DOI:10.1111/j.1750-2659.2010.00180.x
www.influenzajournal.com

Original Article

Rapid detection of the pandemic 2009 H1N1 virus M gene by real-time and gel-based RT-PCR assays

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Accepted 31 August 2010. Published Online 6 October 2010.

Background Since the first pandemic 2009 H1N1 (pH1N1) virus was isolated from humans, it has also been detected in other mammalian (pigs, cats, dogs, ferrets) and avian (turkey) species, most likely because of cross-species transmission from humans. The pH1N1 contains six genes derived from swine influenza viruses (SIVs) currently circulating in North America of human- (PB1), avian- (PB2, PA), and swine- (HA, NP, and NS) origin and two genes (NA and M) derived from Eurasian SIVs. The novel genetic composition of pH1N1 necessitates development of novel molecular and serological assays to differentiate the pH1N1 virus from circulating human, swine, turkey, canine, and feline influenza viruses.

Methods To detect and discriminate the pH1N1 from currently circulating SIVs in North America, we developed and evaluated a TaqMan probe-based real-time and a gel-based RT-PCR assay, both targeting the pH1N1 matrix gene.

Results The real-time and gel-based RT-PCR assays were able to specifically detect the pH1N1 M gene and differentiate it from SIVs circulating in North America, including the classical and novel human-like H1N1 influenza virus as well as H1, H2, and H3 subtype triple reassortant SIVs. Both assays were highly sensitive and specific for the pH1N1 virus.

Conclusions The newly developed pH1N1-specific real-time and gel-based RT-PCR assays can be used to detect and differentiate the pH1N1 virus from currently circulating SIVs in North America. We suggest a combinational diagnostic approach where the real-time RT-PCR is used for high-throughput detection of influenza positive or suspect samples and the gel-based RT-PCR for confirmation and sequencing of the M-gene.

Keywords Differentiation, North America swine influenza virus, pandemic 2009 H1N1, real-time and gel-based RT-PCR.

Please cite this paper as: Ma *et al.* (2010) Rapid detection of the pandemic 2009 H1N1 virus M gene by real-time and gel-based RT-PCR assays. Influenza and Other Respiratory Viruses 4(6), 397–403.

Introduction

The pandemic 2009 H1N1 (pH1N1) virus was first isolated from humans in North America in April 2009. This virus spread very rapidly around the world, resulting in laboratory confirmed infection of humans in more than 70 countries within 2 months; subsequently, the WHO declared it a pandemic on June 11, 2009. As of 14 March 2010, the pH1N1 virus has been confirmed in more than 213 countries and territories and has caused the death of 16 813 persons worldwide.[1] The genome of the pH1N1 contains six genes (PB1, PB2, PA, HA, NP, and NS) from currently circulating North American (N.A.) swine influenza viruses (SIVs) and two genes (NA and M) from Eurasian SIVs.[2] It is a triple reassortant virus containing genes from human (PB1), avian (PB2, PA), and swine (HA, NP, NA, M, NS) influenza viruses. This novel reassortant pH1N1 virus was not found previously in humans and other species.[3,4] The pH1N1 has been transmitted most likely from humans to other species including pigs, ferrets, turkeys, dogs, and cats.[5,6] Importantly, the pH1N1 was shown to be pathogenic and easily transmissible in pigs.[7] Since the first triple reassortant SIV belonging to the H3N2 subtype was isolated in 1998 from pigs in North America,[8] SIVs with this genetic constellation belonging to the H1N1, H1N2, H2N3, H3N1 in addition to the H3N2 subtypes have been isolated from N.A. pigs,[9–14] and the H1N1, H1N2, and H3N2 subtypes have become the majority of SIVs circulating in N.A. swine.[9,15–17] The triple reassortant SIVs contain an internal gene constellation consisting of swine- (NP, M, NS), human- (PB1), and avian-origin (PA and PB2) influenza genes resulting in a virus that is well transmitted and maintained, and also known as the triple reassortant internal gene (TRIG) cassette.[17,18] In contrast, the composition of

