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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-