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Real-time reverse transcription–polymerase chain reaction assays for the detection and differentiation of North American swine influenza viruses

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Abstract. Swine influenza is an acute respiratory disease of swine caused by type A influenza viruses. Before 1998, mainly ''classical'' H1N1 swine influenza viruses (SIVs) were isolated from swine in the United States. Since then, antigenically distinct reassortant H3 and H1 SIVs have been identified as causative agents of respiratory disease in pigs on US farms. Improvement in SIV diagnostics is needed in light of the recently observed rapid evolution of H1 and H3 SIVs and their zoonotic potential. To address this need, real-time reverse transcription–polymerase chain reaction (RT-PCR) assays for the detection of SIVs were developed. A highly sensitive matrix (M) gene–based RT-PCR assay that is able to detect both the H1 and H3 subtypes of SIVs, with a sensitivity per reaction of approximately 2 copies of in vitro–generated M-specific negative-sense RNA molecules and approximately 0.05 $TCID_{50}$ in lung lavage of experimentally SIV-infected pigs, was established. This RT-PCR assay can be performed within a few hours and showed a sensitivity of 94% and a specificity of 85% when compared with virus isolation. In addition, H1-, H3-, N1-, and N2-specific primer and probe sets were designed for use in the differentiation of different SIV subtypes. The hemagglutinin (H)- and neuraminidase (N)-specific primer and probe sets were less sensitive than the M-specific assay, although they were found to be specific for their respective viral genes and able to distinguish between their respective SIV subtypes.

Introduction

Influenza in swine is caused by influenza A viruses. It is an acute respiratory disease, and its severity depends on many factors, including host age, virus strain, and secondary infections.[6] Influenza A viruses are isolated from a number of other animal host species, including birds, humans, horses, whales, and minks. They are generally host specific. Although whole viruses rarely cross the species barrier, individual gene segments can cross this barrier through the process of genetic reassortment. Pigs have been postulated to play an important role in interspecies transmission to humans by acting as the ''mixing vessel'' for reassortment between viruses specific to different host species.[16,18] Swine support the replication of both human and avian influenza A viruses.[10] Type A influenza viruses can be highly variable in their surface glycoproteins hemagglutinin (H) and neuraminidase (N), and to date, 15 different H subtypes and 9 different N subtypes have been identified in avian and mammalian species.[17,27] Three main subtypes of influenza viruses are currently circulating in swine populations in the United States: H1N1, H3N2, and H1N2.[8,9,12,13,25,26,28]

Various diagnostic tests have been used for the direct and indirect detection of swine influenza virus (SIV) in pigs: virus isolation (VI) in embryonated chicken eggs or Madin–Darby canine kidney (MDCK) cells and subsequent H and N subtyping, reverse transcription–polymerase chain reaction (RT-PCR), immunohistochemistry, antigen-capture enzyme-linked immunosorbent assay, indirect fluorescence antibody, and hemagglutination inhibition.[2,3,5,11,19,21,23,24] Recently, a gel-based multiplex RT-PCR assay was developed to detect and identify H1 and H3 subtypes of SIV.[4,5] Traditionally, SIV isolation from field samples has been carried out using embryonated chicken eggs or MDCK cells. Although VI in embryonated eggs or MDCK cells is a sensitive method, it may take 1–2 weeks to obtain results. In contrast to most of the above-mentioned diagnostic assays, real-time RT-PCR is a rapid assay, where results may be available within hours. In addition, it can also be less expensive than VI and gel-based RT-PCR assays. Real-time RT-PCR offers also the advantages of no post–polymerase chain reaction (PCR) sample handling, thus reducing the chance for

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Table 1. PCR primer and hydrolysis probe sequences.