the external genes (HA and NA) is less conserved, which is reflected by multiple reassortant events that have produced viruses with different combinations of HA and NA genes (e.g. H1N1, H1N2, H2N3, H3N1, and H3N2).[17] The triple reassortant H1N1 SIVs have been known to infect humans in the USA sporadically; most cases of zoonotic transmission are directly related to exposure to pigs and can cause severe flu-like illness in some of the infected patients.[19,20] The pH1N1 virus has been shown to cross the species barrier and infect pigs in North America[21] and other pig-rearing countries (e.g. Australia, Canada, Germany, United Kingdom, France, Japan, Italy, Thailand), and has the potential to transmit between pigs and be maintained in swine herds;[22] for this reason, it is important to develop rapid diagnostic tools to detect a potential transmission of the pH1N1 virus from humans or other sources to pigs. As infections with triple reassortant SIVs are very common in N.A. swine herds, it is crucial to discriminate the triple reassortant pH1N1 virus from the currently circulating SIVs. In this study, we developed pH1N1-specific real-time and gel-based PCR assays based on the genetic diversity of the N.A. and Eurasian swine influenza matrix gene; both rapid tests are able to detect the pH1N1 virus M gene in a highly specific and sensitive manner and differentiate them from currently circulating N.A. SIVs including H1N1, H1N2, H3N2, and H2N3 SIV subtypes.

Materials and methods

Virus and bacterial isolates

Two pH1N1 influenza viruses, one derived from a human patient (A/CA/04/09; CA09) and the other one derived from a pig (A/Swine/Alberta/25/2009; Alb09), were propagated in 10-day-old SPF embryonated chicken eggs. In addition, other swine viral and bacterial pathogens were used: porcine reproductive and respiratory disease virus (PRRSV), porcine circovirus type 2 (PCV-2), transmissible gastroenteritis virus (TGEV), porcine parvovirus (PPV), classical H1N1 SIV (cH1N1; A/Swine/IA/15/1930), triple reassortant H1N1 (trH1N1; A/Swine/Kansas/77778/2007) SIV and triple reassortant H3N2 (trH3N2; A/Swine/Texas/4199-2/98) SIV, human-like triple reassortant H1N1 (hu-like H1N1; A/Sw/MN/07002083/07) SIV, triple reassortant H2N3 (A/Swine/Missouri/4296424/2006) SIV. Swine bacterial pathogens included *Actinobacillus pleuropneumoniae* (*A. pleuropneumoniae)* serotypes 1 and 5, *Streptococcus suis* (*S. suis*) and *Haemophilus parasuis* (*H. parasuis)* were used to test the diagnostic specificity of the newly developed RT-PCR assays.

Primers and probes

One set of primer and probe specific for a defined region of the matrix gene of the pH1N1 virus was designed to detect the viruses containing the pH1N1 M gene based on sequence information available in GenBank (Table 1). Probes were labeled with 6-carboxyfluorescein and with 6-carboxytetramethylrhodamine at the 5′ and 3′ ends, respectively, and purchased from a commercial vendor (IDT, Coralville, IA). The primers for the gel-based RT-PCR targeting the matrix gene of the pH1N1 virus were designed based on the sequence information from GenBank. Richt et al have reported in a previous study[23] that the primers and probe for the N.A. SIV real-time RT-PCR showed both a high specificity (85%) and sensitivity (94%) when compared with virus isolation, and this assay has been used in diagnostic settings by various veterinary diagnostic laboratories throughout the USA. Nucleotide information of each primer or probe is summarized in Table 1.

Experimental and clinical samples

Nasal swab (Polyester-Tipped; Fisher Scientific, Hampton, NH) samples were collected from pigs and transported using the viral transport medium based on a formulation recommended by the WHO animal influenza training

Table 1. Primers and probe used in real-time RT-PCR assay and primers used in the gel-based RT-PCR

