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One-step real-time RT-PCR for pandemic influenza A virus (H1N1) 2009 matrix gene detection in swine samples

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A B S T R A C T

In the spring of 2009, a novel (H1N1) influenza A virus began to spread among humans worldwide. Although the 2009 H1N1 is related genetically to swine influenza viruses, human infection has not been connected to pig exposure. Because the virus is now circulating widely in the human population, swine herds are at increased risk of becoming infected. In order to investigate potential outbreaks of the 2009 pandemic virus in pigs, a quantitative real-time reverse transcriptase-polymerase chain reaction (qRT-PCR) for the detection of the (H1N1) 2009 RNA in clinical specimens was developed. To evaluate the applicability of the test as a diagnostic tool in the screening of field specimens from swine, 64 field isolates of North American swine, 5 equine and 48 avian influenza viruses collected during diagnostic investigations were analyzed retrospectively as well as samples collected during an experimental *in vivo* infection with two novel H1N1 isolates, A/California/04/2009 (H1N1)v virus and A/Mexico/4108/2009 (H1N1)v. The sensitivity of the qRT-PCR was shown to be higher with respect to standard techniques such as virus isolation and the reproducibility was satisfactory. The present unique and highly sensitive assay is able to detect as little as 1×10^1 copies of RNA per μl of template and it represents a rapid and useful approach for the screening and quantitation of (H1N1) 2009 RNA in porcine specimens.

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1. Introduction

Swine influenza is an acute respiratory disease caused by influenza A viruses that belong to *Orthomyxoviridae*, a family of enveloped negative-sense, segmented, single stranded RNA viruses. Based upon the major differences within the hemagglutinin (HA) and neuraminidase (NA) proteins, 16 HA and 9 NA subtypes have been identified thus far (Rohm et al., 1996; Webster et al., 1992; Fouchier et al., 2005). It is recognized that influenza viruses evolve by reassortment and/or point mutation, thus giving rise to new viral subtypes with different host tropism. In April 2009, a novel swine-lineage influenza virus capable of rapid human transmission was reported, although infection with (H1N1) 2009 was not connected to pig exposure or to a contemporary infection in the swine population (Dawood et al., 2009). This novel pandemic H1N1 possessed a unique genome arrangement. Six genes, including PB2, PB1, PA, HA, NP and NS, cluster together with those belonging to the viruses identified as triple-reassortant swine influenza viruses of the North American lineage, whereas the M and NA genes are derived from Eurasian lineage swine influenza viruses (Dawood et al., 2009). Other than sporadic transmission to humans (Myers et al., 2007), classical swine influenza A viruses of the H1N1 subtype were historically distinct from avian and other mammalian influenza viruses based on host specificity, serotype, and/or genotype (Vincent et al., 2008). Swine influenza virus was first recognized as an agent of respiratory disease in pigs in 1928 (Shope, 1931), and the North American swine influenza virus-lineage genes of the pandemic virus have its genetic origins with this ancestral H1N1. Three predominant swine influenza virus subtypes are currently circulating in US swine following the emergence of the triple reassortant H3N2 in 1998: reassortant H1N1 (rH1N1), H1N2, and H3N2 and their drift mutant derivatives, all containing the triple reassortant internal gene cassette (TRIG) (for review see Vincent et al., 2008).

The novel (H1N1) 2009 is not known to be circulating widely among swine. Pigs have been shown to be susceptible to the human pandemic (H1N1) 2009 infection (Lange et al., 2009; Vincent, unpublished data). The chance of cross-species transmission may lead to serious consequences in terms of human risk of infection by increasing the reservoir of the virus in addition to dramatic costs for the pork industry. Swine have been shown to possess receptors for avian and human influenza viruses in the tracheal epithelium, leading to the suggestion that the pig is a mixing vessel

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Table 1
Primers and probe sequences for 2009 (H1N1) qRT-PCR. The probe was designed using the sequence of A/California/04/2009 (H1N1) (FJ96513).

