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Development and Preliminary Evaluation of a Rapid Oligochromatographic Assay for Specific Detection of New Human Influenza A H1N1 Virus▽

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A new oligochromatographic assay, Speed-Oligo Novel Influenza A H1N1, was designed and optimized for the specific detection of the 2009 influenza A H1N1 virus. The assay is based on a PCR method coupled to detection of PCR products by means of a dipstick device. The target sequence is a 103-bp fragment within the hemagglutinin gene. The analytical sensitivity of the new assay was measured with serial dilutions of a plasmid that contained the target sequence, and we determined that down to one copy per reaction of the plasmid was reliably detected. Diagnostic performance was assessed with 103 RNAs from suspected cases (40 positive and 63 negative results) previously analyzed with a reference real-time PCR technique. All positive cases were confirmed, and no false-positive results were detected with the new assay. No cross-reactions were observed when other viral strains or clinical samples with other respiratory viruses were tested. According to these results, this new assay has 100% sensitivity and specificity. The turnaround time for the whole procedure was 140 min. The assay may be especially useful for the specific detection of 2009 H1N1 virus in laboratories not equipped with real-time PCR instruments.

Influenza is a highly contagious infectious disease, with annual epidemics that typically occur in the Northern Hemisphere between December and April (12). Major changes in influenza A viruses have led to the periodic emergence of pandemics with increased morbidity and mortality rates (20).

In April 2009, the World Health Organization (WHO) reported a novel H1N1 reassortant virus (2009 H1N1) causing human infection in Mexico and the USA (21). Continued identification of new cases indicated sustained human-to-human transmission of this 2009 H1N1 (15). Spread of the new virus in several countries has led to a pandemic alert situation. In June 2009, the WHO raised the level of influenza pandemic alert from phase 5 to phase 6 (6). Most confirmed cases are characterized by self-limited, uncomplicated febrile respiratory illness, and the symptoms are similar to those of seasonal influenza (8). Some patients have developed severe illness, often characterized by life-threatening pneumonia. In the 58th Pandemic (H1N1) 2009 update, the WHO reported 94,512 laboratory-confirmed cases worldwide, with 429 deaths (22).

This 2009 H1N1 may be detected in samples or viral cultures by direct immunoassays targeting common epitopes of influenza A viruses (9). However, these assays cannot distinguish the novel virus from current seasonal strains. This differentiation can be achieved by reverse transcription-PCR (RT-PCR) targeting specific sequences or by viral culture, followed by hemagglutination inhibition tests or sequencing (only available in certain laboratories). The use of endpoint PCR assays in the clinical setting has been hampered by the laborious and time-consuming methods required to detect PCR products. Oligochromatography, a new technique based on an adaptation of lateral flow immunochromatography, was recently proposed as an easy and rapid alternative for nucleic acid detection (7, 10, 11).

Our laboratory carries out regional influenza surveillance in Andalusia (in the south of Spain) as part of the national influenza laboratory network. In the current pandemic situation, we are investigating and confirming or ruling out 2009 H1N1 in respiratory specimens from Andalusian patients.

The aim of the present study was to describe the development and preliminary evaluation of the utility for 2009 H1N1 detection of Speed-Oligo Novel Influenza A H1N1 test (Vircell, S.L., Granada, Spain), a new assay based on a PCR method coupled to rapid detection of PCR products by means of a dipstick device. Real-time RT-PCR methods for influenza virus typing and subtyping, including specific 2009 H1N1 detection, were used as reference techniques.

MATERIALS AND METHODS

Viral strains and clinical material. To determine the specificity of the assay, RNA or DNA extracted from the following viral strains were tested at 5 ng per reaction: adenovirus, strain Adenoid 71 (ATCC VR-1); influenza A (H5N1) virus, strain A/Vietnam/1194/2004 NIBRG-14 (NIBSC 07/252); influenza A (H1N1) virus, strain A/PR/8/34 (ATCC VR-95); influenza A (H3N2) virus, strain A/Victoria/3/75 (ATCC VR-822); influenza B virus, strain B/Hong Kong/5/72 (ATCC VR-823); parainfluenza 1 virus, strain Sendai/52 (ATCC VR-105); parainfluenza 2 virus, strain Greer (ATCC VR-92); parainfluenza 3 virus, strain C 243 (ATCC VR-93); and respiratory syncytial virus, strain Long (ATCC VR-26).