Specificity and size	Primer/Probe	Sequence*
Matrix gene, 100 base pairs	M-F	5′-AGA TGA GTC TTC TAA CCG AGG TCG-3′
	M-R	5′-TGC AAA AAC ATC TTC AAG TCT CTG-3′
	M-Probe	FAM-TCA GGC CCC CTC AAA GCC GA-TAMRA
Swine H1, 102 base pairs	H1-F	5′-AAT AAT TCA ACY GAC ACT G-3′
	H1-R	5′-GTT TAC ATA GTT TYC CRT-3′
	H1-Probe	TXRED-AAG AAT GTA ACM GTA ACA CAC TCT G-BHQ2
Swine H3, 244 base pairs	H3-F	5′-AAA TTG AAG TGA CTA ATG CTA C-3′
	H3-R	5′-TGA GGC AAC TAG TGA CCT AAG-3′
	H3-Probe	FAM-CAA CAG GTA GAA TAT GCG ACA GTC C-TAMRA
Swine N1, 267 base pairs	N1-F	5′-GTA ATG GTG TTT GGA TAG GAA G-3′
	N1-R	5′-ATG CTG CTC CCA CTA GTC CAG-3′
	N1-Probe	FAM-TGA TTT GGG ATC CTA ATG GAT GGA CAG-TAMRA
Swine N2, 233 base pairs	N2-F	5′-TGG ACA GGG AAC AAC ACT AAA C-3′
	N2-R	5′-ACA AGC CTC CCA TCG TAA AT-3′
	N2-Probe	TXRED-CAA ATG AAA TGG AAC ACC CAA CTC AT-BHQ2

* Y = C, T; M = A, C; R = A, G; FAM = 6-carboxyfluorescein; TAMRA = 6-carboxytetramethylrhodamine; TXRED = Texas Red; BHQ2 = Black Hole Quencher 2.

cross-contamination versus standard RT-PCR. In addition, because the real-time RT-PCR product is detected with a sequence-specific probe, there is confirmation that the correct target was amplified; this reduces the chance for false positives. To date, no real-time RT-PCR assay for the detection of SIV in clinical samples has been described.

In this study, the development of a 1-step real-time RT-PCR assay with hydrolysis-type probes is described for the rapid screening of clinical samples for type A SIVs and for the subsequent identification of SIV subtypes circulating in the United States.

Materials and methods

Ribonucleic acid extraction

The RNA was extracted with the QIAamp Viral RNA Mini kit[a] using a protocol for fluid samples recommended by the manufacturer. In brief, 140 μl of material from clinical samples was applied to the spin column. Ribonucleic acid was eluted in 60 μl of nuclease-free water, and 8 μl per real-time RT-PCR was used for the template.

Hydrolysis probe and primer sets

An influenza virus matrix (M) gene–specific PCR primer set and hydrolysis probe were designed for a region conserved in all type A influenza virus M genes (Table 1). The sequences of primers and probes were based on a recent publication by Spackman et al. (2002). In addition, H1-, H3-, N1-, and N2-specific primer and probe sets for conserved regions of the H1, H3, N1, and N2 gene sequences were developed (Table 1). Because of the sequence variation within the H1, H3, N1, and N2 genes, the probes and primers were primarily targeted to North American influenza viruses. Probes were labeled at the 5′ end either with the 6-carboxyfluorescein or Texas Red reporter dye and at the 3′ end either with the 6-carboxytetramethylrhodamine or Black Hole Quencher 2 quencher dye (Table 1).

Real-time RT-PCR

A OneStep RT-PCR Kit[b] was used with a 25-μl reaction volume. The reverse transcription (RT) step conditions for all primer sets were 30 min at 50 C and 15 min at 95 C. The assays were run under the following conditions.

Matrix assay. One microliter of kit-supplied enzyme mixture (combination of Omniscript Reverse Transcriptase, Sensiscript Reverse Transcriptase, and HotStarTaq DNA polymerase), 0.5 pmol of each primer, 0.1 μM probe, 400 μM each deoxynucleotide triphosphate (dNTP), 5 μg nonacetylated bovine serum albumin, 7.5 mM $MgCl_2$, and 2.5 U of ribonuclease (RNase) inhibitor[c] were used. The PCR cycling protocol was set as follows: 45 cycles of 94 C for 15 sec and 60 C for 60 sec.

