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Performance of a commercial assay for the diagnosis of influenza A (H1N1) infection in comparison to the Centers for Disease Control and Prevention protocol of real time RT-PCR

MARÍA G. BARBÁS[1*], SANDRA V. GALLEGO[1], GONZALO M. CASTRO[1], ELSA BAUMEISTER[2], SILVIA KADEMIAN[1], JUAN DE LEON[1], ANALÍA CUDOLÁ[1]

[1]Laboratorio Central del Ministerio de Salud de la Provincia de Córdoba, Tránsito Cáceres de Allende 421 (5000) Córdoba; [2]INEI-ANLIS Dr Carlos G. Malbrán, Ciudad Autónoma de Buenos Aires. Argentina.
**Correspondence. E-mail. labmolecularcba@yahoo.com.ar*

ABSTRACT

At the time of influenza A (H1N1) emergency, the WHO responded with remarkable speed by releasing guidelines and a protocol for a real-time RT-PCR assay (rRT-PCR). The aim of the present study was to evaluate the performance of the "Real Time Ready Influenza A/H1N1 Detection Set" (June 2009)-Roche kit in comparison to the CDC reference rRT-PCR protocol. The overall sensitivity of the Roche assay for detection of the *Inf A* gene in the presence or absence of the *H1* gene was 74.5 %. The sensitivity for detecting samples that were only positive for the *Inf A* gene (absence of the *H1* gene) was 53.3 % whereas the sensitivity for H1N1-positive samples (presence of the *Inf A* gene and any other swine gene) was 76.4 %. The specificity of the assay was 97.1 %. A new version of the kit (November 2009) is now available, and a recent evaluation of its performance showed good sensitivity to detect pandemic H1N1 compared to other molecular assays.

Key words: pandemic, PCR, influenza

RESUMEN

Evaluación del desempeño de un equipo comercial para el diagnóstico de influenza A (H1N1) en comparación con el protocolo de RT-PCR en tiempo real diseñado por los Centros de Control y Prevención de Enfermedades (CDC). Durante la pandemia de influenza A (H1N1), la OMS recomendó algoritmos y protocolos de detección del virus mediante RT-PCR en tiempo real. El objetivo del presente estudio fue evaluar el desempeño del equipo que comercializa la empresa Roche, *Real Time Ready Influenza A/H1N1 Detection Set* (junio de 2009), en comparación con el protocolo de RT-PCR en tiempo real de los CDC. La sensibilidad global del ensayo de Roche para la detección del gen *Inf A* en presencia o ausencia del gen *H1* fue 74,5 %. La sensibilidad para la detección de muestras positivas solo para el gen *Inf A* (ausencia del gen *H1*) fue 53,3 % y la sensibilidad para la detección de muestras positivas para H1N1 (presencia del gen *Inf A* y cualquier otro gen porcino) fue 76,4 %. La especificidad fue 97,1 %. Existe una nueva versión del equipo (noviembre 2009) que, según se ha descrito, presenta buena sensibilidad en comparación con otros ensayos moleculares para detectar H1N1 pandémica.

Palabras clave: pandemia, PCR, influenza

The World Health Organization (WHO) Collaborating Centre for Reference and Research on Influenza at the Centers for Disease Control and Prevention (CDC), U.S., reported the first two cases of human infection by swine-origin influenza A virus in April 2009 (1). This strain was further characterized by a unique combination of gene segments from swine influenza A and human influenza A (H1N1) viruses that had not been previously identified. Thereafter, in May 2009, new cases of human infection caused by the same novel virus were also identified in Mexico, Canada, and elsewhere around the world. Human infection by *Swine influenza virus* had been previously reported (9, 10, 12); however, no evidence of efficient transmission of these strains between humans had been demonstrated before 2009 (3, 10). In contrast, pandemic H1N1 virus emerged in 2009 as a novel virus subtype easily transmitted among humans. Within a month of the initial identification of the virus, a considerable number of cases were reported throughout 21 countries worldwide (4, 14).

Molecular diagnoses are currently the method of choice for influenza A (H1N1) swine lineage viruses (A/California/4/2009-like viruses). The use of different target gene assays is the most appropriate for the correct identification of this virus subtype. Therefore, soon after the identification of the swine-origin influenza virus, WHO released guidelines and a protocol for a

real-time RT-PCR (rRT-PCR) assay (2). Such guidelines recommended the use of a combination of three primer and probe sets: *Inf A*, designed to amplify a conserved region of the matrix gene from all influenza A viruses; sw*H1*, to specifically detect the hemagglutinin gene segment (subtype H1) from *Influenza A (H1N1) virus*; and sw*Inf A*, targeted to the nucleoprotein (NP) gene segment from all swine influenza viruses. The availability of this rapid and sensitive assay for the detection of *Influenza A (H1N1) virus* was critical for restraining the spread and extension of the pandemia. In Argentina, the National Reference Laboratory (ANRL) confirmed the first case of H1N1 virus infection on May 16th 2009, in a person who had travelled to the USA. The ANRL was initially designated as the unique officially authorized laboratory for diagnosing the pandemic H1N1 2009 virus infections. By the end of June 2009, after a rapid increase in the number of cases (8) and the raise of the pandemic alert level by WHO, the ANRL called for a National Meeting with representatives of some provinces, including Córdoba, in order to implement the diagnosis of H1N1 in the national laboratory network. Technical training on rRT-PCR to detect the novel *Influenza A (H1N1) virus*, following the CDC Protocol, was performed. Moreover, the methodology was transferred to the corresponding public health laboratories all over the country. In Córdoba, the main Mediterranean province of Argentina, the Central Laboratory was the only one officially authorized by *Ministerio de Salud de la Provincia de Córdoba* to immediately carry out the diagnosis of H1N1 in response to the sanitary emergency.