	Primers/probe sequence	Location of primers*	Amplicon (bp)
Real-time RT-PCR pH1N1 specific	Pan-M-Probe: 5′- TTG CAT GGG CCT CAT ATA CAA C-3′		92
	Pan-M-F: 5′- GGT GTC ACT AAG CTA TTC AA-3′	342–361	
	Pan-M-R: 5′- CAA AAG CAG CTT CTG TGG TC-3′	414–433	
Gel-based RT-PCR pH1N1 specific	CA-M-Fw: 5′- GGT GTC ACT AAG CTA TTC AAC-3′	342–362	218
	CA-M-Rw: 5′- TTG CCG TAG TGC TAG CCA GC-3′	540–559	
Real-time RT-PCR N.A. SIVs[23]	NA_SIV-M-Probe: 5′- TCA GGC CCC CTC AAA GCC GA-3′		100
	N.A._SIV-M-F: 5′- AGA TGA GTC TTC TAA CCG AGG TCG -3′		
	N.A._SIV-M-R: 5′- TGC AAA AAC ATC TTC AAG TCT CTG-3′		

*Location of primers was calculated based on the ORF of the Matrix 1 gene.

manual.[24] Fifteen nasal swabs (10 swabs from day 5 post-infection and 5 swabs from day 7 post-infection) were collected from pigs challenged intratracheally with the human-derived pH1N1 CA09 virus. Fifteen nasal swabs (10 swabs from day 5 post-infection and 5 swabs from day 7 post-infection) were collected from pigs challenged with the pig-derived pH1N1 Alb09 virus. Thirty-two clinical samples (nasal swabs and lung tissues) from pigs collected by the Kansas State University Veterinary Diagnostic Laboratory (KSUVDL) were used in this study.

Virus isolation

For virus isolation, 10% lung homogenates from pigs collected by the KSUVDL were centrifuged for 10 minutes at 640 × ***g***. The supernatant was passed through 0·45-μm filters (Fisher Scientific, Denver, CO, USA) to remove any bacterial contamination and was inoculated onto monolayers of Madin-Darby canine kidney (MDCK) cells in 24-well plates. Similarly, nasal swab samples were also passed through 0·45-μm filters to remove any bacterial contamination and were inoculated onto monolayers of MDCK cells in 24-well plates. The MDCK cells were maintained in Eagle's Minimum Essential Medium (MEM) containing 1 μg/ml TPCK-trypsin (Sigma, St. Louis, MO, USA) and 0·3% bovine albumin (Sigma). The plates were incubated at 37°C in a 5% CO_2 incubator and observed daily. Plates were evaluated for cytopathic effects (CPE) after 48–72 hours. After CPEs were observed, the supernatant of infected cells was harvested for the further analysis.

RNA/DNA preparation and RT-PCR

To prepare RNA from nasal swabs, other clinical samples, SIVs (cH1N1, trH1N1, trH3N2, hu-like H1N1, H2N3), PRRSV, and TGEV, 50 μl of RNA was extracted using the QIAamp viral RNA kit (Qiagen, Valencia, CA, USA) from 140 μl of the respective sample. For PPV, PCV2 and swine bacterial pathogens, 200 μl of sample was utilized to extract DNA by QIAamp DNA kit (Qiagen); the extracted DNA was finally diluted in 200 μl of ddH_2O. RNA was extracted from known pH1N1 virus in parallel with other samples as a control to confirm RNA extraction efficiency.

A total reaction volume of 25 μl was used for the real-time RT-PCR assay targeting the pH1N1 matrix gene employing the Qiagen OneStep RT-PCR kit (Qiagen). The assay was performed on a Smart Cycler II (Cepheid, Sunnyvale, CA, USA) using a Qiagen OneStep RT-PCR master mix with modifications: 5 μl of 5× buffer, 2 μl of dNTP mix (10 mm), 0·625 μl of forward primer (20 μm), 0·625 μl of reverse primer (20 μm), 0.375 μl of probe (10 μm), 0·2 μl of BSA (25 μg/μl), 1·25 μl of $MgCl_2$ (50 mm), 0·25 μl of RNase inhibitor (10 U/μl), 2·0 μl of enzyme mix, 4·675 μl of RNase-free water, and 8 μl of template. Eight microliters of RNA extracted from the pH1N1 virus was used as a positive control and 8 μl of RNase-free water used as a negative control. The thermocycling conditions for the pH1N1 M-specific real-time RT-PCR were set as follows: 50°C for 30 minutes, 95°C for 15 minutes, then 40 cycles of 94°C for 15 seconds and 60°C for 1 minute. Based on the detection limit of the assay for the *in vitro* transcribed pH1N1 M-specific RNA, the cutoff for the real-time RT-PCR assay was set at 35 threshold cycles (Ct).

