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Short communication

Comparison of two real-time RT-PCR-based systems for the detection and typing of the pandemic influenza A virus, 2009

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A B S T R A C T

During the 2009 pandemic the Virology Laboratory of L. Spallanzani, Rome, Italy, adopted a real-time RT-PCR developed by the Centers for Disease Control and Prevention (CDC), Atlanta, Georgia to diagnose pandemic influenza A/H1N1 (H1N1pdm). A new multiplex real-time RT-PCR distributed by Astra Diagnostics, coupled with the extraction system developed and commercialized by Siemens Healthcare Diagnostics (referred to as the RealStar system), was tested for the ability to detect and type influenza A in clinical samples, with particular emphasis on influenza A-positive samples untyped by the CDC method. Seventy-six nasopharyngeal swabs, resulting by the CDC method H1N1pdm ($n = 7$), H3N2 ($n = 3$), and not subtyped ($n = 66$), were re-analysed with the RealStar system. All H3N2 and H1N1pdm-positive samples were correctly identified; among the untyped samples, the RealStar system detected 24/66 (36.4%) H1N1pdm and 1/66 (1.5%) seasonal influenza A. In conclusion, the RealStar system confirmed the results of all the influenza A-positive samples subtyped by the CDC method, and was able to type 37.9% of samples untyped by the CDC method. However, 62.1% of samples, detected as influenza A-positive but not subtyped by the CDC method, were found to be negative by the RealStar system. Further investigation is needed to explain this latter, unexpected, finding.

In April 2009, an outbreak of influenza in Mexico was found to be caused by a new strain of influenza virus (CDC, 2009). The novel strain of A/Influenza H1N1 serotype was derived by reassortment of swine, avian and human influenza A viruses. The virus spreads rapidly to a large number of countries and on June 11, 2009 the World Health Organization (WHO) declared that the infections caused by the new strain had reached pandemic proportions. As of end of January, 2010, WHO has reported approximately 14,700 deaths in more than 209 countries resulting from pandemic influenza H1N1 (WHO, 2010). However, given that countries are no longer required to test and report individual cases, the number of reported cases understates significantly the real number of cases. The establishment of adequate rapid methods for the diagnosis of the emerging and potentially challenging viral infections is necessary for the timely identification of cases and for the rapid implementation of public health measures to limit their spread. Real-time RT-PCR is considered as the "gold standard" for molecular detection of influenza viruses due to its high assays specificity, sensitivity and a broad linear dynamic range. With the emergence of the 2009 pandemic influenza A virus (A/H1N1pdm), real-time RT-PCR became rapidly the primary assay for diagnosis and surveillance. The Centers for Disease Control and Prevention (CDC), Atlanta, Georgia, developed a one step real-time RT-PCR protocol for A/H1N1pdm detection shortly after the emergence of the outbreak. The assay includes four parallel real-time RT-PCR, specific for influenza A virus, swine influenza A virus, novel H1 hemagglutinin, as well as a reaction control to assess specimen quality, extraction efficiency and the presence of PCR inhibitors.

According to the CDC protocol, a specimen is considered positive for novel A/H1N1pdm influenza virus if the influenza A virus, swine influenza A virus, novel H1 hemagglutinin reaction growth curves cross the threshold line within 40 cycles (WHO, 2009).

This method was distributed to collaborating centers as well as to national of regional reference centers from other countries, to assist laboratory diagnosis in the early phases of the pandemic, and it has been considered as the reference methods during the first period of the pandemic. Since then, other RT-PCR-based molecular protocols were developed rapidly and used in research and clinical laboratories during the outbreak (Carr et al., 2009; Ellis et al., 2009; Hall et al., 2009; He et al., 2009; Jiang et al., 2010; Lam et al., 2010; Pabbaraju et al., 2009; Poon et al., 2009; Wang

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Table 1
Primers used to detect influenza A virus subtypes.

Primer name	Sequence (5′–3′)	Position	Amplicon length	Reference
H1s	gaa tgg ttt gta ccc aaa cct gag c	510-534 (CY039463.1)	151 bp	Unpublished
H1as	gtg aag aca ctg ca gaga cat aag cat tt	661-633 (CY039463.1)		
H3s	tag agg cat att cgg cgc aat cg	1033-1055 (CY039079.1)	145 bp	Unpublished
H3as	tag ttt ggt tga ttg ctg cct gag t	1178-1157 (CY039079.1)		
N1Hs	aag ggg ttt tca tac agg tat ggt	1039-1062 (CY03359.5)	107 bp	Poddar (2002)
N1Has	tct gtc cat cca tta gga tcc	1145-1125 (CY03359.5)		
N2s	tgg gag tgc ttc agg aca agc tga t	729-753 (CY039521.1)	170 bp	Unpublished
N2as	cta ttg gag cct ttc cag ttg tct ct	899-874 (CY039521.1)		
ANP-3 SW	5′ atg gcg tct caa ggc acc a 3′	1-19 (FJ969512)	320 bp	Lalle et al. (2010)
ANP-2 SW	5′ cct cct gtt ttc tta ggg tc 3′	281-262 (FJ969512)		