H1 assay. One microliter of kit-supplied enzyme mixture (combination of Omniscript Reverse Transcriptase, Sensiscript Reverse Transcriptase, and HotStarTaq DNA polymerase), 1.6 pmol of each primer, 0.1 μM probe, 400 μM each dNTP, 5 μg nonacetylated bovine serum albumin, 6.0 mM $MgCl_2$, and 2.5 U of RNase inhibitor[c] were used. The PCR cycling protocol was set as follows: 45 cycles of 94 C for 15 sec and 54 C for 60 sec.

H3 assay. One microliter of kit-supplied enzyme mixture (combination of Omniscript Reverse Transcriptase, Sensiscript Reverse Transcriptase, and HotStarTaq DNA polymerase), 5.0 pmol of each primer, 0.1 μM probe, 400 μM each dNTP, 5 μg nonacetylated bovine serum albumin, 5 mM $MgCl_2$, and 2.5 U of RNase inhibitor[c] were used. The PCR cycling protocol was set as follows: 45 cycles of 94 C for 15 sec and 62 C for 60 sec.

N1 assay. One microliter of kit-supplied enzyme mixture (combination of Omniscript Reverse Transcriptase, Sensiscript Reverse Transcriptase, and HotStarTaq DNA polymerase), 1.0 pmol of each primer, 0.15 μM probe, 400 μM each dNTP, 5 μg nonacetylated bovine serum albumin, 7.0 mM $MgCl_2$, and 2.5 U of RNase inhibitor[c] were used. The PCR cycling protocol was set as follows: 45 cycles of 94 C for 15 sec and 62 C for 60 sec.

Table 2. Results of real-time RT-PCR with M-, H1-, H3-, N1-, and N2-specific primer and probe sets with various influenza virus isolates.*

Virus isolates†	Subtype/origin	Primer/probe specificity				
		M	H1	H3	N1	N2
A/Sw/IA/3421/90	H1N1	+	+	−	+	−
A/Sw/IA/case 13/99	H1N1	+	+	−	+	−
A/Sw/MN/37866/99	H1N1	+	+	−	+	−
A/Sw/NE/45891/00	H1N1	+	+	−	+	ND
A/Sw/MN/1192/01	H1N2	+	+	−	−	+
A/Sw/TX/4199-2/98	H3N2	+	−	+	−	+
A/Sw/NC/35922/98	H3N2	+	−	+	−	+
A/Sw/CO/23619/99	H3N2	+	−	+	−	+
A/Sw/OK/18089/99	H3N2	+	−	+	−	+
A/Chicken/Scot/59	H5N1	+	−	−	+	−
A/Mallard/OH/184/86	H5N1	+	−	−	+	−
A/Gull/MD/19/77	H2N9	+	−	−	−	−
B/Memphis/12/97	US strain	−	−	−	−	−
PRRSV-VR 2332	US strain	−	−	−	−	−
PCV—type 1	PK15 cells	−	−	−	−	−
PCV—type 2	US strain	−	−	−	−	−

* Sw = swine; PRRSV = porcine reproductive and respiratory syndrome virus; PCV = porcine circovirus; ND = not done.
† Standard 2-letter postal codes are used for US states.

N2 assay. One microliter of kit-supplied enzyme mixture (combination of Omniscript Reverse Transcriptase, Sensiscript Reverse Transcriptase, and HotStarTaq DNA polymerase), 1.0 pmol of each primer, 0.2 μM probe, 400 μM each dNTP, 5 μg nonacetylated bovine serum albumin, 6.0 mM $MgCl_2$, and 2.5 U of RNase inhibitor[c] were used. The PCR cycling protocol was set as follows: 45 cycles of 94 C for 15 sec and 60 C for 60 sec.

The establishment and optimization of all real-time RT-PCR protocols was performed with the Cepheid Smartcycler thermocycler and software[d] performing checkerboard titrations to identify the optimal concentrations for primers, probes, and $MgCl_2$ as well as to define the optimal annealing temperature and extension time for the respective assays. All temperature transition rates were set at the maximum transition rate. Fluorescence data were acquired at the end of each annealing step. Clinical samples for the evaluation of the assay were run using the ABI PRISM® 7900 HT Sequence Detection System[e] with the 96-well format using the same parameters as described above. Positive and negative results of the real-time RT-PCRs were determined by the respective instrument analysis software and rechecked manually.