Specificity	Primer	Sequence 5′–3′
(H1N1) 2009 Matrix Gene	M(76)-For	TCAGGCCCCCTCAAAGCCGA
	M(99)-Probe	FAM[a]-CGCGCAGAGACTGGAAAGTGTC-TAMRA[b]
	M(234)-Rev	GGGCACGGTGAGCGTGAACA

[a] 6-Carboxyfluorescein.
[b] Tetramethylrhodamine.

for the emergence of new subtypes with human pandemic potential (Ito et al., 1998; Scholtissek et al., 1993).

In order to recognize promptly the novel pandemic (H1N1) 2009 in swine, reducing the potential serious economic damage as well as exposure of humans to the virus, the development of a rapid and sensitive test capable of identifying and differentiating the pandemic strain from type A influenza viruses circulating in pigs is necessary. In this manuscript the development of a quantitative real-time reverse transcriptase-polymerase chain reaction (qRT-PCR) using TaqMan technology for the rapid and sensitive detection of pandemic (H1N1) 2009 matrix gene and quantification of viral nucleic acid in diagnostic samples, is reported. For this purpose, field isolates of North American swine, equine and avian influenza viruses were analyzed retrospectively as well as samples (swabs and lavage fluid) collected during an experimental *in vivo* infection with A/California/04/2009 (H1N1)v and A/Mexico/4108/2009 (H1N1)v isolates. Data obtained by the qRT-PCR analysis were compared with those achieved from virus isolation of the clinical samples collected during the *in vivo* study.

2. Materials and methods

2.1. Oligonucleotide design and synthesis

The matrix (M) gene sequences of endemic swine influenza virus isolates, novel pandemic (H1N1) 2009, and sequences from a panel of human and avian type A influenza virus strains, including type A human seasonal strains, were retrieved from the GenBank database (http://www.ncbi.nlm.nih.gov/Genbank/index.html) and aligned using the DNAStar software package (DNAStar Inc., Madison, WI, USA). The primers were designed using the Geneious software (Biomatters, Ltd.) to amplify a conserved 159 bp within the aligned M genes. However, the probe was purposely designed using the sequence of A/California/04/2009 (H1N1) (FJ96513), in a conserved, yet lineage-specific region shared by all pandemic (H1N1) 2009 isolates sequenced thus far. The primers and probe were synthesized by IDT (Coralville, IA, USA). The TaqMan probe was dual-labeled with 6-carboxyfluorescein (FAM) at the 5′ end and with tetramethylrhodamine (TAMRA) at the 3′ end. The position and sequence of primers and probe used for the assay are reported in Table 1.

2.2. Standard RNA for absolute quantification

To obtain a standard for the TaqMan assay, a 1022-bp RT-PCR product containing the full-length M gene of A/California/04/2009 (H1N1) virus was amplified using primer pair M+5 and M-1027 (Hoffmann et al., 2001), and the RT-PCR product was cloned into pGEM®-T easy vector system (Promega, Madison, WI, USA), then linearized and transcribed with RiboMAX™ Large Scale RNA Production System-T7 (Promega, Madison, WI, USA), from the T7 promoter, according to the manufacturer's guidelines. After DNase treatment to remove residues of plasmid DNA, the transcripts were purified using a commercial column (RNeasy kit, Qiagen S.p.A., Germantown, MD, USA) and quantified by spectrophotometric analysis. Tenfold dilutions of the RNA transcript, representing 10^0 to 10^9 copies RNA μl^{-1} of template, were prepared in sterile water, and aliquots of each dilution were frozen at −80 °C. Each aliquot was used only once.

