Application of PCR Assay to Differentiate Two Subtypes of Swine Influenza Viruses

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Summary and Implications

A reverse transcription-polymerase chain reaction (RT-PCR) test was developed to differentiate between H1 and H3 subtypes of swine influenza virus (SIV). The sensitivity and specificity of this test was evaluated by comparing the results of the PCR test with subtyping results of an immunological assay performed at the National Veterinary Services Laboratories (NVSL). The test was performed on 68 egg-derived SIV isolates, as well as directly on 30 lung homogenates. The data suggest that a PCR-based assay may be a reliable screening test for SIV both from egg fluid and directly from lung tissue.

Introduction

The recent isolation of an H3N2 subtype of swine influenza virus (SIV) in association with severe clinical disease in U.S. swine brings a new perspective to the diagnosis and control of swine influenza in this country. The initial report came from North Carolina, but, more recently, influenza outbreaks in vaccinated sows resulting in abortion and death have been reported in Iowa, Minnesota, and elsewhere. Thus, U.S. swine appear to be at risk of infection with a significant new pathogen.

Although H3N2 SIV is present in swine populations in other parts of the world, the only previous evidence for infection in the United States had been a low prevalence in serological surveys and a single instance in 1977 in which H3N2 subtype was isolated from pigs in Colorado without clinical disease. Thus, H3N2 strains were not considered to be a significant cause of influenza in U.S. swine. However, the recent isolation of H3N2 SIV has serious implications. First, the infections have resulted in the death of adult sows, suggesting a high degree of virulence. In addition, epidemics of infection with H3N2 strains have occurred in animals immunized with a vaccine that is efficacious against the classic H1N1 strains routinely found in U.S. swine, suggesting very little cross-protection between the H1N1 and H3N2 strains.

It is critical for diagnosticians to have access to rapid and accurate methods for the diagnosis and differentiation of SIV subtypes. The study presented herein describes a PCR assay that has been used on egg-derived SIV isolates as well as directly on lung tissue for the identification and subtyping of SIV. Our results indicate that this test should be a valuable diagnostic tool.

Materials and Methods

A RT-PCR assay was developed in our laboratory with sets of primers corresponding for hemagglutinin (H) gene of H1N1 and H3N2 SIV, respectively. The H gene was selected because H is the most important viral protein with respect to virus infection and protection. Each set of primers (forward and reverse) was designed using H gene sequences of H1N1 and H3N2 strains deposited in GenBank (National Center for Biotechnology Information located on the internet at www.ncbi.nlm.gov/Genbank/GenbankOverview.html, and diagnostic performance of the RT-PCR was evaluated.

For evaluation, a total of 76 egg fluids was collected from submissions to the Iowa State University Veterinary Diagnostic Laboratory (ISU-VDL). Of these, 68 egg fluids contained SIV at various HA titers and three were spiked with viral agents that are not SIV but cause hemagglutination. The remaining five egg fluids served as negative controls. In addition to egg fluids, 30 homogenates of lung tissues that were positive for SIV by a fluorescent antibody test and/or commercial antigen-capture ELISA kit were obtained from Dr. Gene Erickson at the Rollins Animal Disease Diagnostic Laboratory (RADDL), Raleigh, NC. The isolates and tissue homogenates were tested by RT-PCR in our laboratory. In addition virus isolation was attempted using MDCK cells on the tissue homogenate at RADDL. All isolates from ISU-VDL and RADDL were submitted to NVSL, USDA, Ames, IA, for immunological subtyping. Results of the PCR assay were then compared with those of the immunological subtyping on all isolates.

For RT-PCR, viral RNA was extracted using the QIAamp Viral RNA Mini Kit (Qiagen, Valencia, CA) by following the procedure recommended by the manufacturer. Viral RNA was reverse-transcribed to cDNA with forward primers for H1 and H3, respectively, and M-MLV reverse transcriptase (GIBCO/BRL, Grand Island, NY). Further amplification was conducted with reverse primers for H1 and H3, respectively, and *Taq* polymerase (Boehringer Mannheim) in the following reaction conditions: 94°C for 1 min followed by 35 cycles of 94°C for 30 s, 46°C for 30 s and 72°C for 45 s. PCR products were analyzed by agarose gel electrophoresis with ethidium bromide. Gel samples with an approximately 600-bp product were considered as H1 and those with a 350-bp product as H3 as shown in Figure 1.