The gel-based RT-PCR assay was conducted using a two-step RT-PCR as follows: 20 μl RNA extracted from either virus stocks or nasal and clinical samples was transcribed into cDNA using Superscript II (Invitrogen, Carlsbad, CA, USA) according to the protocol provided by the manufacturer utilizing 1 μl (20 μm) of Uni-12 (5′-AGCAAAAGCAGG-3′) primer in a 50-μl reaction volume. The reverse transcription (RT) reaction was performed at 42°C for 50 minutes. Ten microliters of total 50 μl of the RT-product (or 10 μl DNA extracted from PPV, PCV2 and the swine bacterial pathogens) was used for the PCR. The cDNA (RNA viruses) or DNA (DNA viruses and bacterial pathogens) was amplified by utilizing *Taq* DNA polymerase (Invitrogen) according to the manufacturer's protocol. The reaction volume was 25 μl total: 2·5 μl of 10× PCR buffer, 1 μl of dNTPs (10 mm), 1 μl of $MgCl_2$ (50 mm), 0·5 μl of forward primer (20 μm), 0·5 μl of reverse primer (20 μm), 0·5 μl of *Taq* enzyme (5 U/μl), 9 μl of ddH_2O and 10 μl of RT-product. The amplification condition consisted of 94°C for 3 minutes followed by 30 cycles of 94°C for 30 seconds, 62°C for 45 seconds, and 72°C for 60 seconds. A final extension step at 72°C for 7 minutes was performed. Ten microliters of 25 μl total of PCR product was analyzed on a 1% agarose gel containing ethidium bromide.

Amplification, cloning and *in vitro* transcription of the M-gene

To produce *in vitro* transcribed pH1N1 M-specific RNA for testing the sensitivity of the developed RT-PCR assay, the M gene from the pH1N1 CA09 virus was amplified employing a two-step RT-PCR using the universal primers as described previously[25] and the amplicon cloned into the pCRII-Topo Vector (Invitrogen). The cloned DNA was called Topo-CA09-M, and the correct sequence of the insert was confirmed by conventional sequencing. After linearization of the Topo-CA09-M plasmid with *Hind* III, the linearized plasmid was transcribed using T7 *in vitro* transcription kit (Promega, Madison WI, USA). The *in vitro* transcribed RNA was treated with DNase to remove all residual plasmid DNA and subsequently purified by Trizol extraction. The concentration of the transcribed RNA was determined by Nanodrop spectrophotometry (Thermo Scientific, Asheville, NC, USA), and copy numbers were

calculated based on an OD_{260} value and the molecular weight of the M-specific RNA (Qiagen: October 2004, *QuantiTect probe PCR handbook*, p. 41).

Assessment of test performance

The analytic specificity of the newly developed real-time and gel-based RT-PCR assays targeting the pH1N1 matrix gene was evaluated for cross-reactivity with common swine viral pathogens (PRRSV, PCV-2, PPV, TGEV, cH1N1 SIV, trH1N1 and trH3N2 SIVs, hu-like H1N1 SIV, H2N3 SIV) and bacterial pathogens (*A. pleuropneumoniae* serotypes 1 and 5, *S. suis* and *H. parasuis*). The analytic sensitivity of the assays was assessed by using *in vitro* transcribed RNA of the M gene. Thirty nasal swab samples collected from pigs challenged with either the pH1N1 CA09 or Alb09 viruses, and 32 clinical samples collected by the KSUVDL were used to assess the diagnostic performance of the newly developed pH1N1 M-specific real-time RT-PCR assay and compared with the results obtained from gel-based RT-PCR and conventional virus isolation.

