Original Article

Effectiveness of two rapid influenza tests in comparison to reverse transcription-PCR for influenza A diagnosis

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Abstract

Introduction: The influenza A virus is responsible for high morbidity and mortality in children and adults worldwide. Thus, a rapid, sensitive, and specific diagnosis tool is required.

Methodology: An immunofluorescence assay (DFA) and a lateral-flow immunochromatographic assay were compared with RT-PCR for detection of the influenza A virus in 113 nasopharyngeal wash samples obtained from pediatric patients. Samples were collected between July and December 2009, during the pandemic outbreak of influenza A H1N1/09.

Results: The sensitivity, specificity, and positive and negative predictive values obtained for the DFA were 68.97%, 76.63%, 75.47%, and 70%, respectively, while the values obtained for the immunochromatographic assay were 58.62%, 81.82%, 77.27%, and 65.22%, respectively. The frequency of the influenza A virus was 51.33%, and a total of 27 samples were positive for the pandemic influenza A H1N1/09.

Conclusions: DFA and the immunochromatographic assay can be important tools for patient care during influenza season and in outbreaks as they usually provide results within 45 minutes. Furthermore, positive results in conjunction with the patient's symptoms could provide a correct diagnosis, thus facilitating appropriate patient management. Nonetheless, the results of these assays still require confirmation by RT-PCR.

Key words: influenza; diagnostic; rapid; effectiveness; outbreak

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Introduction

Influenza A is a member of the *Orthomyxoviridae* family and has a linear, segmented, negative-sense, single-stranded RNA genome. The virus is classified according to the hemagglutinin and neuraminidase subtypes, which define its antigenicity, present in its envelope. The epidemiological success of influenza A is mainly attributed to its ability to carry out genetic reassortment, which is responsible for the generation of new strains of viruses with pandemic potential [1,2].

It has been estimated that between 25 and 50 million cases of interpandemic influenza A occur annually in the United States alone, leading to 150,000-200,000 hospitalizations and 30,000-40,000 deaths. Extrapolating these values to the world, approximately 600 million cases and 250,000-500,000 deaths occur annually in the general population [3]. It is important to keep in mind that the number of deaths attributed to influenza A is difficult to determine because infections caused by the virus are usually not confirmed. Furthermore, many deaths associated with influenza occur due to secondary complications, when the virus is no longer detectable [4]. In addition to the number of cases resulting from interpandemic outbreaks, influenza pandemics occur in all age groups and lead to a high number of cases in a short amount of time.

In early April 2009, a new influenza A virus of subtype H1N1 emerged unexpectedly among humans in California and Mexico, rapidly expanding worldwide through direct transmission from human to human, generating the first pandemic of the 21st century [3]. Between then and August 2010, a total of 18,449 confirmed deaths were caused by the influenza A H1N1/09 strain [5].

Due to the high mortality caused by the influenza A virus, a rapid, sensitive, and specific diagnostic tool that facilitates appropriate management of patients with these infections is required. However, laboratory

techniques used for viral diagnosis are varied and differ in their sensitivity, cost, and time to obtain results.

Virus isolation in cell culture has been considered the gold standard for diagnosis of the influenza virus. However, this method presents some limitations, such as the time required to obtain results, because the characteristic cytopathic effect (CPE) caused by the virus can take days to occur. For this reason, there are some different rapid techniques based on immunofluorescence and immunoassays that provide results in minutes, although these techniques vary widely in sensitivity and specificity. Therefore, since 2000, molecular biology-based techniques such as reverse transcription-PCR (RT-PCR) have been implemented to identify viral pathogens such as influenza A. RT-PCR offers results that are just as sensitive and specific as those obtained from viral isolation in cell culture [6-8].

In this study, we evaluated the sensitivity, specificity, and predictive values of a direct immunofluorescence assay (DFA) and a lateral-flow immunochromatographic (IC) assay for detection of the influenza A virus, in comparison with RT-PCR, during the pandemic outbreak of influenza A H1N1/09 in Mexico.

We also evaluated the presence of respiratory syncytial virus (RSV) and human metapneumovirus (hMPV) in most samples using DFA, as these viruses are recognized as important causes of respiratory illnesses in infants and young children. To our knowledge, this is the first study reporting the effectiveness of rapid influenza diagnostic tests in children during the outbreak of the pandemic influenza A H1N1/09 virus.