et al., 2009; Wang and Taubenberger, 2010; Whiley et al., 2009). However, the majority of these assays are not designed for the contemporary detection and subtyping of influenza A viruses in the same assay. As regional reference center for influenza surveillance, the Virology laboratory of the National Institute for Infectious Diseases "L. Spallanzani", Rome, Italy, used the kit provided by CDC since the first appearance of the pandemic to test approximately 2700 samples from May 2009 to January 2010. The specimens were collected from patients consulting directly the facility or sent to the Institute by other hospitals. The diagnostic procedure used in the laboratory was based on the CDC method, applied to nucleic acids extracted by the MDx BioRobot platform with the QIAamp Virus BioRobot MDx kit (Qiagen, Valencia, CA, USA). The overall prevalence of influenza A in the study period was 35.9%, of which 32.2% resulted A/H1N1pdm, 0.3% H3N2 and 3.5% untyped. Only 2 cases of influenza B were detected in the study period. One of the difficulties encountered in the diagnostic activity was that the sensitivity of the CDC primer–probe set to detect the influenza A virus (targeting the matrix gene, M) is superior to that of the primer-probe sets to assign the subtype (targeting the nucleoprotein, NP, and the hemagglutinin, HA, genes). Therefore, a number of influenza A-positive samples, with a low viral load (Ct > 35) could not be typed by this method. To subtype the strains identified only as influenza A by the CDC kit, conventional subtype-specific end point One-Step RT-PCR (Qiagen, Chatsworth, CA) (Table 1), or sequencing of the NP gene were used (Lalle et al., 2010). Using these additional steps, approximately 10% of the samples not typed by the CDC method were subtyped. However, a large number of samples (accounting for a total of 3.5%) remained untyped.

Recently, an assay has been developed and distributed by Astra Diagnostics (Hamburg, Germany), named RealStar Influenza Screen & Type RT-PCR Kit 5.0, which is able to detect and differentiate simultaneously seasonal influenza A and B viruses, as well as the A/H1N1pdm, in a single reaction. This method, based on multiplex real-time one step RT-PCR, consists of 3 primer and probe sets, targeting the M gene of human seasonal influenza A, human influenza B and A/H1N1pdm, and includes also as a primer and probe set specific for the Internal Control (IC). The probes specific for human seasonal influenza A, influenza B, A/H1N1pdm and Internal Control are labeled with different fluorescent dyes, to allow the simultaneous and differential detection of four targets in a single reaction. The details on primer–probe composition, as well as the cycling conditions, are specified in the manufacturer instruction manual. During the 2009–2010 influenza season, this system was commercialized in Italy in conjunction with the automatic extraction platform developed by Siemens Healthcare Diagnostics, Deerfield, USA (Versant Sample Preparation 1.0 on Versant kPCR Molecular System). Throughout the paper, the combination of the Versant extraction + Astra RT-PCR will be referred to as RealStar system.

The aim of the present study was to establish whether the RealStar system may offer advantages over the protocol used in the diagnostic routine, i.e. CDC method in conjunction with the automatic MDx BioRobot extraction platform, for detecting and subtyping influenza A-positive samples.

The analytical sensitivity of the two systems against influenza A was compared using serial dilutions of viral stock preparations of A/H1N1pdm (clinical isolate kindly provided by R. Azzi, Florence) and of seasonal H3N2 (A/Port Chalmers/1/73), grown in MDCK cultures and in embryonated eggs, respectively.

More in detail, 6 replicates of 10 fold dilutions of A/H1N1pdm and H3N2 preparations were extracted in parallel by (1) the QIAamp Virus BioRobot MDx kit (Qiagen, Valencia, CA, USA) using the MDx BioRobot platform and (2) the Versant Systems. The nucleic acid preparations obtained with either extraction methods were analysed with the CDC and the multiplex real-time RT-PCR, respectively.

The number of viral genomes in the stock preparations of A/H1N1pdm and H3N2 was assessed first using the CDC real-time RT-PCR, modified to provide quantitative results. In particular, a reference standard curve was prepared using serial dilutions of nucleic acid extracts from a reference influenza A/H1N1pdm virus preparation (strain A/Hamburg/4/2009) supplied by the European Network for Diagnostics of Imported Viral Diseases (ENIVD), according to Meschi et al. (2011). The resulting number of viral genomes, referred to the M gene, was 3.98×10^9 and 1.58×10^9 copies/ml for A/H1N1pdm and H3N2 viral stock preparations, respectively.

The results indicate that the RealStar system is able to detect the A/H1N1pdm and the H3N2 in 6/6 (100%) replicates down to a 10^{-8} dilution of A/H1N1pdm (containing 35 copies/ml), and down to a 10^{-7} dilution of H3N2 virus (containing 150 copies/ml); the CDC test was positive in 6/6 (100%) replicates for the dilution of A/H1N1pdm virus containing 350 copies/ml. For H3N2, the dilution showing 100% positivity was coincident with the RealStar system.