In vitro transcription of control RNA

In vitro–transcribed M, H1, H3, N1, or N2 gene RNA was used as positive control and for the determination of the analytical sensitivity of the assay. The following influenza A isolates were used for amplification of the respective genes: M gene from Ck/PA/13552/98 (H7N2), H1 gene from Sw/IA/case #13/99 (H1N1), H3 gene from Sw/TX/4199-20/98 (H3N2), N1 gene from Sw/IN/1726/89 (H1N1), and N2 gene from Tk/MO/24093/99 (H1N2). Sequencing was performed with the ABI BigDye terminator system[f] to verify the insert sequences.

In vitro transcription was performed using the Ribomax™ Large Scale RNA production system.[g] The influenza M, H1, H3, N1, and N2 genes were cloned into either pCRII-TOPO[h] (H1 gene) or pAMP1 vector[i] (other genes) and were transcribed with the Ribomax system[g] from either T7 or SP6 promoter, depending on orientation, in accordance with the kit instructions, and quantified by spectrophotometry.

Gel-based RT-PCR assay

A 2-step RT-PCR assay for the detection of SIV in clinical samples was used. The RNA was extracted with the QIAamp Viral RNA Mini kit,[a] according the manufacturer's protocol. The RT was performed in a total volume of 20 μl and PCR in a total volume of 100 μl. The reaction mixture contained 10× PCR reaction buffer,[j] 5 mM $MgCl_2$, 1 mM each dNTP,[k] 30 U RNase inhibitor,[l] and 50 U of M-MuLV reverse transcriptase.[m] The primers were specific for the nucleoprotein (NP) of influenza A viruses: SIV-N-F: 5′-AAG CAG GGT AGA TAA TCA CTC-3′; SIV-N-R: 5′-GAG CAC CAT TCT CTC TAT TGT TA-3′. The RT conditions using the SIV-N-F primer were as follows: 20 min at 42 C, 5 min at 99 C, and 3 min at 5 C (step to add PCR master mix). The PCR cycling protocol was set as follows: 35 cycles of 94 C for 30 sec, 53 C for 30 sec, and 72 C for 45 sec. After the final 8-min extension cycle at 72 C, the samples were stored at 4 C. After completion of thermocycling, samples were electrophoresed and viewed under ultraviolet light. A positive sample revealed an amplification product of about 250 base pairs (bp).

Specificity of primer and probe sets for real-time RT-PCR

Real-time RT-PCR with the M gene primer and probe set was performed with template RNA from influenza virus isolates representing various H subtypes (Table 2), including isolates of avian and swine origin, to demonstrate specificity for type A influenza virus. Real-time RT-PCR with the H1,

H3, N1, and N2 subtype–specific probe sets was also performed with template RNA from various H subtypes listed in Table 2 to demonstrate specificity for their respective subtypes.

Virus titration

Ten-fold serial dilutions of clinical samples (nasal swabs, lung lavage, and lung homogenates) starting at 1:10 were prepared in cell culture infection medium (McCoy medium[n] without serum, supplemented with 5 μg/ml trypsin[o]). The MDCK cells (with medium plus trypsin) were inoculated with the dilutions and incubated in microtiter plates at 37 C for 72 hr. Plates were examined for cytopathic effects after 72 hr. Virus titer was determined by the Reed-Muench method.[14] The RNA for real-time RT-PCR was extracted from virus dilutions at the time of cell inoculation as described above.