Results

Analytic performance of pH1N1-specific real-time and gel-based RT-PCR assays

The pH1N1 M-specific real-time RT-PCR assay is highly specific for the pH1N1 virus. No positive results were obtained when the pH1N1-specific real-time assay was used on RNA or DNA derived from samples containing PRRSV, PCV-2, TGEV, PPV, cH1N1 SIV, trH1N1 and H3N2 SIVs, huH1N1 SIV, H2N3 SIV, *A. pleuropneumoniae* serotypes 1 and 5, *S. suis* and *H. parasuis* (Table 2). Only samples derived from the CA09 and Alb09 virus stocks were positive using the real-time RT-PCR assay (Table 2). These results demonstrate that the real-time RT-PCR assay is highly specific for the pH1N1 virus and does not react with other relevant swine pathogens including other SIVs of various subtypes currently circulating in the USA. Under the reaction conditions used in the current study, it was possible to detect approximately two copies of the matrix gene as determined with *in vitro* transcribed RNA molecules (Table 3). Similarly, the pH1N1-specific gel-based RT-PCR assay also targets the matrix gene and reacted only with samples derived from the pH1N1 virus stocks (CA09 and Alb09); no cross-reactivity with currently circulating SIVs in North America and other relevant viral and bacterial swine pathogens was detected using the newly established pH1N1-specific gel-based RT-PCR assay (Figure 1). The gel-based assay was less sensitive than the real-time RT-PCR assay and was able to detect approximately 200 copies of the matrix gene as determined with *in vitro* transcribed RNA molecules (Table 3).

Table 2. Specificity of the pH1N1 M-gene-specific real-time and gel-based RT-PCT assays

	pH1N1		Other swine viral bacterial pathogens										
	CA09	Alb09	cH1N1	trH1N1	trH3N2	Hu-like H1N1	H2N3	PPV	PRRSV	TGE	PCV2	*Streptococcus suis*	*APP**	*Haemophilus parasuis*
Real-time RT-PCR	+	+	–	–	–	–	–	–	–	–	–	–	–	–
Gel-based RT-PCR	+	+	–	–	–	–	–	–	–	–	–	–	–	–

+, positive; –, negative.
**APP, Actinobacillus pleuropneumoniae* serotypes 1 and 5.

Table 3. The analytical sensitivity of the pH1N1 M-gene-specific real-time and gel-based RT-PCR assays

RNA molecules of matrix gene	Real-time RT-PCR	Gel-based RT-PCR
2×10^8	+	+
2×10^7	+	+
2×10^6	+	+
2×10^5	+	+
2×10^4	+	+
2×10^3	+	+
2×10^2	+	±*
2×10^1	+	–
2×10^0	±	–
2×10^{-1}	–	–

*Both positive and negative results were obtained.

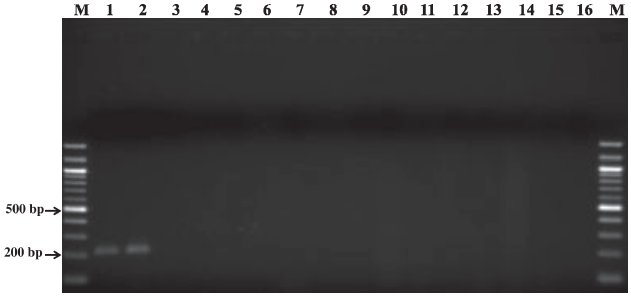

Figure 1. Detection of different swine pathogens by the pH1N1-specific gel-based RT-PCR assay. M, DNA ladder; lane 1, CA09; lane 2, Alb09; lane 3, cH1N1; lane 4, trH1N1; lane 5, trH3N2; lane 6, hu-like H1N1; lane 7, H2N3; lane 8, PRRSV; lane 9, TGEV; lane 10, PCV-2; lane 11, PPV; lane 12, *Streptococcus Suis*, lane 13, *Haemophilus parasuis*; lane 14, *Actinobacillus pleuropneumoniae* serotypes 1; lane 15, *A. pleuropneumoniae* serotypes 5; lane 16, negative control.