The clinical utility of the new assay was evaluated by using RNA from 103

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respiratory samples (nasal/throat swabs and/or nasopharyngeal aspirates) previously tested for the presence of 2009 H1N1 (from 26 April to 1 June 2009). We also used DNA or RNA (or their cDNA products, when available) previously extracted from 150 respiratory samples that tested positive for other respiratory viruses (two adenoviruses, two bocaviruses, 1 human metapneumovirus, 1 coronavirus HKU1, 1 coronavirus NL63, 1 coronavirus OC43, 30 seasonal influenza A H1N1 viruses, 47 seasonal influenza A H3N2 viruses, 63 seasonal influenza B viruses, one parainfluenza 3 virus, and one respiratory syncytial virus). All DNAs and RNAs had been frozen at −80°C after routine virological investigation (see below).

Construction of plasmid cDNA. The analytical sensitivity of the new kit was tested by constructing the pH1(531-1076) plasmid, derived from the amplification of cDNA from a clinical sample that had tested positive for 2009 H1N1 with the reference real-time PCR. Primers were designed with Beacon Designer (Premier Biosoft International, Palo Alto, CA), using the strain A/California/04/2009(H1N1) (accession no. FJ966082) as the target sequence. The primer sequences were as follows: pNew-531-F, 5′-CTCAGCAAATCCTACATTAATG-3′, and pNew-1076-R, 5′-GTCCACCCCCCTTCAATG-3′. After conventional PCR, a 545-bp PCR product was purified from a 0.8% agarose gel with NucleoSpin Extract II (Macherey-Nagel, Düren, Germany) and subsequently cloned into the pGEM-T vector system (Promega Biotech Ibérica, SL, Alcobendas, Madrid, Spain), according to the manufacturer's instructions, to obtain pH1(531-1076). To confirm that specific H1 fragments were integrated into the plasmid and that pH1(531-1076) was purified, DNA sequencing was performed in both directions with M13 forward and M13 reverse primers at the López Neyra Institute of Parasitology and Biomedicine (CSIC, Granada, Spain). Plasmid DNA was quantified by measuring the A_{260} (considering a mean extinction coefficient for double-stranded DNA of 50 $\mu g\ ml^{-1}\ cm^{-1}$). The number of copies was estimated from the DNA concentration and plasmid size (3,550 bp) by assuming an average molecular mass of 660 Da for 1 bp of double-stranded DNA. The analytical sensitivity of the new assay was evaluated by performing serial dilutions down to 1 copy of pH1(531-1076)/μl.

RNA extraction and cDNA synthesis. RNA for the 2009 H1N1 investigation was obtained from 200 μl of respiratory samples (nasal and/or throat swabs in viral transport medium or nasopharyngeal aspirates) using the automated bioMérieux NucliSENS easyMAG nucleic acid extraction platform (bioMérieux, Marcy l'Etoile, France), according to the manufacturer's instructions. External lysis was carried out to inactivate clinical samples. Clinical material was managed under biosafety conditions according to safety guidelines (4). Total cDNA was synthesized with random primers from 10 μl of extracted RNAs using the iScript Reverse Transcriptase System (Bio-Rad Laboratories, Hercules, CA).

Reference method. Two routine real-time RT-PCRs 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 2009 H1N1 cases. The screening assay included primers and TaqMan probes targeted at a fragment of the NP gene of both influenza A and B viruses. No mismatches had been found with published sequences of 2009 H1N1 within complementary sequences of the primers and probes (2). 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 viruses H1 and H3, as described by Suwannakarn et al. (16). 2009 H1N1 was found to have several mismatches with the 3′ ends of the primers used in this PCR assay; hence, a case of 2009 H1N1 would yield a positive result for influenza virus A with the screening assay and a negative result with this subtyping assay.

The presence of 2009 H1N1 was confirmed in nonsubtypeable influenza A cases by real-time PCR carried out with SW H1 forward and SW H1 reverse primers and TaqMan SW H1 probe targeted at the H1 gene of this virus, as previously recommended (23). Real-time PCR was performed on cDNAs from nonsubtypeable influenza virus A specimens with the LightCycler FastStart DNA Master^{Plus} Hybridization Probes kit (Roche Diagnostics GmbH, Mannheim, Germany), 0.5 μM concentrations of the primers, and 0.2 μM TaqMan probe. The amplification was performed in a LightCycler 2.0 Instrument (Roche Diagnostics GmbH): 95°C for 10 min and 45 cycles of 95°C for 10 s plus 58°C for 40 s. A single fluorescence reading was taken in each cycle. A readout was performed through channel 530. Inhibition of the PCR was ruled out by real-time PCR of human RNase P, carried out on negative samples, by using RNase P forward and reverse primers and RNase P probe as recommended (23).