Evaluation and comparison of real-time RT-PCR with VI and gel-based RT-PCR

One hundred and eighteen samples were obtained from pigs submitted to the Veterinary Diagnostic Laboratory at Iowa State University. The samples consisted of lung lavage, lung homogenates, and nasal swabs. Isolation of influenza virus from these samples was performed in MDCK cell cultures as described above. For gel-based and real-time RT-PCR, RNA was extracted from each sample as described above. The RNA from each sample was isolated and tested independently for the presence of SIV with real-time RT-PCR using the influenza virus M primer and probe set (National Animal Disease Center) and with the gel-based RT-PCR using the NP-specific primer set (Veterinary Diagnostic Laboratory at Iowa State University). The results of the real-time RT-PCR assay were compiled and statistically analyzed (using the statistical software JMP 5.0.1) with the results of the gel-based RT-PCR and the VI assay by an independent third party.

Results

Analytical sensitivity and specificity of the swine influenza type A–specific real-time RT-PCR assay. The M gene was chosen as the target gene for the influenza A–specific assay. This viral gene is highly conserved in influenza A isolates from various animal species. As previously reported,[22] a M-specific real-time RT-PCR assay was able to detect influenza A viruses from a variety of animal species. Using the reaction conditions described in Materials and Methods, it was possible to reproducibly detect approximately 2 copies of in vitro–transcribed M gene RNA species per reaction (Fig. 1A). When RNA was isolated from lung lavage ($10^{5.5}$ $TCID_{50}$/ml) from an experimentally infected pig, the sensitivity of the assay was 2.5 $TCID_{50}$/ml or 0.05 $TCID_{50}$/reaction. The M gene primer and probe set was tested with RNA obtained from SIV isolates representing all H subtypes circulating in the United States (H3N2, H1N1, and H1N2) and also influenza isolates of avian origin (Table 2). The M primer set was able to detect all type A influenza viruses tested but not other unrelated swine viruses or influenza B virus (Table 2).

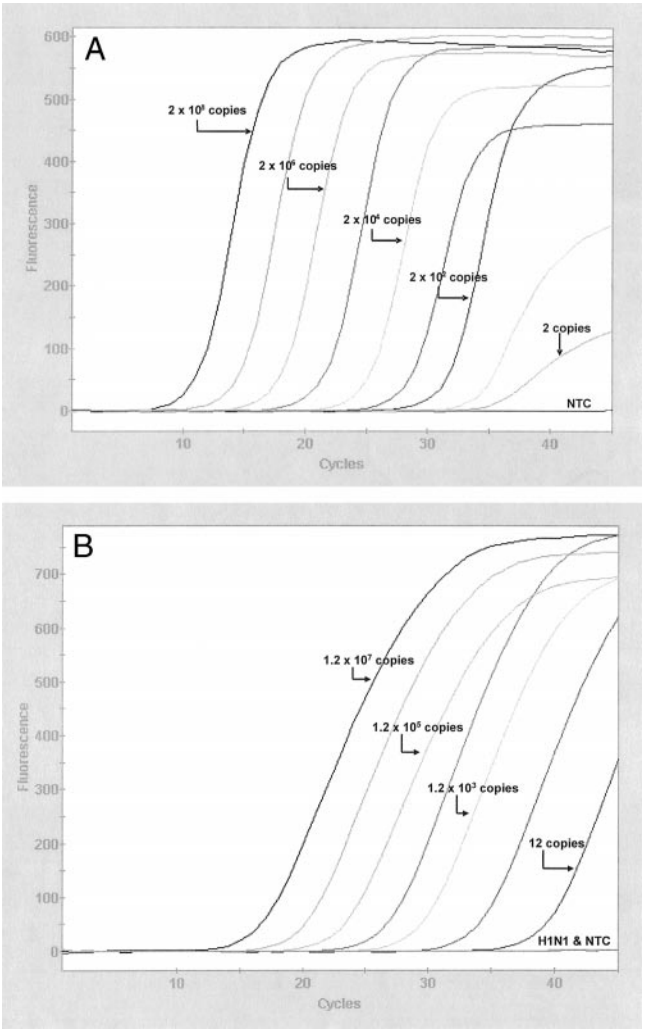

Figure 1. The RT-PCR amplification curves. **A**, RT-PCR amplification curves of 9 concentrations (10-fold dilutions) of M-specific RNA and a no-template control (NTC), and **B**, RT-PCR amplification curves of 7 concentrations (10-fold dilutions) of N2-specific RNA, an NTC, and an H1N1 SIV control.

