Short communication

Design and clinical application of a molecular method for detection and typing of the influenza A/H1N1pdm virus

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A B S T R A C T

In March/April 2009, Mexico experienced an outbreak of respiratory illness, due to a new influenza of swine origin virus, which spread rapidly via human-to-human transmission, and became pandemic (A/H1N1pdm). Because of its unique genome composition, which includes gene segments of swine, avian and human origin, and to the considerable differences to the human influenza A viruses that have circulated so far, the currently used molecular methods proved inadequate.

Based on published sequences, a primer set targeting the nucleoprotein gene was designed, which provided enhanced sensitivity for the new strain and proved suitable for sequence-based strain identification. The novel nucleoprotein reverse-transcription-PCR showed higher sensitivity for A/H1N1pdm than a commercial test for influenza A, and was comparable to the real-time-based method developed by the Centers for Disease Control and Prevention. It was used to screen 177 clinical samples referred to the laboratory for suspected A/H1N1pdm infection, detecting 17 (9.6%) infections that were confirmed by sequence analysis (100% sensitivity as compared to the real-time kit).

The novel method is suitable for the diagnosis of A/H1N1pdm, and is also suitable, at least in the screening phase, for laboratories not equipped with the real-time PCR technology.

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Infections with swine influenza viruses have been detected sporadically in humans since the 1950s and the human disease is usually clinically similar to the disease caused by the human influenza viruses (Myers et al., 2007). Many of the swine viruses circulating today are composed of human and/or avian virus genes as a result of the genetic reassortment that takes place when human and avian influenza viruses occasionally transmit to pigs and exchange genetic material, leading to the emergence of new "hybrid" strains (Van Reeth, 2007).

Between late March and early April 2009, Mexico experienced outbreaks of respiratory illness, with increased reports of patients with influenza-like illness in several areas of the country. On April 23, the swine origin of this novel influenza A/H1N1 virus was identified and communicated to the Pan American Health Organization (PAHO). This virus, which spread rapidly to many other countries via human-to-human transmission, is a novel strain, undetected previously in either pigs or humans (Novel Swine-Origin Influenza A (H1N1) Virus Investigation Team, 2009). In fact, the majority of genes, including the hemagglutinin (HA), are similar to those of swine influenza viruses that have been circulating among pigs in the U.S. for a decade, while the genes coding for the neuraminidase (NA) and matrix (M) proteins are similar to the corresponding genes of swine influenza viruses of the Eurasian lineage (Smith et al., 2009).

On June 11, the World Health Organization (WHO) raised the level of influenza pandemic alert to phase 6 (Zarocostas, 2009), meaning that the world was on the brink of a new influenza pandemic. The name of this pandemic virus is influenza A/H1N1pdm.

Establishment of adequate methods for the diagnosis of emerging viruses with the potential of spreading rapidly is necessary for the timely identification of cases and for the implementation of public health measures to limit their spread.

Molecular diagnosis of influenza is generally achieved through a two-phase process: a screening phase targeting conserved internal genes, and subsequent strain identification achieved by either subtype-specific reverse-transcription Polymerase Chain Reaction (RT-PCR) or entire/partial genome sequencing (Petric et al., 2006). The M gene is used commonly as PCR target for the screening phase, as well as the nucleoprotein (NP) whose sequences are highly conserved among the subtypes of A influenza viruses, and are significantly distinct from those of type B (Wright et al., 1995; Minosse et al., 2007). These screening methods, which target conserved internal genes, have been so far designed on human seasonal
influenza strains, therefore false-negative results may occur in the case of A/H1N1pdm infections as a consequence of sequence variation in primer and probe targets, with obvious detrimental effects on public health.

For this reason, the use of new molecular tools is mandatory when dealing with a newly emerged viral strain. In fact, the NP gene hemi-nested PCR currently used in the laboratory for the diagnosis of influenza (Minosse et al., 2007), was not suitable for rapid A/H1N1pdm screening, for two main reasons: (i) the presence in the primers of several mismatches to the published A/H1N1pdm sequences and (ii) the long turnaround times, due to the hemi-nested nature of the routine method. Another assay available in the laboratory for the differential diagnosis of respiratory infections was based on a commercial multiplex RT-PCR kit (Seeplex RV12 ACE Detection, Seegene, Seoul, Korea), able to detect 12 different respiratory viruses, including influenza A and B. On the basis of the influenza A primer sequences (targeting the M gene) and the manufacturer’s specifications, this method was predicted to be able to detect the new A/H1N1pdm, although with an unknown sensitivity. In addition, because at the beginning of the outbreak the manufacturer had not yet disclosed the sequence of the M primer set, the amplicon could not be sequenced for strain identification.

The aim of this study was to develop a new one-step RT-PCR assay able to detect the novel H1N1 influenza virus within a few hours. Based on A/H1N1pdm sequences published on the Global Initiative on Sharing Avian Influenza Data (GISAID) website, a primer set targeting the NP gene was designed, producing an amplicon large enough to allow strain identification by subsequent sequencing. Primers designed were: ANP-3 SW: 5′-ATG GCC TTG AAT GCA GAT CTG-3′ and ANP-2 SW: 5′-CCT CCC TCG ACA ACC-3′. The optimal sensitivity of NP RT-PCR for A/H1N1pdm was 5 NP gene Ct if not processed immediately. Viral...
detected another seasonal influenza A, typed as H3N2 by type-specific RT-PCR. Notably, the 2 non-typable influenza A cases identified by the CDC kit (C7 values: 37.1 and 37.8, respectively with the M-specific generic primer set), and not detected by either Seeplex RV12 or the new NP RT-PCR, resulted also RT-PCR-negative with the routine M primer set (Minosse et al., 2007), as well as with all the type-specific H1N1 and H3N2 primer sets used for typing. These data suggest either a very low viral load, as supported by the high C7 values in InfA real-time RT-PCR, or unspecific results by the CDC kit.

In conclusion, the NP RT-PCR assay described above has enhanced sensitivity for the H1N1pdm strain, and is suitable for the screening phase of laboratory diagnosis. In addition, the amplicon size is large enough to allow strain identification, based on sequence analysis. The high sensitivity of NP RT-PCR allows strain definition also of samples that are not typable with the CDC method (positive to the influenza A generic primers but negative to the A/H1N1pdm-specific primer sets). This novel NP RT-PCR is suitable for the screening of A/H1N1pdm in laboratory settings which are not equipped with the real-time PCR technology, referring to reference laboratories for strain identification, on the basis of nucleotide sequence analysis.

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References


