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Short communication

Development of a real-time RT-PCR for the detection of Swine-lineage Influenza A (H1N1) virus infections

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ABSTRACT

Background: A novel influenza A virus, subtype H1N1 of swine-lineage (H1N1 swl) has transmitted rapidly to many regions of the world with evidence of sustained transmission within some countries. Rapid detection and differentiation from seasonal influenza is essential to instigate appropriate patient and public health management and for disease surveillance.
Objectives: To develop a rapid and sensitive real-time reverse transcriptase polymerase chain reaction (rtRT-PCR) for confirmation of H1N1 swl.
Study design: A one-step rtRT-PCR approach was employed to target the matrix gene of the novel influenza A/H1N1 swl and validated against a panel of seasonal influenza A (H1N1 and H3N2), swine influenza A/H1N1 and avian influenza A/H5N1 viruses. The assay following validation was then used prospectively to detect H1N1 swl positive specimens from the recent outbreaks in the UK and the Republic of Ireland.
Results: The one-step H1N1 swl matrix rtRT-PCR successfully detected H1N1 swl clinical specimens and did not cross-react with seasonal influenza A, subtypes H1N1 and H3N2 viruses and swine influenza A (H1N1). The H1N1 swl matrix assay did cross react with H5N1. The H1N1 swl matrix assay was then compared to two other assays using a dilution series and a panel of untyped influenza A positive clinical samples. These experiments found the assay to have a comparable sensitivity to the established universal influenza A rtRT-PCR and was more sensitive than the H1N1 swl specific assay that targeted the H1 region.
Conclusions: The results demonstrate that the rtRT-PCR is sensitive and should be used alongside existing universal influenza A assays to rapidly detect the novel H1N1 swl virus.

1. Background

On April 15th and 17th 2009, a novel swine-lineage influenza A (H1N1 swl) infection was reported to the World Health Organisation (WHO) by the Centers for Disease Control and Prevention (CDC) in Atlanta in two children presenting with febrile respiratory illness from adjacent counties in southern California.[1,2] These cases were not epidemiologically linked and neither child had exposure to swine.[2] Subsequent phylogenetic characterisation of H1N1 swl from the U.S. index case (A/California/04/2009) showed that the virus had a unique genome composition that had not been previously identified. Six genes (PB2, PB1, PA, HA, NP and NS) were similar to viruses previously identified in triple-reassortant swine influenza viruses in North American pigs.[2] The remaining two genes (NA and M) were derived from Eurasian swine influenza viruses and this particular gene constellation has never been previously identified in humans or other reservoirs.[2]

Since the original identification of H1N1 swl in the U.S. and Mexico, sustained human to human transmission has been seen in other countries raising concerns of a future pandemic.[3–5] The symptoms seen in U.S. and cases outside of Mexico resemble those normally seen in influenza, with fever, cough, sore throat, rhinorrhea, headache and myaglia, however, approximately 25% of patients had vomiting or diarrhea, which is unusually high compared with infections with other strains of influenza.[2] The H1N1

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Table 1
Primer and probes sequences for the generic influenza A and the H1N1 swl rtRT-PCR assays.

Virus target	Target gene	Forward primer	Reverse primer	Probe
Generic Influenza A	Matrix	AAGACAAGACCAATYCTGTCACCTCT	TCTACGYTGCAGTCCYCGCT	**FAM**-TYACGCTCACCGTGCCCAGTG-**BHQ**
Influenza A H1N1 swl	Matrix	TGTGCCACTTGTGAACAGATTG	CTGATTAGTGGATTGGTGGTAGTAGC	**HEX**-5′ TGATTCACAGCATCGGTCTCACAGACAG 3′-**BHQ**

swl NA gene segments sequenced to date suggests that the virus is fully susceptible to the neuraminidase inhibitors, oseltamivir and zanamivir; however, M gene sequencing has shown resistance to the adamantanes.[6]

The emergence of the H1N1 swl virus has ramifications for existing diagnostic and typing PCR methods as the genetic differences mentioned above may result in a failure to detect and/or type this new virus. Most influenza A PCR assays in use in the United Kingdom target conserved regions of the M gene and therefore should detect influenza A from all established subtypes, including the newly emergent H1N1 swl. However, such methods need to be complimented with a rapid typing test to distinguish seasonal influenza A from H1N1 swl.

This article describes the development of an M gene-based H1N1 swl real-time reverse transcriptase polymerase chain reaction (rtRT-PCR) assay that does not cross-react with human seasonal influenza A viruses (subtypes H1N1 and H3N2). We have used this technique to confirm H1N1 swl infection and to identify transmission events of this novel virus.