2.3. Field and experimental samples collection, preparation and virus isolation

To evaluate the applicability of the test as a diagnostic tool for the screening of field specimens, 64 field isolates of North American swine, 5 equine and 48 avian influenza viruses, collected during diagnostic investigations and 100 samples collected during an experimental *in vivo* study were examined. The *in vivo* study was conducted in two separate groups of 4-week-old pigs inoculated with two pandemic (H1N1) 2009 isolates, A/California/04/2009 (H1N1)v (pigs 551–565) and A/Mexico/4108/2009 (H1N1)v (pigs 581–595), respectively, kindly provided by the Centers for Disease Control and Prevention (CDC). All pigs came from a herd free of swine influenza virus and porcine reproductive and respiratory syndrome virus (PRRSV). They were treated with ceftiofur crystalline free acid (Pfizer, New York, NY, USA) to reduce bacterial contaminants preceding the start of the study. The two groups were housed in individual isolation rooms at A-BSL3 and cared for in compliance with the Institutional Animal Care and Use Committee of the National Animal Disease Center. Pigs were humanely euthanized with a lethal dose of pentobarbital (Sleepaway, Fort Dodge Animal Health, Fort Dodge, IA, USA) at the appropriate time during the course of the study. Thirty pigs, 15 per group, were inoculated intratracheally with 2×10^5 $TCID_{50}$ of A/California/04/2009 (H1N1)v and 2×10^5 $TCID_{50}$ of A/Mexico/4108/2009 (H1N1)v, both isolated and prepared on MDCK cells. Five pigs remained non-challenged as negative controls. The pigs were anesthetized by intramuscular injection of a cocktail of ketamine (8 mg/kg), xylazine (4 mg/kg) and Telazol (6 mg/kg, Fort Dodge Animal Health, Fort Dodge, IA, USA) followed by virus inoculation. Pigs were observed daily for clinical signs and sample collection. Nasal swabs were taken and placed in 2 ml minimal essential medium (MEM) on 0, 3, 5, and 7 dpi to evaluate nasal virus shedding and stored at −80 °C until the end of the study. Five inoculated pigs per group were euthanized on 3, 5, and 7 dpi and five control pigs were euthanized on 7 dpi. After euthanasia, each lung was lavaged with 50 ml of MEM to obtain bronchioalveolar lavage fluid (BAL fluid). Each nasal swab sample was subsequently thawed and vortexed for 15 s, centrifuged for 10 min at 640 × *g* and the supernatant passed through 0.45 μm filter. Subsequently, 200 μl of the nasal swab sample was then placed on confluent MDCK cells in 24-well plates to incubate for 1 h. After 1 h of incubation the sample was removed and 400 μl MEM w/TPCK trypsin was added. The plate was checked at 24 and 48 h for cytopathic effects. After 48 h, 200 μl of cell culture supernatant from each well of the 24-well plate was subsequently passed onto a confluent 48-well plate after a freeze and thaw cycle. After 48 h evidence of cytopathic effects was evaluated and presence of virus antigen confirmed by immuno-cytochemical staining with an anti-influenza A nucleoprotein monoclonal antibody as described previously (Kitikoon et al., 2006). Tenfold serial dilutions in serum-free MEM supplemented with TPCK trypsin and antibi-

otics were made with each BAL fluid sample. Each dilution was plated in triplicate in 100 μl volumes onto PBS-washed confluent MDCK cells in 96-well plates. Plates were evaluated for CPE between 48 and 72 h post-infection. At 72 h, plates were fixed with 4% phosphate-buffered formalin and stained using immunocytochemistry as above.

RNA isolation was carried out from cell culture-grown virus isolates, nasal swab filtrates in MEM, and BAL fluid with the MagMAX™-96 Total RNA isolation kit (Ambion, Austin, TX, USA) in accordance with the manufacturer's protocol. Template RNA was eluted in 60 μl of buffer and stored at −80 °C prior to use.

2.4. One-step real-time RT-PCR

Duplicates of each RNA sample and standard were amplified by the qRT-PCR assay performed on a 7500 Real-Time PCR System (Applied Biosystem) with the AgPath-ID™ One-Step RT-PCR Kit (Ambion, Austin, TX, USA) with ROX added as passive reference dye. The 25 μl reaction volume for each sample contained 8 μl of extracted RNA, 12.5 μl of AgPath Kit 2× buffer, 1 μl of AgPath 25× enzyme mix, 500 nM of primers M(76)-For and M(234)-Rev, 100 nM of M(99)-probe, 1.67 μl of AgPath Detection Enhancer and 1.08 μl of ultrapure DNase–RNase-free distilled water. The thermal profile consisted of a single cycle of reverse transcription for 10 min at 45 °C and 10 min at 95 °C for reverse transcriptase inactivation and DNA polymerase activation. The amplification of cDNA was performed by 45 cycles including denaturation at 95 °C for 15 s, annealing for 1 min at 54 °C and extension at 72 °C for 15 s. The increase in fluorescent signal was registered during the annealing step of the reaction and the data were analyzed with sequence detector software (7500 System Software v.1.3.1, Applied). Data reported represent the average of the duplicates for each sample and standard.