Methodology

Samples

A total of 113 nasopharyngeal washes were obtained from pediatric patients with typical signs of influenza (*e.g.*, fever, headache, cough, sore throat, stuffy nose) who were hospitalized at the Pediatric Hospital of Sinaloa (HPS) and the Mexican Institute of Social Security (IMSS, Hospital No. 35), located in the city of Culiacan Sinaloa, Mexico, between July and December 2009. Approval was obtained from the institutional human research ethics committees. Once obtained, samples were transported to the Research Department in viral transport medium MicroTest M4 (Remel, Dartford, UK). The samples were washed with PBS (pH 7.4) and centrifuged at 2,500 rpm for 5 minutes. The supernatants were stored at -70°C for later use, while the cell pellets were fixed with sterile acetone for 10 minutes at room temperature, washed with PBS, and stored at 4°C for later use.

Viral RNA isolation

Viral RNA was extracted from 200 µL of supernatant samples using the PureLink Viral RNA/DNA Mini Kit (Invitrogen, Carlsbad, CA, USA). Extracted RNA was used immediately for RT-PCR.

RT-PCR

Viral RNA was processed using the SuperScript III One-Step RT-PCR System with Platinum Taq DNA Polymerase (Invitrogen, Carlsbad, CA, USA). Previously described M52C (5'-CTTCTAACCGAGGTCGAAACG-3') and M253R (5'-AGGGCATTTTGGACAAA(G/T)CGTCTA-3') primers [9], which amplify a 244 bp product corresponding to the matrix gene of the influenza A virus, were used. RT-PCR was performed in a Mastercycler gradient thermocycler (Eppendorf, Hamburg, Germany) under the following conditions: cDNA synthesis at 55°C for 30 minutes, an initial denaturation step at 95°C for 2 minutes, 40 cycles of 95°C for 15 seconds, 45°C for 30 seconds, and 68°C for 1 minute, followed by a final extension at 68°C for 5 minutes. RT-PCR products were visualized by 2% agarose gel electrophoresis and ethidium bromide (10 mg/mL) staining in a UV transilluminator. The positive control used was viral RNA obtained from the influenza A/Swine/1976/31 (H1N1) strain (ATCC VR-99^{M}) (ATCC, Manassas, VA, USA), and the negative control included template-free reaction tubes.

Identification of influenza A H1N1/09 by qRT-PCR

Influenza A positive samples confirmed by RT-PCR were processed according to the CDC protocol for the identification of new subtypes of influenza A virus. Viral RNA was isolated using the MagNA Pure LC Total Nucleic Acid Isolation Kit, which is designed to be used with the MagNA Pure LC Instrument LC 2.0 (Roche Applied Science, Mannheim, Germany). The SuperScript III Platinum One-Step Quantitative Kit (Invitrogen, Carlsbad, CA, USA) and the 7500 Real Time PCR System (Applied Biosystems, Carlsbad, CA, USA) were used for qRT-PCR. The primers and probes used were as follows: InfA Forward (5'GACCRATCCTGTCACCTCTGAC-3'), InfA Reverse (5' AGGGCATTYTGGACAAAKCGTCTA-3'), InfA

Probe (5'TGCAGTCCTCGCTCACTGGGCACG-3'), SW InfA Forward (5'-GCACGGTCAGCACTTATYCTRAG-3'), SW InfA Reverse (5'-GTGRGCTGGGTTTTCATTTGGTC-3'), SW InfA Probe (5'-CYACTGCAAGCCCA"T" ACACACAAGCAGGCA-3'), SW H1 Forward (5'-GTGCTATAAACACCAGCCTYCCA-3'), SW H1 Reverse (5'-CGGGATATTCCTTAATCCTGTRGC-3'), SW H1 Probe (5'-CAGAATATACA"T"CCRGTCACAATTGGARAA-3'), RnaseP Forward (5'-AGATTTGGACCTGCGAGCG-3'), RnaseP Reverse (5'-GAGCGGCTGTCTCCACAAGT-3'), and RnaseP Probe (5'-TTCTGACCTGAAGGCTCTGCGCG-3').