Evaluated assay. The Speed-Oligo Novel Influenza A H1N1 test is a PCR-based method coupled to a dipstick device that enables a rapid detection of 2009 H1N1 in clinical samples. The PCR mix (supplied in lyophilized format) contains PCR reagents and a specific oligonucleotide pair for the amplification of a fragment in the H1 gene. An internal control, a plasmid containing a fragment of the *Legionella pneumophila* heat shock protein (*dnaJ*) gene, is also included.

TABLE 1. Accession numbers and strain references of the first available 2009 H1N1 sequences and latest sequences used for the design and validation of primers and probes included in the Speed-Oligo Novel Influenza A H1N1 kit

Influenza A virus strain	GenBank accession no.
First available sequences	
A/California/07/2009(H1N1)	FJ966974
A/California/04/2009(H1N1)	FJ966082
A/Texas/04/2009(H1N1)	FJ966982
A/Texas/05/2009(H1N1)	FJ966959
A/California/06/2009(H1N1)	FJ966960
A/California/05/2009(H1N1)	FJ966952
Rechecked sequences	
A/Shiga/1/2009(H1N1)	GQ261277
A/Texas/05/2009(H1N1)	GQ168861
A/New York/3100/2009(H1N1)	CY041621
A/Moscow/03/2009(H1N1)	GQ255901
A/New York/3313/2009(H1N1)	CY041645
A/California/04/2009(H1N1)	GQ280797
A/Mexico/InDRE4487/2009(H1N1)	GQ303340
A/Arizona/09/2009(H1N1)	GQ323486
A/Illinois/04/2009(H1N1)	GQ323530
A/Washington/14/2009(H1N1)	GQ323509
A/Fuzhou/01/2009(H1N1)	GQ288372
A/Italy/49/2009(H1N1)	GQ283484
A/Finland/554/2009(H1N1)	GQ283488
A/Yokohama/1/2009(H1N1)	GQ287627
A/South Australia/2001/2009(H1N1)	GQ258462
A/New York/3307/2009(H1N1)	CY041637
A/Paris/2591/2009(H1N1)	GQ249333
A/Philippines/2006/2009(H1N1)	GQ243760
A/Israel/644/2009(H1N1)	GQ166760

The dipstick device carries two specific probe pairs for the detection of 2009 H1N1 and internal control PCR products. Each probe pair consists of one probe conjugated with colloidal gold (gold probe) and another probe immobilized onto nitrocellulose (nitrocellulose probe) (see the procedure below). Primers and probes were designed so that only 2009 H1N1 was detected. For the specific 2009 H1N1 detection, primers and probes were designed using the first available hemagglutinin sequences from 2009 H1N1 (accession numbers FJ966974, FJ966082, FJ966982, FJ966959, FJ966960, and FJ966952) (Table 1) (2). These sequences were aligned with the closest sequences found in the database from swine influenza virus strains reported in other seasons (accession numbers EU604689, EU139831, CY028780, AY038014, AF222030, AF455680, CY027515, EU798787, and EU139828). The oligonucleotides were designed so that the 3′-terminal nucleotides did not match with swine influenza virus sequences different from the current pandemic 2009 H1N1. Three different oligonucleotide combinations (Table 2) were evaluated with an initial set of 16 positive and 14 negative respiratory samples. Oligonucleotides were synthesized at the Analytical Services Department of DNA Technology (Risskov, Denmark).

The procedure of the assay was as follows: 2-μl aliquots of the cDNAs were subjected to PCR in a Labcycler instrument (Sensoquest, Göttingen, Germany). Amplification conditions were as follows: 92°C for 1 min, followed by 40 cycles of 92°C for 20 s plus 55°C for 20 s plus 72°C for 20 s, and then a final cycle of 72°C for 1 min and 95°C for 1 min. After amplification, PCR products were detected by means of the dipstick according to the kit instructions. Briefly, 5 μl of denatured PCR product is diluted in 35 μl of running solution and placed in a thermal block set at 55°C. When placed in contact with the dipstick, the PCR product flows into the strip to react, in a first instance, with the gold probe. The PCR product-gold probe complex reaches the line with the nitrocellulose probe and a second hybridization takes place. At the top of the strip, a third control line monitors the flow of the liquid along the strip. This line reacts with the excess of colloidal gold. The final reading was visually accomplished after 5 min of incubation. Reactivity was confirmed by visualization of a red line (Fig. 1, left).

