 70 minutes), and results were confirmed by other PCR tests.

One of the assays used for comparison was deployed by the National Animal Health Laboratory Network (NAHLN). This organization, established in 2002, is a cooperative effort between the Animal and Plant Health Inspection Service (APHIS) and National Institute of Food and Agriculture (NIFA) and the American Association of Veterinary Laboratory Diagnosticians (AAVLD). Initially, the network was established with 12 core diagnostic laboratories; there are currently 62 member laboratories. The mission of NAHLN is to safeguard animal health by providing standardized protocols and training for detection of animal diseases, coordinating proficiency testing within the member laboratories and overseeing surveillance testing for the early detection of various veterinary agents of concern. More information on this organization is available at http://www.aphis.usda.gov/animal_health/nahln/.

The PCR protocol deployed by NAHLN is a series of two RT-PCR tests (one for general detection using M gene and one to differentiate between pandemic and endemic SIV using N gene). The M gene assay was originally described by Spackman *et al.*[30] and was developed for the detection of avian influenza. To update the assay to detect the pH1N1 virus, an additional reverse primer was incorporated.[28] ISU-VDL's in-house HA subtyping PCR is able to differentiate between H1 and H3 subtypes and between N1 and N2 subtypes but cannot distinguish between endemic and pandemic H1N1 viruses (Table 3). The CDC protocol[23] differentiates pH1N1 from human influenza virus and may be useful in monitoring the emergence of reassortant influenza viruses, but cannot differentiate pH1N1 from endemic H1 SIVs (Table 3), raising a concern on its differential diagnostic utility at public health laboratories as incidence of SIV infection in humans has been reported.[31] The differential matrix assay described herein is able to detect and differentiate pandemic from endemic influenza A in a single multiplexed reaction and is currently being used by the ISU-VDL for monitoring the appearance of pH1N1 in samples submitted to the laboratory. The assay may be a good supplement to the CDC testing protocol and to the NAHLN PCRs.

The study observation emphasizes that primers and/or probes should be designed with good bioinformatics support. Like other RT-PCR assays, monitoring the variation of sequences in field strains and updating the primers and/or probes is necessary to ensure reliable performance of the assay. This assay will be useful in observing the appearance of pH1N1 in various species and helpful in monitoring the potential for interspecies transmission of this virus.

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