2.5. Evaluation of qRT-PCR performance

In order to exclude cross-reactivity between pandemic (H1N1) 2009 and other viruses responsible for respiratory diseases of pigs, (H1N1) 2009 qRT-PCR test specificity was evaluated by analysis of the following: endemic swine influenza virus, avian and equine influenza viruses, coronaviruses (porcine respiratory coronavirus (PRCoV), transmissible gastroenteritis virus (TGEV)), PRRSV, porcine circovirus type 2 (PCV2), porcine adenovirus, porcine parvovirus, blue-eye paramyxovirus and pseudorabies virus. Nasal swab and BAL fluid samples collected from five uninfected pigs as well as sterile water were also included in the analysis as negative controls and no-template controls, respectively. To determine the detection limit of the (H1N1) 2009 qRT-PCR assay, 10-fold dilutions of a BAL fluid sample containing 1×10^7 copies of A/Mexico/4108/2009 (H1N1)v RNA μl^{-1} were made and subsequently analyzed. Serial 10-fold dilutions of standard RNA which contained from 10^1 to 10^9 copies of RNA transcript and the corresponding C_T values were used to plot the standard curve for the pandemic (H1N1) 2009 RNA absolute quantification.

Reproducibility of the assay was evaluated by testing several clinical samples containing A/California/04/2009 (H1N1)v RNA quantities that included the full range covered by the qRT-PCR. The intra-assay reproducibility was measured by testing the same samples 10 times in the same experiment, whereas the inter-assay reproducibility was confirmed by testing the same samples in 10 independent experiments. Coefficients of variation (CVs) were calculated by dividing the standard deviation of each tested sample by its mean and multiplying that result by 100 (Decaro et al., 2004, 2005).

2.6. USDA-validated qRT-PCR

Swine and equine influenza virus isolates and the clinical samples from pigs infected experimentally with 2009 (H1N1)v were subjected to the USDA-validated qRT-PCR procedure for the general detection of type A influenza virus RNA (matrix screening assay), following procedures described previously (Spackman and Suarez, 2008).

3. Results

3.1. Performance of the (H1N1) 2009 qRT-PCR assay

The no-template controls and 2009 (H1N1) negative specimens did not produce detectable fluorescence signal. The detection limit of the assay was assessed as 1×10^1 RNA copies μl^{-1}, whereas, in general, gel-based RT-PCR is limited generally to detect to 1×10^2 copies μl^{-1} of template. Tenfold dilutions of standard RNA were used to create a standard curve representing 10^1 to 10^9 copies of viral RNA standards and linearity was observed over the entire quantitation range (slope = −3.45). The coefficient of linear regression (R^2) was 0.998. In order to verify the reproducibility of the assay, intra-assay and inter-assay CVs were calculated and satisfactory results were obtained. Intra-assay CVs ranged from 22% (samples containing 5×10^7 RNA copies) to 44% (samples containing 2×10^2 RNA copies), whereas the inter-assay CVs ranged from 27% (2×10^3 RNA copies) to 51% (2×10^2 RNA copies). North American swine influenza virus isolates as well as equine influenza virus isolates were successfully detected by the USDA-validated qRT-PCR (data not shown). All endemic North American swine influenza virus isolates were negative for (H1N1) 2009 specific matrix gene RNA using the present qRT-PCR assay, whereas the (H1N1) 2009 strains used as positive control were positive. Cross-reactivity with other extant swine viral pathogens was not detected. Amplification of equine and avian influenza virus for (H1N1) 2009 remained below detection threshold, although weak cross-reactivity was observed at later cycle numbers. A single avian influenza isolate (A/Mynah/Mass/71 H4N8) was detected by (H1N1) 2009 qRT-PCR.