Diagnostic performance of pH1N1-specific real-time and gel-based RT-PCR assays

Twenty-seven of 30 nasal swabs from pigs inoculated with either the CA09 or Alb09 pH1N1 viruses were positive using the matrix real-time RT-PCR assay (Table 4). Twenty-four of 30 nasal swabs were positive for pH1N1 RNA using the gel-based RT-PCR assay; the latter results were confirmed by sequencing of the amplicon. Twenty-one of 30 nasal swabs from pH1N1 infected pigs were positive by conventional virus isolation (Table 4). All 30 nasal swabs were negative (Table 4) using the real-time RT-PCR assay targeting the N.A. swine matrix gene.[23] Importantly, no evidence for the presence of the pH1N1 virus was found in the 32 clinical samples collected from swine farms in the Midwest and sent to the KSUVDL with both real-time and gel-based pH1N1-specific RT-PCR assays. Thirty-one of 32 samples were positive by the N.A. SIV-specific real-time RT-PCR assay and 28 of them were confirmed to be positive by virus isolation (Table 4).

A total of 62 samples were tested by the newly developed pH1N1-specific real-time and gel-based PCR assays. Twenty-four samples were positive and 35 were negative by both assays, and results of both assays were in agreement with 56 samples (Table 4). The three discordant samples were positive by the real-time RT-PCR and negative by the gel-based RT-PCR, a discrepancy most likely because of the higher sensitivity of the real-time RT-PCR assay when compared to the gel-based RT-PCR (the sensitivity and specificity of the real-time RT-PCR relative to the gel-based RT-PCR was 100% and 95%, respectively). When testing nasal swab samples collected from the pH1N1-inoculated pigs, six or three discordant samples were positive using the real-time or gel-based RT-PCR assays, respectively; however, they were negative by the virus isolation. When compared to the virus isolation, the diagnostic sensitivity of both the real-time and gel-based RT-PCR assays was 100%; the diagnostic specificity of the real-time and gel-based RT-PCR assay was 83% and 88%, respectively.

Discussion

Currently, various subtypes of triple reassortant SIVs (H3N2, H1N1, H1N2) are co-circulating in swine in North

Table 4. Summary of the pH1N1 M-gene-specific real-time and gel-based RT-PCR results compared with virus isolation

Samples	Real-time RT-PCR (pH1N1)	Gel-based RT-PCR (pH1N1)	Real-time RT-PCR (N.A. SIV)	Virus isolation
Nasal swabs*	27/30**	24/30	0/30	21/30
Clinical samples***	0/32	0/32	31/32	28/32

*Swabs from pigs challenged with either the CA09 or Alb09 pH1N1 virus.
**Number of positive samples/number of samples tested.
***Samples collected from pigs by the Kansas State University Veterinary Diagnostic Laboratory.

America. Notably, the pH1N1 virus has been isolated from swine in many swine producing countries including the USA and Canada.[26,27] Therefore, it is critical for veterinary diagnostic laboratories all over the world to have access to assays employing rapid, reliable and affordable technology, which are able to differentiate the pH1N1 virus from currently circulating SIVs. The efficient use of a rapid and accurate diagnosis of pH1N1 in pigs will be critical for the control and eradication of pH1N1, if it enters the swine populations and also for the prevention of pH1N1 transmission from infected pigs to humans and other susceptible animals. In this study, we developed a one-step real-time RT-PCR and a two-step gel-based RT-PCR assay targeting the matrix gene of the pH1N1 virus; both assays are able to specifically detect pH1N1 viruses and do not detect currently circulating SIVs in North America and other important swine viral and bacterial pathogens. The matrix gene was chosen as the target gene for both assays because it is derived from Eurasian SIVs in pH1N1 viruses and genetically different from the matrix gene carried by N.A. SIVs. The reason for not choosing the neuraminidase gene (in pH1N1 viruses also derived from Eurasian SIVs) as the target gene for the pH1N1-specific real-time or gel-based RT-PCR assays is based on the knowledge that the neuraminidase gene is not highly conserved among influenza A viruses when compared with the matrix gene. Indeed, when the neuraminidase was chosen as the target for a real-time RT-PCR assay, the sensitivity and specificity of the assays dropped significantly compared to the matrix-based assay (data not shown). Besides its high specificity, the newly developed pH1N1-specific real-time RT-PCR assay targeting the matrix gene is extremely sensitive; it has the ability to detect approximately two copies of an M-specific RNA molecule (Table 3) which is comparable or more sensitive than previous reported real-time assays for the pH1N1 virus.[28,29] Importantly, when combined with the N.A. SIV-specific matrix-based real-time assay used throughout veterinary diagnostic laboratories in the USA, the newly developed pH1N1-specific real-time RT-PCR assay will be a very useful tool for surveillance for currently circulating SIVs as well as for pH1N1 viruses in swine herds. In addition to the real-time RT-PCR assay, we also developed a pH1N1-specific gel-based RT-PCR assay targeting the matrix gene; this assay was able to specifically detect the pH1N1 virus and did not react with other relevant viral or bacterial pathogens of swine (Table 2). The newly developed gel-based RT-PCR assay gives the user the opportunity to sequence the amplified matrix gene to confirm the specificity of the reaction and the presence of the pH1N1 virus. We suggest to first use the pH1N1-specific real-time RT-PCR assay for analysis of diagnostic samples and employ the pH1N1-specific gel-based RT-PCR assay with positive and suspect samples to confirm the initial findings.