Influenza A DFA

A total of 25 µL of the cell pellet was added to a Teflon-coated glass microscope slide with a single 6 mm diameter well (OXOID, Cambridge, UK) and air-dried. Cell spots on each slide were stained with 25 µL of reagent included in the IMAGEN Influenza A and B Kit (OXOID, Cambridge, UK) at 37°C for 15 minutes in a moist chamber. Excess reagent was washed with 1X PBS, the slide was air-dried, and a drop of mounting fluid was added. Finally, the slides were examined at x100 magnification with a DM1000 fluorescence microscope (Leica Microsystems, Heidelberg, Germany). Samples showing either cytoplasmic or nuclear apple-green fluorescence were considered positive.

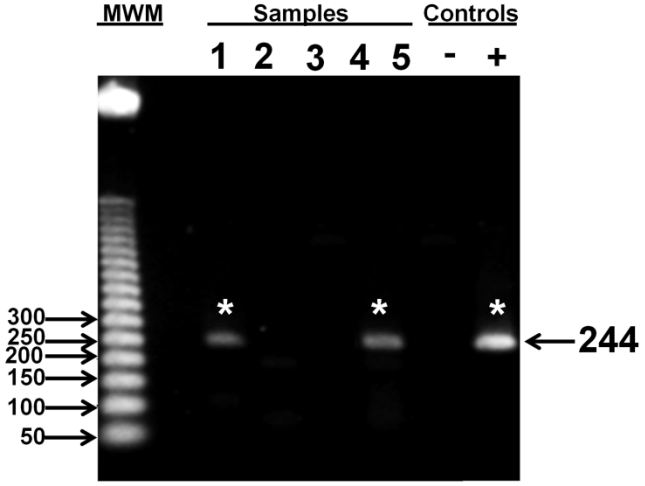
Lateral-flow immunochromatographic assay

The Xpect Flu A&B Kit (Remel, Lenexa, KS, USA) was used according to the manufacturer's instructions. A total of 100 µL of supernatant (previously obtained) was mixed with 100 µL of specimen diluents into a dilution tube provided with the kit. A total of 100 µL of the homogenized mixture was transferred with a transfer pipette to the test device. The results were read after 15 minutes and were considered positive if they showed two blue bands, one in the detection (T) region and the other in the control (C) region of the test device.

Respiratory syncytial virus and human metapneumovirus DFA

A total of 77 samples were evaluated for RSV and hMPV using DFA. A total of 25 µL of the cell pellet was evaluated according to the manufacturer's instructions, similar to the procedure for the influenza A DFA. The kits used were IMAGEN Respiratory Syncytial Virus (RSV) (OXOID, Cambridge, UK) and IMAGEN hMPV Kit (OXOID, Cambridge, UK). Samples showing either cytoplasmic or nuclear apple-green fluorescence were considered positive.

Figure 1. Detection of the influenza A virus by RT-PCR. 2% agarose gel electrophoresis. Lanes: molecular weight marker (sizes from 50 bp to 800 bp, in increments of 50 bp); 1-5, samples; 6, negative control; 7, positive control (influenza A/Swine/1976/31 H1N1). Specific amplicon for the influenza A virus corresponds to a size of 244 bp, as is indicated.



Determination of sensitivity, specificity and predictive values

Sensitivity, specificity and positive and negative predictive values were determined using the public domain statistical package Epi Infoversion 3.5.1 developed by the CDC.

Results

A total of 113 samples were received between July and December 2009, with the highest numbers received in August (32; 28.31%) and September (33; 29.2%). A total of 54% of the samples were from males, while the remaining 46% were from females; the patient's sex was not specified in two samples. A total of six age groups were formed: (1) less than one year of age (n = 18); (2) 1 to 3 years (n = 31); (3) 4 to 6 years (n = 23); (4) 7 to 9 years (n = 12); (5) 10 to 12 years (n = 10); and (6) over 12 years (n = 10). The patient's age was not specified in nine samples.