Analytical sensitivity and specificity of the H- and N-specific real-time RT-PCR assays. The sensitivity of the influenza virus H1, H3, N1, and N2 real-time RT-PCR assays relative to virus titer detectable by standard VI in MDCK cells was determined. The detection limit for the H1 and H3 gene assays was approximately 1 $TCID_{50}$/reaction, for the N1 assay approximately 2 $TCID_{50}$/reaction, and for the N2 assay approximately 0.5 $TCID_{50}$/reaction.

The RNA detection limits for the H- and N-specific probe sets were determined by detection of in vitro–transcribed RNA templates. The minimum copy number of H1 gene RNA that could be detected was approximately 35 gene copies, for the H3 gene RNA

Table 3. Summary of real-time RT-PCR and VI results for 118 individual samples from pigs tested for type A influenza virus by the M-specific real-time RT-PCR assay.

		VI		
		Positive	Negative	Total
Real-time RT-PCR	Positive	17	15	32
	Negative	1	85	86
Total		18	100	118

approximately 100 gene copies, for the N1 gene RNA approximately 300 gene copies, and for the N2 gene RNA approximately 12 gene copies per reaction (Fig. 1B).

The H- and N-specific primer and probe sets were tested with swine and avian influenza isolates representing viruses from relevant H and N subtypes. The H- and N-specific primer and probe sets detected RNA only from virus isolates of their respective subtypes and did not recognize unrelated swine viruses or influenza B virus (Table 2).

Comparison of M-specific real-time RT-PCR with VI and gel-based RT-PCR. The performance of the M-specific real-time RT-PCR assay was compared with the VI assay on MDCK cells with 118 clinical samples (nasal swabs, lung lavage, and lung homogenates) from pigs. Eighty-five samples were negative and 17 were positive by both assays (Table 3). The results of the 2 assays agreed on 102 samples and disagreed on 16 samples; thus, overall apparent agreement was 86%. Most of the discordant samples were positive in real-time RT-PCR and negative in VI (94%). The overall relative sensitivity of the real-time RT-PCR test compared with VI on MDCK cells was 94%, and the relative specificity was 85%. When the real-time RT-PCR was compared with the NP-specific gel-based RT-PCR, the relative sensitivity was 88% and the relative specificity was 87% (data not shown).

Discussion

This study describes a real-time RT-PCR assay for the detection of type A SIVs and the H and N subtypes (H1N1, H3N2, and H1N2) circulating in the United States. This is the first report using this technology specifically for the detection of SIVs. There have been reports on the use of real-time RT-PCR for the detection of human and avian influenza viruses.[20,22] Real-time RT-PCR is less expensive than VI in embryonated eggs or MDCK cells, and the results are available within hours. The M-specific real-time RT-PCR assay was able to detect 2 copies of in vitro–transcribed RNA or 0.05 $TCID_{50}$ per reaction. This remarkable sensitivity of the M-specific real-time RT-PCR assay may be because $TCID_{50}$ reflects only infectious particles, whereas nucleic acid amplification also detects noninfectious virion particles.[7,22] It was not possible to detect such a low copy number with the subtype-specific real-time RT-PCR assays. This is most likely because the amplification product was >200 bp for the H3-specific and both N-specific primer and probe sets (Table 1); this is in contrast to the 100-bp amplicon detected in the M-specific assay (Table 1). It is known that the real-time RT-PCR using hydrolysis-type probes is most efficient when the DNA amplification product size is between 50 and 150 bp.[p,1] Because H and N genes of influenza A viruses are less conserved than the M gene, it was not possible to design nondegenerative H- or N-specific primer and probe sets that amplify a ≤150-bp amplicon and are still specific for their respective subtypes. Interestingly, when a degenerative primer was designed for the amplification of a 109-bp piece of the H1 gene, the sensitivity of the assay was still about 1 log less than the M-specific assay. The M-specific real-time RT-PCR assay showed an overall relative sensitivity of 94% and a relative specificity of 85% when compared with the VI on MDCK cells. Only a single sample was negative by real-time RT-PCR but positive by VI, whereas 15 samples were positive by real-time RT-PCR and negative by VI (Table 3). Although VI was the performance standard used for comparison, this technique does not provide definitive proof that a pig is infected because there are a number of causes (virus inactivated during shipping or by disinfectants; some influenza viruses may not grow to detectable titers in cells or eggs) for false negatives with VI. Therefore, the relative specificity of the real-time RT-PCR may be artificially lower, and some of the real-time RT-PCR–positive discordant samples may be true positives. The H- and N-specific primer and probes sets were found to be specific for their respective viral genes and able to distinguish between their respective SIV subtypes (Table 2). None of these SIV-specific primer and probe sets reacted with unrelated swine viruses or an influenza B virus isolate (Table 2). Therefore, it can be concluded that these assays provide a rapid and feasible alternative to VI or gel-based RT-PCR techniques.