The results testing experimental and clinical samples showed that both the pH1N1-specific real-time and gel-based RT-PCR assays were more sensitive when compared to conventional virus isolation tests because some nasal swabs collected from inoculated pigs on day 7 post-infection contained low amounts of virus, only detectable using the newly developed RT-PCR assays but not by virus isolation. The low virus titers in nasal swabs at day 7 post-infection confirm our results in previous studies where nasal swabs and lung lavages of pigs infected with various SIV subtypes in the majority of animals were negative for the presence of SIV.[14,30,31] All clinical samples tested in this study were negative for the pH1N1 virus M gene using both pH1N1-specific real-time and gel-based RT-PCR assays, indicating that infection of USA pigs with the pH1N1 viruses is a rare event. It should be noted, however, that the pH1N1 virus has been found in USA swine herds and has been shown to be able to infect, transmit and maintain in swine herds and also cause severe clinical symptoms.[26,32] Interestingly, when using our in-house real-time RT-PCR specific for SIV neuraminidase genes on all 32 diagnostic samples, 13 of the 31 positive samples represent the classical N1 subtype and none of them contained a human-like N1 subtype (data not shown), suggesting that the other 18 samples are either N2 or other neuraminidase subtypes.

The pandemic H1N1 virus has an ability to infect pigs and replicate in swine shown in several experimental studies.[7,33] Furthermore, it has the capacity to infect swine and cause respiratory diseases in pigs similar to other currently circulating N.A. H1N1 SIVs.[27] It is also clear that the pH1N1 virus can be transmitted and maintained in pigs[22] and, therefore, reassortment events with currently circulating SIVs and other mammalian (e.g. human seasonal influenza A viruses) or avian viruses (e.g. H5N1) can be expected in the future. Such events could lead to the production of a 'novel' influenza virus which might be able to cross species barriers to human and other mammalian species and might have the potential to cause a new pandemic. The rapid diagnosis of pH1N1 cases in pigs will allow swift measures to control and eventually eradicate this virus from the pig populations. Our newly developed pH1N1-specific real-time and gel-based RT-PCR assays will be an important support tool to rapidly identify and molecularly characterize such a cross-species transmission event. Both assays are able to specifically detect and differentiate the pH1N1 viruses from currently circulating SIVs in North America. Therefore, these newly developed diagnostic assays might be important tools for surveillance, control, and eradication of the pH1N1 virus in N.A. swine herds.

Acknowledgements

The authors thank Audree Gottlob, Darlene Sheffer, and Jake Elder for their technical support and Dr. M. M. Chengappa for providing the bacterial isolates and Dr. Richard Webby for providing the Alb09 pH1N1 swine virus, and grant support from the National Institute of Allergy and Infectious Diseases, National Institute of Health, Department of Health and Human Services (Contract No. HHSN266200700005C) and the Kansas Bioscience Authority.

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