3.2. Isolation from clinical specimens

In the group of pigs infected with A/California/04/2009 (H1N1)v, 29/30 nasal swabs (Table 2) and 10/15 BAL fluids (Table 3) were positive for isolation on cell culture. One nasal swab sample as well as all BAL fluid samples at 7 dpi were negative by virus isolation. In the group infected with A/Mexico/4108/2009 (H1N1)v virus, virus was isolated from 18/30 nasal swabs and from 10/15 BAL fluid samples. Seven nasal swabs collected at 3 dpi, 5 collected at 7 dpi, and all BAL fluids collected at 7 dpi were negative for virus isolation.

3.3. qRT-PCR analysis of clinical samples

The qRT-PCR results from the clinical specimens from the *in vivo* study are summarized in Tables 2 and 3. When tested with the (H1N1) 2009 qRT-PCR assay, 78 clinical specimens were positive with similar C_T values as the USDA-validated qRT-PCR assays (data not shown). Briefly, 29/30 nasal swabs collected from the group infected with A/California/04/2009 (H1N1) virus and 19/30 nasal swabs collected from the group infected with A/Mexico/4108/2009 (H1N1) virus were positive by (H1N1) 2009 qRT-PCR. All BAL fluids samples were positive for both groups (15/15). The number of samples in agreement between the (H1N1) 2009 qRT-PCR and virus isolation (VI) were 63/78 including nasal swab and BAL fluid samples. In addition, 14 samples were positive by using (H1N1)

Table 2
Quantity of viral RNA copies per μl template extracted from nasal swabs collected at day (d) 3, 5, 7 p.i. from pigs infected with A/California/04/2009 (H1N1)v (pigs 551–565) and A/Mexico/4108/2009 (H1N1)v (pigs 581–595). Nasal swabs collected at day 0 p.i. with negative results are not reported in the table as well as negative control groups. VI, virus isolation; NTC, no-template control; NS, nasal swab.

Pig number	VI	2009 (H1N1) qRT-PCR
551 d3	+	8.49×10^1
552 d3	+	1.06×10^2
553 d3	+	7.79×10^2
554 d3	+	2.66×10^2
555 d3	+	1.78×10^2
556 d3	+	6.92×10^2
557 d3	+	2.65×10^2
558 d3	+	1.27×10^2
559 d3	+	7.19×10^2
560 d3	+	–
561 d3	+	1.00×10^1
562 d3	+	1.00×10^1
563 d3	+	1.80×10^2
564 d3	+	1.00×10^1
565 d3	+	1.00×10^1
556 d5	+	6.87×10^3
557 d5	+	1.13×10^4
558 d5	+	9.51×10^3
559 d5	+	6.07×10^3
560 d5	+	2.50×10^4
561 d5	+	3.86×10^2
562 d5	+	2.01×10^3
563 d5	+	4.29×10^3
564 d5	+	9.14×10^3
565 d5	+	1.18×10^3
561 d7	+	1.00×10^1
562 d7	–	2.00×10^1
563 d7	+	1.00×10^1
564 d7	+	1.00×10^1
565 d7	+	2.98×10^1
NTC		–
581 d3	+	3.85×10^2
582 d3	+	1.30×10^2
583 d3	–	1.00×10^1
584 d3	–	1.00×10^1
585 d3	+	6.59×10^3
586 d3	–	–
587 d3	–	–
588 d3	–	–
589 d3	+	1.00×10^1
590 d3	–	–
591 d3	+	6.60×10^2
592 d3	+	9.01×10^2
593 d3	–	1.40×10^1
594 d3	+	–
595 d3	+	2.00×10^1
586 d5	+	1.61×10^2
587 d5	+	7.75×10^2
588 d5	+	2.10×10^2
589 d5	+	2.85×10^3
590 d5	+	–
591 d5	+	2.75×10^2
592 d5	+	2.16×10^2
593 d5	+	1.00×10^1
594 d5	+	1.59×10^1
595 d5	+	4.25×10^1
591 d7	–	–
592 d7	–	–
593 d7	–	–
594 d7	–	–
595 d7	–	–
NTC		–