A rapid detection and subtyping method is important to obtain detailed information on the prevalence of defined subtypes of influenza A virus in the US swine population in order to establish effective control and diagnostic measures for the swine industry. It is noteworthy to mention that the real-time RT-PCR assays in this report were able to detect all SIV subtypes currently circulating in the United States. For example, these real-time RT-PCR assays identified recently emerged swine H3N2 isolates that are associated with 3 phylogenetically distinct human-like hemagglutinin molecules, designated cluster I, II, and III (cluster I,

Sw/TX/4199-2/98; cluster II, Sw/CO/23619/99; and cluster III, Sw/OK/18089/99).[15,25] A recent study showed that H3N2 cluster I and cluster III viruses share common epitopes, whereas a cluster II virus showed only limited cross-reactivity.[15] In addition to the H3N2 subtype, newly emerged reassortant H1N1 and H1N2 SIVs were recently found in US pigs, both reassortants between the classical H1N1 and H3N2 viruses (R. Webby, personal communication). The real-time RT-PCR assays in this study identified classical (A/Sw/IA/3421/90, A/Sw/MN/37866/99) and reassortant H1N1 SIVs (A/Sw/NE/45891/00) as well as an H1N2 reassortant SIV (A/Sw/MN/1192/01). This emphasizes the necessity of update surveillance tools in the United States for detection of the increased diversity of SIVs. Although human influenza A viruses do not spread in birds and usually avian influenza A viruses do not spread in humans, the species barrier in pigs is relatively low; pigs might function as mixing vessels for the creation of new pandemic reassortant viruses.[17,18] Therefore, it is of particular interest to know which subtypes are prevalent in local swine populations and to compare them with those prevalent in the human population.

The real-time RT-PCR assays developed in this study could provide a rapid influenza virus diagnosis with easier identification and more rapid subtyping than other methods. These assays are also able to detect concurrent infections with different influenza A virus subtypes in an individual animal. A multiplex real-time RT-PCR with the detection and subtyping of SIV in 1 reaction would be desirable; such an assay should be feasible in the near future with the development and availability of new reporter and quencher dyes.

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Sources and manufacturers

a. QIAamp Viral RNA Mini kit, Qiagen Inc., Valencia, CA.
b. OneStep RT-PCR Kit, Qiagen Inc., Valencia, CA.
c. RNase inhibitor, Promega, Madison, WI.
d. Smartcycler thermocycler and software, Cepheid, Sunnyvale, CA.
e. ABI PRISM® 7900 HT Sequence Detection System, Applied Biosystems, Foster City, CA.
f. BigDye terminator system, Applied Biosystems, Foster City, CA.
g. Ribomax™ Large Scale RNA production system, Promega, Madison, WI.
h. pCRII-TOPO, Invitrogen, Carlsbad, CA.
i. pAMP1 vector, Life Technologies, Rockville, MD.
j. Boeheringer Mannheim, Indianapolis, IN.
k. Amresco Inc., Solon, OH.
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