

Evaluation of Vircell SPEED-OLIGO® NOVEL INFLUENZA A H1N1

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INTRODUCTION

Influenza virus is the causal agent of influenza or flu, an infectious disease with characteristic symptoms in adults: high fever, headache, photophobia, sore throat, cough, malaise and myalgia. Elderly patients suffering from chronic bronchopathy often present tracheobronchitis and bronchiolitis. Infants can present severe respiratory infection together with convulsions and encephalitis. It is often difficult to achieve a clinical diagnosis, since it can be confused with other respiratory diseases.

There are three types of influenza viruses: A, B and C. Among these, influenza A viruses are subdivided according to their hemagglutinin (H) and neuraminidase (N) surface protein subtypes. The current subtypes of circulating influenza A viruses are H1N1 and H3N2. Influenza virus present a high capacity for genetic variation: the fact that its viral genome is segmented, allowing for the reassortment of these segments in mixed infection, together with the high variability of its surface proteins gives the virus this unique characteristic. On April 2009, a novel H1N1 (nH1N1) reassortant causing human infections in Mexico and USA was identified by the CDC. Continued identification of new cases indicates sustained human-to-human transmission of this novel influenza A virus. Most confirmed cases have been characterized by self-limited, uncomplicated febrile respiratory illness and symptoms similar to those of seasonal influenza; in addition, however, vomiting or diarrhea was common. Some patients required hospitalization due to more severe disease.

This new H1N1 virus may be detected either in direct samples or cultures by direct immunoassays targeting common epitopes of influenza A viruses. Yet, these assays can not distinguish the novel virus from the current circulating seasonal strains. This differentiation can be only achieved by sequencing (only available in a limited number of laboratories) or by RT-PCR targeting specific sequences of the new virus.

OBJECTIVE

The aim of this study was to evaluate the utility of SPEED-OLIGO® NOVEL INFLUENZA A H1N1 kit from Vircell (Spain), a commercial new assay based on a PCR method coupled to rapid detection of PCR products by means of a dipstick device. An in-house Real Time RT-PCR was used as reference method.

METHODS

Materials

RNAs from 103 respiratory samples (nasal/throat swabs in viral transport medium and nasopharyngeal aspirates) collected from April 26 to June 1, 2009, and previously tested for the presence of the nH1N1, were used.

Additionally, DNAs or RNAs (or their cDNA products, when available) previously extracted from 150 respiratory samples that tested positive for other respiratory viruses (see Table 1) were assayed. All DNAs and RNAs had been frozen at -80°C after the routine virological investigation.

Adenovirus	2
BoV	1
BoV + hMPV	1
CoV HKU1	1
CoV NL63	1
CoV OC43	1
Influenza A H1 (07-08 season)	29
Influenza A H1 (08-09 season)	1
Influenza A H3 (08-09 season)	47
Influenza B (07-08 season)	39
Influenza B (07-08 season) + BoV	3
Influenza B (08-09)	21
hMPV	1
Parainfluenza-3	1
RSV	1
Total	150

To determine the specificity of the assay, RNA or DNA extracted from cultures of the following strains (Table 2) was also tested at 5 ng per reaction:

Virus	Strain	Source
Adenovirus	Adenoid 71	ATCC VR-1
Influenza A (H5N1)	A/Vietnam/1194/2004 NIBRG-14	NIBSC 07/252
Influenza A (H1N1)	A/PR/8/34	ATCC VR-95
Influenza A (H3N2)	A/VICTORIA/3/75	ATCC VR-822
Influenza B	B/HONG KONG/5/72	ATCC VR-823
Parainfluenza 1	Sendai/52	ATCC VR-105
Parainfluenza 2	Greer	ATCC VR-92
Parainfluenza 3	C 243	ATCC VR-93
Respiratory Syncytial Virus	Long	ATCC VR-26

Analytical sensitivity was evaluated by testing dilutions of known concentration of a purified plasmid containing a sequence from the H1 gene of the nH1N1 virus comprising 545 nucleotides (531-1076) in a pGEM[®]-T vector. The number of copies was estimated from the DNA concentration and the size of the plasmid (3550 pb), by assuming an average molecular mass of 660 Da for 1 bp of double-stranded DNA.