A total of 58 samples (51.33%) were positive for the influenza A virus by RT-PCR (Figure 1); positive samples were predominant from July to September (82.75%; 48/58). There were no differences in the number of positive samples between the sexes; there were 29 positive samples for each sex. The following age groups showed higher numbers of cases for both genders: less than one year (17.24%, 10/58); 1 to 3

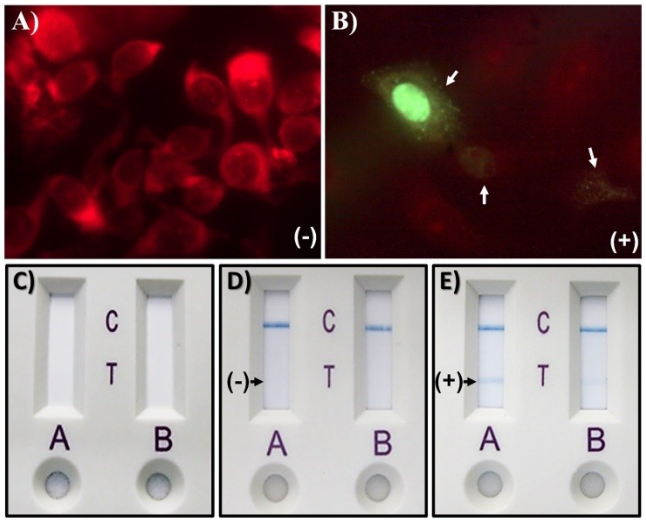
years (24.13%, 14/58); and 4 to 6 years (18.96%, 11/58).

The 58 influenza A positive samples were processed by qRT-PCR to identify influenza A H1N1/09. A total of 27 samples (46.55%, 27/58) were positive for the pandemic strain and were received mostly in August (48.14%). The positive samples were predominantly from male patients (51.85%, 14/27), and the age groups that showed an increased number of cases of the pandemic virus were 1 to 3 years and 4 to 6 years, with a total of seven and five positive samples, respectively (Table 1).

With DFA, 53 influenza A positive samples (46.9%, 53/113) and 60 negative samples (53.1%, 60/113) were obtained (Figure 2: panel A for a negative result and panel B for a positive result); with the IC assay, 44 positive samples (38.94%, 44/113) and 69 negative samples (61.06%, 69/113) were obtained (Figure 2: panel D for a negative result and panel E for a positive result for influenza A). The concordance determined between the DFA and RT-PCR (standard) assays was 72.56%, whereas the concordance determined between the IC and RT-PCR assays was 69.91%. The DFA had a sensitivity of 68.97% and a specificity of 76.36%, while the IC assay exhibited a sensitivity of 58.62% and a specificity of 81.82% (Table 2).

Samples were also tested for the presence of hMPV and RSV using DFA. From 77 tested samples, 17 (22.07%, 17/77) were positive for RSV, and 20 (25.97%, 20/77) were positive for hMPV. For RSV, positive cases were high in the male population (58.82%, 10/17) and in the 1 to 3 years age group (29.41%, 5/17). For hMPV, positive cases were high in the male population (55%, 11/20) and in the 1 to 3 years (35%, 7/20) and 4 to 6 years (30%, 6/20) age groups (Table 3). A total of 19 samples (24.67%, 19/77) showed some type of coinfection based on DFA. The frequency of coinfection with RSV and influenza A was 6.49% (5/77); with hMPV and influenza A, it was 10.38% (8/77); and with RSV and hMPV, it was 2.59% (2/77). Four samples (5.19%, 4/77) showed concurrent infection by all three viruses (Table 4).

Figure 2. Images obtained by fluorescence microscopy and lateral immunochromatographic assay.



(A) Respiratory epithelial cells present in a nasopharyngeal wash sample, x40
(B) Respiratory epithelial cells from a nasopharyngeal wash sample infected with the influenza A virus; white arrows indicate infected cells, x100. Images obtained by lateral immunocromatografic assay.
(C) Typical aspect of the test
(D) Aspect for a negative sample
(E) Aspect for a positive sample

Discussion

The influenza A virus is associated with severe respiratory complications such as bronchiolitis and pneumonia [10]. However, the infections caused by influenza are often confused with those caused by the respiratory syncytial virus. For this reason, the early diagnosis of influenza A infection could have an impact on patient care, specific antiviral therapy, and other aspects of clinical management. Furthermore, for hospitalized adults and children, a rapid diagnosis could significantly reduce their hospital stay and avoid the complications of secondary bacterial pneumonia or mixed pneumonia. To that end, the development and use of rapid viral diagnosis tests could allow physicians to make more accurate decisions about treatment and reduce the unnecessary use of antibiotics. However, the laboratory techniques used to diagnose respiratory viruses are diverse and differ in sensitivity, cost, and time to obtain results [11-13].