TABLE 2. Combinations of primer and probe sequences tested for the development of the Speed-Oligo Novel Influenza A H1N1 assay

Condition	Sequence (5′→3′)		
	PCR primer	Nitrocellulose probe sequence	Gold probe sequence
A	^{531}CTCAGCAAATCCTACATTAATG^{552} ^{649}AAACATATGYATCTGCATTCTGA^{627}	^{570}CCTCGTGCTATGGGGCATTCA^{590}	^{592}CATCCATCTACTAGTGCTGACCAAC^{616}
B	^{922}CCATTTCAGAATATACATCCGA^{943} ^{1024}GAATAGACGGGAYATTCCTC^{1005}	^{98}CAAAATTGAGACTGGCCACAGG^{1001}	^{957}ATGTCCAAAATATGTAAAAAGC^{978}
C	^{1068}GGGGTGGACAGGGATGG^{1084} ^{1195}CAGAATTTACTTTGTTAGTAATYA^{1172}	^{1117}GAGCAGGGGTCAGGATATGCAGCC^{1140}	^{1093}TGGTACGGTTATCACCATCAAA^{1114}
Control[a]	GAAGTTGAAATTACCGTTCC TGAATTCTGACTTGCCCCATG	GTACTGTTTGTTAGGGATCGGG	GCTAAAAAAGGCACAAGCCCAA

[a] That is, the internal control.

RESULTS

Selection of primers and probes for the oligochromatographic test. The three combinations of primers and probes were evaluated with samples previously tested for the detection of 2009 H1N1 virus by the reference real-time PCR method. No signal was observed with the new assay with samples that yielded a negative result with the reference real-time PCR method. The 16 previously positive 2009 H1N1 samples gave a positive result with the new assay. However, very weak signals were obtained with condition C in 16 positive samples tested, whereas weak signals were obtained with condition A in 3 of these 16 samples. A stronger signal was observed with condition B in all samples. Primers and probes of condition B were finally selected for the evaluation of the assay (Fig. 1, right).

Analytical sensitivity and specificity. The lower limit of detection of the kit was determined by preparing dilutions of pH1(531-1076) from 2.56×10^8 copies to 1 copy. A positive result was obtained down to 1 copy (2 ag) of pH1(531-1076) per reaction. No influenza virus strains from other seasons or other viral strains tested positive with the new assay.

Diagnostic performance. Of the 103 RNAs from respiratory specimens collected during the 2009 H1N1 epidemic, the reference method yielded 40 positive results. All results were both positive with the screening real-time PCR (targeting the NP gene) and the confirmation real-time PCR (targeting the H1 gene of 2009 H1N1). No discrepant results were obtained with these two PCR assays. The cycle threshold (C_T) values obtained in the confirmation real-time PCR ranged from 18.8 to 35.21 (Table 3). All positives were confirmed with the new assay. In 39 of these positive cases, the specific line was clearly visible. The remaining case was also considered positive with the new assay, although only a very faint band was seen in the strip. The new assay showed no cross-reactivity with the 150 RNAs or DNAs from samples with other respiratory viruses. No invalid results were obtained with either the reference technique or the new assay.