Results and Discussion

SIV typing results by PCR were compared with those obtained by a standard immunological method. Comparison of results of two tests on the 76 egg fluids is summarized in Table 1. Eight samples containing no SIV or other viral agents were negative for SIV by both PCR and immunological subtyping. Sixty-seven of the remaining 68 samples were determined to be positive for SIV by immunological testing, whereas PCR determined that 60 were positive, demonstrating a sensitivity of approximately 88.2%. The specificity of the PCR assay was calculated to be 100% compared with virus isolation results. PCR subtyping results on the 60 positive samples were in agreement with the immunological subtyping results except one sample. This sample was determined to be H3 by the immunological testing and type H1 by PCR. At this time, we have no explanation for this discrepancy.

Diagnostic performance of PCR on 30 lung homogenates also was determined (Table 2). Twenty-one of the 30 homogenates were positive by PCR, revealing a sensitivity of 70%. PCR identified the subtypes of 20 of the 21 samples correctly compared with the immunological subtyping on isolates from those 21 lung homogenates, demonstrating 95.2% agreement. The one sample was determined to contain both H1 and H3 by the immunological method, but only H3 by PCR.

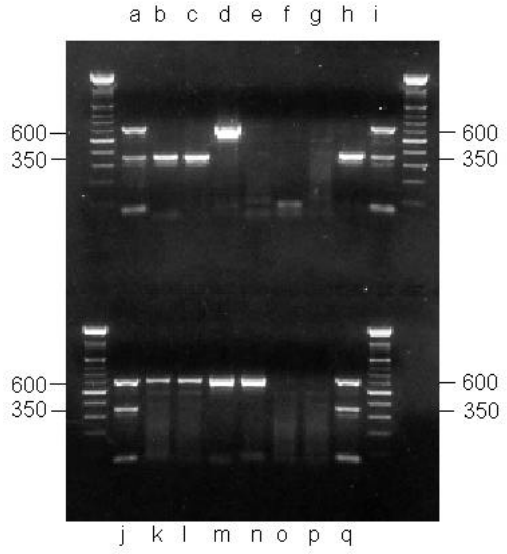

Figure1. Typical results of SIV RT-PCR. Lanes a, i, j and q are the standard containing both H1 and H3 SIV virus. Other lanes are samples being tested. The outermost lanes contain molecular weight standards. Numbers on side refer to molecular weight in base pairs. Note bands of approximately 600 and 350 base pairs for H1 and H3 SIV, respectively.

The RT-PCR test is rapid, with results usually available within 6 to 8 h of test initiation. The test is also highly specific. Sensitivity of testing egg fluid is more sensitive than directly from lung samples, although testing directly from clinical specimens eliminates the 4 to 5 days required for propagating the virus in eggs for further tests. Additionally, there has been at least one case in which RT-PCR performed directly from lung homogenate resulted in a positive test for H3 SIV even though the corresponding egg fluid tested negative.

At current a nondifferential RT-PCR assay is being developed in our laboratory. Preliminary results indicate that this test may be more sensitive on lung homogenates than the differential PCR described in this report. When applied to the lung homogenate samples in this study, all 30 samples tested were positive for SIV (data not shown), compared with only 21 by the differential test. It may be useful to use this test for identification of SIV positive samples, followed by the differential test to determine subtype of SIV.

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Table 1. Comparison of typing of egg fluids by immunological and PCR methods.

		Immunological typing results		
		H1	H3	Neg
PCR typing Results	H1	24	1	0
	H3	0	34	1
	neg	7	1	8

Table 2. Performance of PCR typing on lung homogenates compared with immunological method.

		Immunological typing results[a]		
		H1	H3	Pos[b]
PCR typing results	H1	4	0	0
	H3	0	16	1
	neg	3	5	1

[a]Results of subtyping on isolates from lung tissues.
[b]Subtyped as both H1 and H3 by immunological assay.