2009 qRT-PCR and negative by using VI whereas only 3 samples were positive by VI and negative by (H1N1) 2009 qRT-PCR. The samples analyzed by the (H1N1) 2009 qRT-PCR contained a wide range of 2009 (H1N1) RNA copies per μl of template, from 2.56×10^2 to 1.14×10^7 (BAL fluid), and from 1×10^1 to 2.5×10^4 (nasal swabs).

Table 3
Quantity of viral RNA copies per μl template extracted from BAL fluids at day (d) 3, 5, 7 p.i. from pigs infected with A/California/04/2009 (H1N1)v (pigs 551–565) and A/Mexico/4108/2009 (H1N1)v (pigs 581–595). BAL fluid samples collected from the negative control group with negative results are not reported in the table. VI, virus isolation; NTC, no-template control.

Pig number	VI	2009 (H1N1) qRT-PCR
551 d3	+	2.90×10^6
552 d3	+	2.00×10^6
553 d3	+	9.67×10^4
554 d3	+	2.29×10^5
555 d3	+	1.48×10^6
556 d5	+	9.24×10^5
557 d5	+	2.65×10^5
558 d5	+	3.12×10^5
559 d5	+	9.00×10^4
560 d5	+	1.41×10^6
561 d7	–	1.50×10^4
562 d7	–	2.20×10^3
563 d7	–	4.78×10^3
564 d7	–	6.19×10^3
565 d7	–	5.71×10^5
NTC		–
581 d3	+	3.77×10^6
582 d3	+	1.73×10^5
583 d3	+	8.66×10^5
584 d3	+	2.08×10^5
585 d3	+	1.81×10^6
586 d5	+	1.21×10^5
587 d5	+	7.00×10^5
588 d5	+	5.00×10^5
589 d5	+	1.00×10^5
590 d5	+	1.14×10^7
591 d7	–	1.26×10^4
592 d7	–	1.77×10^3
593 d7	–	4.00×10^3
594 d7	–	3.50×10^4
595 d7	–	2.56×10^2
NTC		–

4. Discussion

The (H1N1) 2009 is of significant human and swine health concern and the future role of pigs in the ecology of this newly emerged virus remains unknown. There is an immediate and critical need for a rapid differential diagnostic method for pandemic (H1N1) 2009 virus detection in swine. Pandemic (H1N1) 2009 isolated from humans has had limited detection in the swine population so far (http://www.oie.int/eng/en_index.htm). However, pigs are susceptible to the infection, as demonstrated by the clinical signs and viral loads that found in nasal swabs and BAL fluids at day 3, 5, and 7 dpi. Similar results have been described by others (Lange et al., 2009). It is likely that the virus will continue to jump from humans to naïve pigs and may become established as an endemic infection in the swine population. In that case, two consequences will be obvious: first, a reservoir of H1N1 virus in the swine population poses an elevated risk for human infection via aerosol transmission from clinically ill pigs, and second, dramatic economic losses for the pork industry due to direct disease related costs as well as indirect market losses. The long-term consequence is the increased chance for novel reassortment between endemic swine influenza viruses and the novel H1N1 in the swine host, posing further human and animal health risks. It is apparent that pigs may be infected at least transiently with wholly avian and/or human viruses, allowing reassortment with swine viruses to acquire avian and/or human virus gene segments (Karasin et al., 2000; Ma et al., 2007). The (H1N1) 2009 underscores the potential risk to the human population of other influenza virus subtypes and genotypes with the swine influenza virus TRIG backbone. Increased surveillance and monitoring for the (H1N1) 2009 as well as other swine influenza virus in both the swine and human populations are critical to under-

stand the dynamic ecology of influenza A viruses in susceptible host populations.