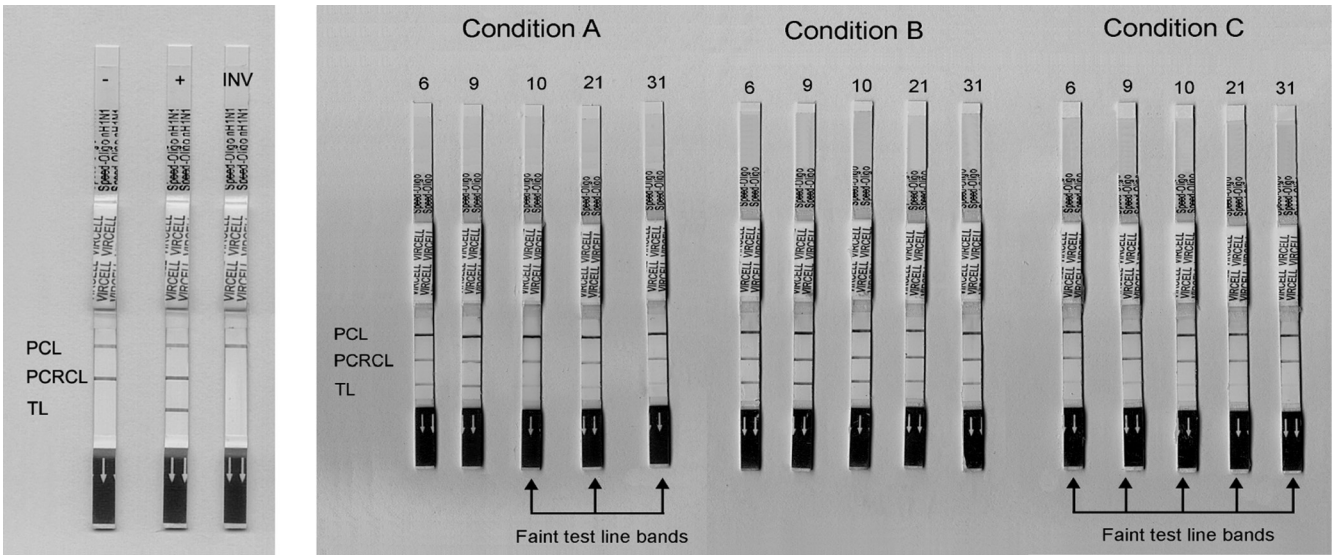

FIG. 1. (Left) Strips of Speed-Oligo Novel Influenza A H1N1 test. Negative (−), positive (+), and invalid results (INV) due to PCR inhibition are indicated. The specific 2009 H1N1 test line (TL), PCR amplification control line (PCRCL), and product control line (PCL) are also indicated. (Right) Strip detection of samples 6, 9, 10, 21, and 31, in which the differential performances between conditions A, B, and C are visible. Very faint test line bands appear in samples 10, 21, and 31 tested with condition A and in all of the samples tested with condition C.

TABLE 3. C_T values obtained with positive samples in the specific 2009 H1N1 real-time PCR assay used as the reference method

Sample no.	C_T
1	18.88
2	31.17
3	22.22
4	25.09
5	20.32
6	20.67
7	27.07
8	29.38
9	24.2
10	33.1
11	22.25
12	28.1
13	21.57
14	21.26
15	22.87
16	25.51
17	21.75
18	27.53
19	32.66
20	22.85
21	26.84
22	32.6
23	35.21
24	19.26
25	24.01
26	24.58
27	28.64
28	33.8
30	21.75
31	27.28
32	25.63
33	21.87
34	27.83
35	18.27
36	22.50
37	21.29
38	32.23
39	25.01
40	28.18

These results yield a 100% sensitivity and specificity for the detection of 2009 H1N1 virus. The total turnaround time required to obtain a result with the new assay was 140 min: 40 min for the RNA extraction, 40 min for the RT step, and 60 min for the PCR and strip detection.

Sequence analysis of new strains. The new test was designed according to the first available H1 sequences of the 2009 H1N1. We rechecked primer and probe sequences used in the assay with 19 currently published H1 sequences of 2009 H1N1 strains from different geographical areas (Table 1) (2). No mismatches with these new sequences were detected in the regions selected for the design of the oligonucleotides.

DISCUSSION

Sequences of 2009 H1N1 genes from isolates collected in different geographical areas are continuously being published. The 2009 H1N1 sequences show ca. 99% homology among strains from different geographical areas, 95% homology with swine origin H1N1 strains from other seasons and 76% homology with seasonal human H1N1. The new virus shows an original and exclusive configuration; six genes (PB2, PB1, PA, HA, NP, and NS) are similar to viruses previously identified in triple-reassortant swine influenza viruses in North American pigs. The other two genes (NA and M) derive from Eurasian swine influenza viruses (8).