Reference technique

RNAs for the investigation of the nH1N1 were obtained from 200 µL of respiratory samples using the automated BioMérieux NucliSENS[®] easyMAG[®] nucleic acids extraction platform (BioMérieux, Marcy l'Etoile, France), following the manufacturer's instructions. External lysis was carried out to inactivate clinical samples. Total cDNA was synthesized with random primers from 10 µL of extracted RNAs using the iScript[™] Reverse Transcriptase System (Bio-Rad Laboratories, Hercules, USA). Two routine real-time RT-PCR were carried out in parallel for the screening of influenza A and B viruses, and for the subtyping of influenza A viruses in the 103 specimens from suspected cases of nH1N1. The screening assay includes primers and a

Taqman[®] probe targeted at a fragment of the NP gene of both influenza A and B viruses. No mismatches have been found with published sequences of nH1N1 within complementary sequences of the primers and probe. Subtyping of influenza A was carried out with a real-time PCR protocol using primers and Taqman[®] probes targeted at the hemagglutinin genes of influenza A H1 and H3, as previously described (J Virol Methods. 2008; 152: 25-31). nH1N1 was found to have several mismatches with the 3' ends of the primers used in this PCR assay; thus, a case of nH1N1 would yield a positive result for influenza A with the screening assay and a negative result with this subtyping assay.

The presence of nH1N1 was confirmed in non-subtypeable influenza A specimens by real-time PCR carried out with primers SWH1 Forward and SWH1 Reverse, and a Taqman[®] SWH1 probe targeted at the H1 gene of this virus following WHO recommendations. The technique was performed with the LightCycler FastStart DNA Master^{PLUS} Hybridization Probes kit (Roche Diagnostics GmbH, Mannheim, Germany), 0.5 µM of primers and 0.2 µM of the Taqman[®] probe. The amplification was performed in a LightCycler 2.0 Instrument (Roche Diagnostics GmbH): 95 °C/10 min and 45 cycles of 95°C/ 10 sec + 58°C/40 sec. A single fluorescence reading was taken in each cycle. Readout was performed through channel 530.

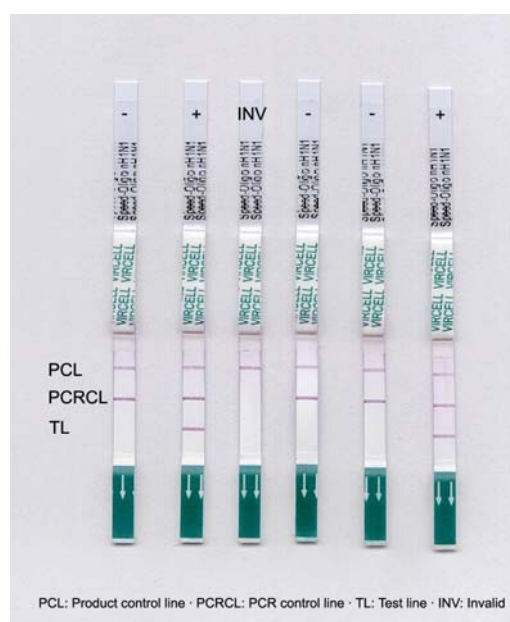
Evaluated assay

SPEED-OLIGO[®] NOVEL INFLUENZA A H1N1 is a PCR-based method coupled to a dipstick device that enables a rapid detection of the nH1N1 influenza A virus. The supplied PCR mix (supplied in lyophilized format) contains a specific oligo pair for the amplification of a fragment in the H1 gene region, together with the Taq polymerase, dNTPs and other elements required for the PCR amplification; a DNA fragment for an internal amplification control and the corresponding primers are also included. cDNAs (2 µl) previously synthesized (see above) and stored at -80°C were used for the PCR reaction according to the manufacturer's instructions. The thermocycler (Labcyler, Sensoquest, Germany) was programmed according to the following parameters:

1 cycle	92°C	1 min
40 cycles	92°C	20 sec
	55°C	20 sec
	72°C	20 sec
1 cycle	72°C	1 min
1 cycle	95°C	1 min
	4°C	

After amplification, PCR products were detected by means of the dipsticks included in the kit, following the supplied instructions. Briefly, 5 µl of denaturalized PCR product is diluted in a running solution and placed in a thermal block set at 55°C. When put in contact with the dipstick, the amplicons flow into the strip to react, in a first instance, with a gold conjugates bearing complementary probes for the specific amplicon and the internal control. The complex between PCR products and gold conjugates reaches the lines (test line and PCR amplification control line) where specific probes have been immobilized, and a second hybridization takes place. A third control line monitors the flow of the liquid along the whole length of the dipstick. Final reading of the results was accomplished visually after 5 minutes incubation of the dipstick. A red line indicated a positive result (see figure 1 for examples and interpretation). The primers and probes have been designed so that only the nH1N1 Influenza A virus is detected.

Figure 1



RESULTS

Analytical sensitivity

The test was able to detect 1 copy of the H1 plasmid used to assay the lower detection limit of the test:

Table 3

Plasmid concentration	Volume per reaction	Amount per reaction	Moles per reaction	Copy number per reaction
0.25 ng/μl	4 μl	1 ng	0.426 fmol	$2.56 \cdot 10^8$
0.025 ng/μl	4 μl	10 pg	$4.2 \cdot 10^{-3}$ fmol	$2.56 \cdot 10^6$
0.5 pg/μl	2 μl	1 pg	$4.0 \cdot 10^{-4}$ fmol	$2.56 \cdot 10^5$
0.5 fg/μl	2 μl	1 fg	$4.0 \cdot 10^{-4}$ amol	256
0.5 ag/μl	2 μl	1 ag	$4.0 \cdot 10^{-4}$ zmol	<1

A positive result was obtained with all dilutions.

Diagnostic performance

Results of the 103 RNA extracts collected during the novel influenza epidemic are shown in Table 4

		SPEED-OLIGO® Novel Influenza A H1N1		
		NEGATIVE	POSITIVE	Total
Reference qRT-PCR	NEGATIVE	63	0	63
	POSITIVE	1*	39	40
	Total	64	39	103

* A faint band was read for this sample. The corresponding Ct obtained in the qRT-PCR assay was 34.

97.5% sensitivity, 100% specificity and 99.0% total agreement values were calculated for SPEED-OLIGO® versus the reference assay.

No SPEED-OLIGO® positive results were obtained with the nucleic acids from 150 respiratory samples collected during previous seasons, giving a 100% diagnostic specificity to the test.

Cross-reactions

None of the nucleic acid preparations from the other respiratory viruses tested in SPEED-OLIGO® gave a positive result. The assay did not present cross-reactivity with the assayed viruses.

CONCLUSIONS

- SPEED-OLIGO® NOVEL INFLUENZA A H1N1 proved to be a reliable, easy to handle and quick test, based on an end-point RT-PCR reaction, able to detect the nH1N1 Influenza A virus.
- The test showed 99% agreement with the validated “in house” qRT-PCR used as reference test in the 103 RNA extracts (40 positive and 63 negative) analyzed with both assays. The only discrepant false negative result shows a faint band on the test line and corresponds to a high Ct in the qRT-PCR. The fact that Speed-Oligo was performed on a previously frozen cDNA material may explain this difference.
- The test showed no false positive results on acid nucleic extracts from 150 respiratory samples collected during 2007-2009, before the onset of the nH1N1 Influenza A outbreak.
- No cross-reactivity with other influenza viruses or other viruses was found when purified acid nucleic preparations were assayed in the new test.