2. Objectives

To develop a rapid and sensitive rtRT-PCR for confirmation of H1N1 swl. To validate the potential for cross reactvity to seasonal influenza A/H1N1 and H3N2, enzootic swine A/H1N1 and avian A/H5N1. To compare the end point detection limit of the newly developed assay against an established diagnostic assay that detects all influenza A viruses and a recently published H1N1 swl specific typing method. To prospectively test specimens from recent confirmed cases of H1N1 swl in the United Kingdom and the Republic of Ireland and finally to compare the newly developed assay to an H1N1 swl specific typing assay on 52 clinical samples that were influenza A positive but untyped at the time of testing.

3. Study design

The H1N1 swl rtRT-PCR assay was designed to target segment 7 encoding the matrix protein 1 gene using the sequence of A/California/04/2009 (FJ969513) and Primer Express version 3.0 (Applied Biosystems). The chosen primers (see Table 1) amplify an 80-bp amplicon and bioinformatics analysis using BLAST and Clustal alignments showed no significant homology to human gene sequences and numerous mutations present in the homologous M gene region in seasonal influenza A, subtypes H1N1 and H3N2 viruses.

The specificity of the assay was assessed retrospectively using a panel of seasonal influenza A, H1N1 and H3N2 samples ($n = 7$) and swine A/H1N1 and avian A/H5N1 viruses were also included in the panel. A pool containing the following commonly encountered respiratory pathogens was also tested: influenza B, influenza C, parainfluenza 1–4, human metapneumovirus, respiratory syncytial virus, *Mycoplasma pneumoniae*, rhinovirus and coronaviruses 229E, OC43 and NL63.

The end-point detection limit of the new H1N1 swl rtRT-PCR was directly compared to a widely used universal influenza A rtRT-PCR assay and a H1N1 swl H1 specific rtRT-PCR using a dilution series of a H1N1 swl clinical sample. The universal influenza A rtRT-PCR assay targets the matrix region of the virus and is in use as the frontline diagnostic test for influenza A virus detection at both Glasgow and Dublin virology laboratories. Participation in various EQA schemes has shown this assay to detect influenza A viruses from humans and animals with high sensitivity. The H1N1 swl H1 specific rtRT-PCR has been implemented by most Health Protection Agency affiliated laboratories in England.[7]

The H1N1 swl matrix assay was then assessed prospectively from the end of April 2009. In the West of Scotland Specialist Virology Centre the assay was ran alongside the routine influenza A rtRT-PCR and was used to test all samples from suspected cases and contacts of H1N1 swl cases. In Dublin the assay was used to test any samples that were influenza A positive that could not be typed by a influenza A H1 or H3 rtRT-PCR. Any samples tested as H1N1 swl positive using the H1N1 swl assay were then tested by sequencing or submitted to the Health Protection Agency for confirmation purposes.

The final assessment comprised of comparing the H1N1 swl matrix assay with the H1N1 swl H1 specific assay on 52 samples that were influenza A positive using the universal influenza A rtRT-PCR. All samples were sent to the WOSSVC during the first week of June, 2009 and were taken from contacts with confirmed cases of H1N1 swl. All were untyped at the time of testing. Total nucleic acid was extracted from respiratory specimens using QIAamp Viral RNA Mini kit (Qiagen, Crawley, United Kingdom) according to the manufacturer's instructions. The oligonucleotide primers and probe (TIB-MOLBIOL, Berlin, Germany) for the both the H1N1 swl and the universal influenza A rtRT-PCR assays are outlined in Table 1. Both assays used the primers at a final concentration of 400 nM and the probe at 200 nM in a 15 μl or 25 μl reaction volume. One-step rtRT-PCR was performed on 6 μl or 5 μl of RNA extract with the Platinum One-step qRT-PCR kit (Invitrogen) on an ABI Prism 7500 SDS real-time platform (Applied Biosystems). The following thermal profile was used: a single cycle of reverse transcription for 15 min at 50 °C, 2 min at 95 °C for reverse transcriptase inactivation and DNA polymerase activation followed by 40 amplification cycles of 15 s at 95 °C and 34 min at 60 °C each (annealing-extension step). The H1N1 swl H1 specific rtRT-PCR was carried out as described elsewhere.[7] Data acquisition occurred at the annealing step of each cycle and the threshold cycle (Ct) for each sample was calculated by determining the point at which the fluorescence exceeded the threshold limit.

4. Results

The specificity of the H1N1 swl matrix protein 1 gene assay was evaluated using a panel comprising seasonal influenza A (H1N1 and H3N2), avian (H5N1) and swine (H1N1) influenza A viruses (Table 2). No cross-reaction with seasonal influenza A (H1N1 and

Table 2
Evaluation of the specificity of the H1N1 swl rtRT-PCR assay.

Influenza A subtype	Influenza A universal	H1N1 swl
H3N2	28.19	Neg
H3N2	25.00	Neg
H3N2	30.05	Neg
H3N2	28.20	Neg
H1N1	27.18	Neg
H1N1	27.38	Neg
H1N1	31.81	Neg
Avian H5N1	26.41	37.02
Swine H1N1	21.95	Neg