In Mexico, according to data from the Ministry of Health in 2008, acute respiratory infections were the leading cause of disease among the general population. These infections were predominantly found in one- to four-year-old children, with a total of 5.3 million cases (22.25%) out of 24.1 million cases in the general population.

Table 1. Number of positive and negative samples for influenza A by RT-PCR and positive samples for the H1N1/09 strain by qRT-PCR, according to sex and age group

Age group (years)	Sex								
	Male			Female			Not specified		
	Positive	Negative	Positive for A H1N1/09 (n = 14)	Positive	Negative	Positive for A H1N1/09 (n = 13)	Positive	Negative	Positive for A H1N1/09
< 1	8	4	3	2	4	1	-	-	-
1-3	6	9	2	8	8	5	-	-	-
4-6	6	9	3	5	3	2	-	-	-
7-9	4	3	3	3	2	1	-	-	-
10-12	2	2	2	5	1	2	-	-	-
> 12	3	2	1	3	2	1	-	-	-
Not specified	-	2	-	3	2	1	-	2	-
Total	**29**	**31**	**14**	**29**	**22**	**13**	**-**	**2**	**-**

Table 2. Sensitivity, specificity, and positive and negative predictive values of DFA and the lateral-flow immunochromatographic (IC) assay in comparison with RT-PCR

Assay	No. of specimens				Sensitivity (%)	Specificity (%)	PPV[a] (%)	NPV[a] (%)
	True positive	True negative	False positive	False negative				
DFA	40	42	13	18	68.97	76.63	75.47	70
IC assay	34	45	10	24	58.62	81.82	77.27	65.22
RT-PCR	58	55	0	0	100	100	100	100

[a]PPV, positive predictive value; NPV, negative predictive value

Table 3. Number of positive and negative samples for hMPV and RSV by DFA, according to sex and age group

Age group (years)	Sex											
	Male				Female				Not specified			
	hMPV (+)[a]	hMPV (-)[a]	RSV (+)[b]	RSV (-)[b]	hMPV (+)	hMPV (-)	RSV (+)	RSV (-)	hMPV (+)	hMPV (-)	RSV (+)	RSV (-)
< 1	1	7	3	5	-	4	-	4	-	-	-	-
1-3	4	6	2	8	3	8	3	8	-	-	-	-
4-6	3	7	1	9	3	1	1	3	-	-	-	-
7-9	-	4	-	4	-	4	-	4	-	-	-	-
10-12	1	2	2	1	2	3	1	4	-	-	-	-
> 12	1	2	1	2	-	5	1	4	-	-	-	-
NS	1	-	1	-	1	2	-	3	-	2	1	1
Total	**11**	**28**	**10**	**29**	**9**	**27**	**6**	**30**	**-**	**2**	**1**	**1**

NS: Not Specified
[a]hMPV (+), positive sample for human metapneumovirus; hMPV (-), negative sample for human metapneumovirus
[b]RSV (+), positive sample for respiratory syncytial virus; RSV (-), negative sample for respiratory syncytial virus

Table 4. Samples with some level of coinfection by influenza A, hMPV, and RSV by DFA

	Type of coinfection				
	Influenza A + RSV	Influenza A + hMPV	Influenza + RSV + hMPV	RSV + hMPV	Total
No. of specimens (n = 77)	5/77 (6.49%)	8 (10.38%)	4 (5.19%)	2 (2.59%)	19 (24.67%)

The frequency of the influenza A virus found in this study was 51.33%. It is important to note that the pandemic outbreak of influenza A H1N1/09 occurred during the study period; for this reason, the frequency is higher than that reported in other studies (10% [14], 16.63% [7], 21.6% [10], 37.6% [15] and 38.6% [16]). Nonetheless, Habib-Bein *et al*. found a similar frequency when they analyzed 238 respiratory specimens using qRT-PCR [17].