The circulation and spread of 2009 H1N1 has shown the need for an accurate diagnosis that enables the rapid detection of new cases (1). Although culture of 2009 H1N1 on MDCK cells followed by sequencing has proved to be a valuable tool to identify the new virus (24), its use in clinical laboratory settings shows evident technical disadvantages. Many molecular "in-house" and commercial methods for the simultaneous detection of seasonal H1N1, H3N2, and influenza B viruses have been used (17). However, some of these methods have been unable to detect 2009 H1N1 (5). In the present study, the real-time PCR used for seasonal H1 detection was not useful to confirm 2009 H1N1 cases, due to mismatches in the sequences complementary to the primers and TaqMan probe of the real-time PCR.

New RT-PCR assays for the specific identification of the 2009 H1N1 have arisen in this scenario as the best diagnostic alternative, targeting different influenza virus genes (hemagglutinin, neuraminidase, and matrix) (3, 13, 14, 19). Cross-reaction with H5N1 has been observed in some of these RT-PCR methods (3). The Centers for Disease Control and Prevention (CDC) (23) recently published recommended sequences for primers and probes against matrix and hemagglutinin genes and real-time PCR protocols to be used to specifically detect 2009 H1N1. Many laboratories have adapted this protocol and included it for confirmation of 2009 H1N1 cases.

New real-time PCR assays have overcome disadvantages of classic end-point PCR assays but imply higher reagent and equipment costs. The Speed-Oligo Novel Influenza A H1N1 test brings together low-cost conventional PCR and rapid detection. Several techniques with this new format have been described (7, 10, 11). At least one of the reactions takes place through a tag-/antitag system (such as biotin/streptavidin) in the reported techniques, whereas reactions occur through direct hybridization with the conjugate and line in the Speed-Oligo test. This double hybridization reinforces the specificity of the assay.

Three different oligonucleotide combinations were initially tested during the development of the test. No false-positive results or cross-reactions were observed with any of them. However, condition C showed a lower sensitivity, and only very weak bands were observed on the strips. That difference was due to a better performance of the PCR amplification in conditions A and B (data not shown). Although conditions A and B showed the same sensitivity in the initial testing, 3 of 16 positive samples yielded stronger signals using condition B. This improved performance was attributed to a better compatibility of the probes during the strip hybridization.

The Speed-Oligo test showed a good analytical sensitivity. It proved possible to detect down to one copy per reaction of pH1(531-1076). The analytical sensitivity of the assay was not tested with cDNA from 2009 H1N1 strains, although we assume that no significant deleterious effect on the sensitivity must appear. The new assay also demonstrated a good diagnostic performance compared to the reference method. All positive cases with the reference technique were confirmed

with the new assay. In just one out of the 40 positive results, a faint signal could be detected in the strip, although it was sufficient to be considered as positive. A cycle threshold (C_T) value of 35.21 had been obtained for this sample in the real-time PCR targeting the H1 gene. With this high C_T value, we assume that a relatively low viral load was present in the specimen. Moreover, the assay was evaluated with previously frozen RNAs. Hence, the appearance of a faint signal in this case might be explained by the low amount of viral RNA in the sample and by the use of frozen RNAs. The remaining 39 positive cases yielded C_T values with the same real-time PCR assay ranging from 18.8 to 33.1. In all cases, the strip revealed a clear positive result. A 100% specificity was obtained when negative samples collected for 2009 H1N1 investigation were assayed. No cross-reactions were observed when DNAs or RNAs from other respiratory viruses were assayed.

One of the advantages of oligochromatographic tests is that the detection step takes only a few minutes. With the assay described here, PCR is completed within 55 min and detection requires no more than 5 min.

Although this new test was developed when only the first few sequences of the 2009 H1N1 were available, comparison with more recent sequences from diverse geographical areas showed no changes that could affect the sensitivity of the test. The new test was not assayed with the H1 influenza virus of swine origin, but no cross-reactivity would be expected. In contrast, other methods for 2009 H1N1 detection have shown that different influenza A viruses of swine origin can also be amplified (14, 18).

In addition to the tests described here, the assay was subjected to stability, reproducibility, and traceability tests in order to fulfill the CE requirements of European Guideline 98/79/EC. This new assay is now commercially available under the CE mark for *in vitro* diagnostic products.

In summary, the new assay is simple, rapid, and flexible and provides a good sensitivity for the specific detection of 2009 H1N1. The oligochromatographic method used for the detection of the PCR amplicons avoids the manipulation requirements, as well as the technical and material requirements, normally associated with the classical agarose gel methods. This assay might be an alternative to real-time PCR assays for laboratories not equipped with real-time PCR instruments. The use of automatic extraction systems further simplifies the whole procedure.

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