To establish whether the increased sensitivity of the RealStar system for A/H1N1pdm was due partly to the difference in extraction method, the CDC assay was applied on A/H1N1pdm and H3N2 dilution series extracted with the Versant system. The M gene Ct values obtained in these experiments are reported in Table 2, where the combination of extraction/detection methods is as follows:

The RealStar system (Versant extraction + multiplex real-time RT-PCR detection, A); MDx extraction + CDC detection (B); Versant extraction + CDC detection (C). As can be seen, the mean Ct values for each dilution tested with the CDC kit is lower in Versant (A and C) vs MDx extraction method (B), suggesting higher efficiency of extraction by the Versant system.

Based on the higher sensitivity of the RealStar system, and on the fact that detection and typing were coincident in this assay, it was possible that influenza A samples remained untyped by the CDC assay, due to the low viral load, may be detected and differentiated by the RealStar system.

In fact, in the laboratory experience, the subtyping capability of the CDC system for A/H1N1pdm virus, based on NP and HA genes, is equal to about 10^3 copies/ml (data not shown). So, the subtyping sensitivity appears to be lower than the detection sensitivity, based

Table 2
The Ct values obtained with different combination of extraction/detection methods.

	Dilutions	(A) Mean Ct Versant extraction/multiplex real-time PCR (M gene)	(B) Mean Ct MDX extraction/CDC (M gene)	(C) Mean Ct Versant extraction/CDC (M gene)
A/H1N1pdm	10^{-8}	38.69	>40	37.18
	10^{-7}	31.00	39.90	33.11
	10^{-6}	26.67	34.82	29.50
	10^{-5}	23.61	30.41	26.72
	10^{-4}	21.19	26.71	23.77
	10^{-3}	18.83	24.79	20.58
H3N2	10^{-8}	>40	>40	>40
	10^{-7}	36.10	36.8	37.08
	10^{-6}	28.95	33.4	32.51
	10^{-5}	24.87	30.22	28.22
	10^{-4}	21.56	26.59	24.88

on M gene (100% at 350 copies/ml), while for the RealStar system the sensitivity of both detection and typing appears to be equal to 35 copies/ml. Therefore, in the next step the RealStar system was applied to clinical samples resulted untyped influenza A-positive by the CDC method (Ct > 35). To this aim, 66 samples, whose replicates had been stored at −80 °C immediately after arrival to the laboratory and never thawed thereafter, were thawed and tested by the RealStar system. As positive control, 7 nasopharyngeal swabs positive to A/H1N1pdm and 3 positive to the seasonal H3N2 influenza were analysed.

To verify the ability of the RealStar system to detect influenza B virus, 5 additional sputum samples, collected in previous influenza seasons and kept stored at −80 °C, and resulted positive to influenza B by end-point RT-PCR (Minosse et al., 2007), were included in this experiment.

All the samples positive for H3N2 and A/H1N1pdm were detected and typed correctly by the RealStar system; in addition, detection/typing was successful in 25/66 (37.9%) samples classified as untyped influenza A-positive by the CDC method, resulting A/H1N1pdm and seasonal influenza A in 24 (36.4%) and 1 (1.5%) cases, respectively, while 41 of these samples (62.1%), remained negative to all viral targets although positive to the Internal Control.

Only 2 out of 5 (40%) influenza B-positive samples were identified.

In conclusion, the RealStar system demonstrated a significantly higher analytical and clinical sensitivity for detection and subtyping of influenza A/H1N1pdm virus as compared to MDx/CDC system, while a similar performance was observed for the two assays for detecting H3N2. However, a significant proportion of untyped samples, positive only to the M gene by the CDC assay, were not detected by the RealStar system. This finding was unexpected, as the detection and typing sensitivity of the new system, as established on the basis of the experiments on limiting dilutions of in vitro grown viral strains, was about 10 times higher than that of the CDC for A/H1N1pdm, and similar for H3N2. This contradiction may be due to specimen degradation during the storage time, but it could also be explained on the basis of the differences on the genetic material integrity between clinical samples and in vitro grown strains, with respect to the target region amplified in the PCR reactions. In addition, the possibility of non-specific amplification, or low level contamination, could not be ruled out in the samples with a high Ct value for M gene and negative in all the additional influenza-specific primer/probe sets in the CDC test. It is also possible that probe break down with the CDC M gene detection set may have generated some false positive results.

Another intriguing point is the fact that RealStar system did not detect all influenza B-positive specimens. This failure should be due either to specimen degradation during the storage time or to the different type of samples (sputum vs nasopharyngeal swabs). Obviously, a comparison between RealStar system and CDC assays could not be performed, since the latter assay does not include influenza B virus detection.

More detailed investigation is necessary in the future to elucidate the puzzling features highlighted by the present study.

Conflict of interest

Emilio Patella was an employee of Siemens Healthcare Diagnostics srl. In no way the contribution of Siemens Healthcare Diagnostics srl to the completion of the study influenced the study design and the analysis of results. There are no additional conflicts of interest for this study.

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