In the event of the pandemic virus spread in the swine population, the assessment of a specific innovative diagnostic tool that permits a rapid identification of the pandemic (H1N1) 2009 in pigs is an absolute necessity. In fact, a sensitive and specific diagnostic test is critical for the implementation of response measures to outbreaks in swine to reduce human risk of infection. The qRT-PCR assay described is able to detect the new pandemic (H1N1) 2009 viral RNA with the ability to differentiate the new lineage from the extant swine influenza viruses circulating in the North American swine population. The assay was shown to be reproducible and linear over a range of 9 orders of magnitude, from 10^1 to 10^9 RNA copies, thus ensuring an accurate measurement of (H1N1) 2009 viral loads in clinical samples. If compared with the classical gel-based RT-PCR protocol, the processing time required by TaqMan RT-PCR is shorter, the contamination risks are lower because of the lack of post-amplification steps, and the specificity is enhanced by the probe hybridization. The specificity of the (H1N1) 2009 qRT-PCR was assessed against a set of viruses associated with respiratory disease in swine, including endemic North American swine influenza virus isolates, PRCoV, TGEV, PRRSV, porcine circovirus type 2, swine adenovirus, porcine parvovirus, blue-eye paramyxovirus and pseudorabies virus. Cross-reaction was not identified in swine specimens, thus providing evidence for high fidelity of the assay for the exclusive detection of (H1N1) 2009 in clinical samples from swine. There is a potential to detect matrix genes not of the classical swine lineage, especially those of avian lineage including the avian-like Eurasian lineage viruses. Since no Eurasian avian-like matrix genes have been reported in the US, any identification of avian-like or equine-like matrix genes would be a novel finding and should be investigated by further molecular diagnostics such as sequencing. Importantly, no endemic US swine influenza viruses tested here were shown to have the Eurasian swine influenza virus-lineage matrix gene, indicating the (H1N1) 2009 virus was not circulating in the US prior to 2009 based on current knowledge. Further testing of swine influenza virus repositories at veterinary diagnostic laboratories is warranted to rule out the existence of this lineage of viruses in North America prior to 2009.

The (H1N1) 2009 qRT-PCR was shown to be more sensitive with respect to VI in pigs inoculated experimentally over the duration of the shedding period. Indeed, 14 samples were demonstrated to be negative by VI but positive by qRT-PCR. However, 3 nasal swabs positive by VI were negative when tested by both qRT-PCR assays. The qRT-PCR may not be more sensitive than VI early in the course of infection when viral titers are extremely low, but it is more rapid and more specific and was more sensitive later in the course of infection. Additional testing in the diagnostic laboratory setting is necessary to compare further the (H1N1) 2009 qRT-PCR with VI. Indeed, the titers of the three 2009 qRT-PCR-negative samples were very low, $10^{0.5}$, $10^{0.7}$ and $10^{1.3}$ $TCID_{50}$/ml. VI is recognized as the gold standard for the detection of influenza viruses but is time-consuming and labor intensive, and lacks specificity; thus the qRT-PCR assay described here can be useful in (H1N1) 2009 outbreaks, experimental challenge studies, and vaccine trials as well. Although a large collection of (H1N1) 2009 virus isolates were not available in our laboratories for testing, analysis of published M-gene sequences of strains from worldwide geographical areas allowed us to pinpoint a novel lineage-specific nucleotide sequence for diagnostic development. The sensitivity of the (H1N1) 2009 qRT-PCR is comparable to the canonical USDA-validated type A influenza virus assay reported by Spackman and Suarez (2008), thus encouraging the use of both assays, first for influenza A screening, followed by differentiation and quantification of pandemic (H1N1) 2009 RNA in clinical samples as described here. In fact, while the type A influenza virus real-time RT-PCR matrix screening assay is able to detect all viral isolates tested in this study, the (H1N1) 2009 qRT-PCR selectively detects only the novel pandemic (H1N1) 2009 viral RNA. This assay can be a powerful tool in the diagnostic laboratory setting for specific simultaneous analysis of up to 96 samples on the same plate in minimal time.

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