Respiratory viruses have commonly been detected using virus isolation in cell culture, with a variety of cell lines. However, this technique is hampered by the need to quickly inoculate clinical samples in multiple cell lines, and it requires time to yield reliable results due to the variability in the time that the virus takes to cause a cytopathic effect (in the case of influenza A, this can range from two days to two weeks [17]).

One of the most important aspects to consider during the identification of the influenza A virus is the collection of specimens and transport to the laboratory. These are considered to be the cornerstones for rapid and accurate diagnosis of the acute respiratory infections caused by this virus. Indeed, the identification of influenza viruses in clinical patient samples is highly dependent on the source of the specimens [18]; samples of nasopharyngeal aspirates and nasopharyngeal washes are superior to other types of samples for the detection of this virus because they yield a large number of epithelial cells during the collection process [19].

The sensitivity of DFA found in this study was 68.97%, which is higher than the 62% found in some evaluations [20] but lower than that reported in some other studies (83% [7], 98.7% [17], and even 100% [21]). In these other studies, the specificities reported were greater than 95%, higher than the 76.63% found in this study. The positive predictive value (PPV) and negative predictive value (NPV) found in our study were 75.47% and 70% respectively. Other studies reported PPVs of 57% [22], 62.8% [6], and 88.1% [23]; these same studies reported NPVs above 79%.

The identification of the influenza A virus or other viruses by DFA depends greatly on the type and quality of the specimens used because samples containing fewer cells are difficult to interpret, and experience is required for interpretation to avoid false positives or negatives that could affect decisions about patients.

The sensitivity for the IC assay found in this study was 58.62%; other studies that used the same technique found sensitivities of 43.6% [10], 44% [25], 55% [26], 56% [27], and over 94% [21,24]. In these studies, the specificities found were above 99%, while the specificity found in our study was 81.82%. The PPVs and NPVs in our study were 77.27% and 65.22%, respectively, while other studies have reported PPVs from 73% [28] and 83% [21] to 100% [29], and NPVs from 56% to 95%. Because immunoassay-based techniques require high viral loads to produce positive results, it is possible that the differences in the sensitivity found in our study and the other ones using this technique could be due to the viral load present in the samples. Another aspect that should be considered when using this kit is that the results can be subject to misinterpretation because they depend on what is observed by the human eye, so the values could vary depending on the skill of the technician.

Importantly, RSV and hMPV were detected in the samples analyzed. However, because the presence of these viruses was demonstrated by DFA, the results should be evaluated with caution and confirmed using techniques such as RT-PCR. Based on the DFA, a total of 19 samples showed some type of coinfection. Influenza A and RSV coinfection was found in 5 of 77 samples (6.49%); this value is higher than reported in other studies, which are in the 3%-4% range [30-32]. The frequency of influenza A and hMPV coinfection was 10.38% (8/77). Again, these cases of confections should be confirmed by RT-PCR.

The DFA and IC assays had a sensitivity and specificity of at least 60%, which indicates that these techniques possess some clinical utility. One advantage of IC assays is that this test can be performed in a doctor's office in 30 minutes; meanwhile, DFA and RT-PCR require specialized equipment, special training, and more time to perform. Making a time/cost analysis between DFA and RT-PCR (not shown), it seems that DFA could be more expensive than RT-PCR if the institution does not have equipment for each test. Each laboratory must assess the optimal methods for its situation and the best application of each technique, taking into account numerous factors including its budget, equipment, staff expertise, the patient population that it serves, the needs of its submitting clinicians, and its surveillance and public health responsibilities.

Based on the results obtained in this study, we speculate that a diagnosis based on the DFA test together with the criterion from clinicians, may give an opportune and correct diagnosis during a new pandemic caused by a novel influenza strain in hospitalized and immunosuppressed patients who require a rapid treatment, before generation of new

primers for a specific RT-PCR. To our knowledge, this is the first study reporting a diagnostic evaluation of rapid influenza tests in children during the outbreak of the pandemic influenza A H1N1/09 in Mexico.

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