Real time RT-PCR based on the CDC protocol was implemented in this laboratory. At the time of the emergency, the "Real Time ready Influenza A/H1N1 Detection Set"-Roche (June 2009 version) (5) was commercially available for H1N1 virus detection in Argentina. Afterwards, a new FDA approved version of the Roche assay became available in November, 2009 (6). The present study evaluated the performance of the Roche marketed kit (June version) in comparison to the CDC assay for the diagnosis of *Influenza A (H1N1) virus*.

This study was carried out in respiratory samples received at the Central Laboratory between July 6th and November 11th, 2009. All specimens were collected from patients with influenza-like illness who were suspected cases of influenza A (H1N1). A total of 1,341 combined nasal and/or pharyngeal swabs or other nasopharyngeal specimens were studied for *Influenza A (H1N1) virus* by rRT-PCR, using the CDC protocol (2) on a BioRad Chromo 4 Thermal-Cycler. The QIAamp Viral RNA Mini Kit (Qiagen, Valencia, CA, USA) was used to obtain the viral RNA. Criteria to define positive or negative samples were adopted from CDC guide-lines (2) and subsequently, from the modifications introduced by the ANRL in July 2009. Accordingly, a sample was defined as positive for *Influenza A (H1N1) virus* if both, the *Inf A* and the respective growth curves of sub-type sw*Inf A* reaction crossed the threshold line within 40 cycles, regardless of the reactivity for sw*H1*. Due to the high prevalence of the virus during the epidemic**,** it was assumed that the only viral strain of swine origin circulating at that time corresponded to the new pandemic virus. Besides, any specimen positive for *Inf A* and sw*H1* subtype was considered presumptively positive for *Influenza A (H1N1) virus*.

The clinical performance characteristics of the Real Time Ready Influenza A/H1N1 Detection Set on the LightCycler 2.0 (Version 4.1 Software) instrument were established by comparing the respective test results with those obtained with the rRTPCR based on the CDC protocol. In this study, 136 samples (10 %) selected from the 1,341 total samples studied by the CDC protocol during the epidemic (July - November 2009) were tested by the Roche assay, strictly under the manufacturer's instructions. The RNA from these samples was purified and conserved at -70 ºC until used.

Of the 136 samples studied, 34 negative and 30 positive samples for influenza A gene alone were randomly selected. On the other hand, the 72 positive samples for *Influenza A (H1N1) virus* studied were selected according to their reactivity (Ct value). Thus, they were classified as high-positive (32/72) if they resulted positive for three viral genes and as low-positive (40/72) when they resulted positive for two viral genes and crossing the threshold line after cycle 34. The 30 positive samples for influenza A gene alone were typified as non-H3N2 seasonal influenza A using an rRT-PCR protocol (7).

Roche´s assay is based on the detection of the conserved matrix protein 2 gene (*M2*) while the respective subtype identification is based on the detection of hemagglutinin H1 gene (5). Total nucleic acids from clinical samples were isolated by using the High Pure Viral Nucleic Acid Kit (Roche), according to the manufacturer's instructions. The results obtained for these samples when tested by the rRT-PCR - Roche assay are shown in Table 1.

Performance parameters were calculated and analysed by means of EPIDAT 3.1 software. The commercial assay showed global sensitivity for *Inf*

Table 1. Comparison of Real time ready Influenza A/H1N1 Detection Set (Roche) gene detection *vs.* rRT-PCR based on CDC protocol results

Roche assay gene detection	CDC protocol results		
	Influenza A (H1N1) positive	Influenza A[(1)] positive	Negative
$A^+/H1^+$	52	6	0
$A^+/H1^-$	8	10	1
$A^-/H1^-$	9	14	32
$A^-/H1^+$	3	0	1
Total	72	30	34

(1) Samples were typified as non-H3N2 seasonal Influenza A using rRT-PCR

A gene detection, regardless of the presence of the *H1* gene, of 74.5 % (95 % CI: 65.6 % to 83.5 %) and specificity of 97.1 % (95 % CI: 89.9 % to 100.0 %) considering that one out of 34 samples that resulted negative for *Influenza A (H1N1) virus* by CDC protocol resulted positive for the influenza A gene alone ($A^+/H1^-$) when evaluated by Roche's commercial kit. Regarding the concordance of the methods compared, the *kappa* index was 57.5 % (95 % CI: 44.3 % to 70.7 %; $p < 0.0000001$) and the overall value of the test was 80.1 % (95 % CI: 72.3 % to 86.3 %).

The sensitivity and specificity for detecting samples that were positive for the *Inf A* gene alone (absence of the *H1* gene) was 53.3 % (95 % CI : 33.8 % to 72.8 %) and 97.1 % (95 % CI: 89.9 % to 100 %) respectively; whereas the sensitivity and specificity for H1N1 positive samples (presence of any swine gene) was 76.4 % (95 % CI : 65.9 % to 86.9 %) and 89.1 % (95 % CI : 80.6 % to 97.5 %), respectively.

Some discordant results were found: 8 samples resulted positive for pandemic H1N1 by the CDC protocol and positive only for the *Inf A* gene by Roche; whereas 6 samples that were positive for *Inf A* gene according to CDC resulted positive for both genes, *Inf A* and *H1*, when tested by the Roche assay. Moreover, of two samples that were negative by the CDC protocol, one resulted positive for the influenza A gene alone and the other one was positive for the *H1* gene ($A^-/H1^+$) by the Roche assay. Of the 32 samples classified as high-positive, one was negative by the Roche assay and of the 40 low-positive ones, 8 were not detected by using the Roche assay.

A sudden emergence and unpredictable progress are common features of influenza pandemics. During the recent outbreak of the *Influenza A (H1N1) virus*, it was essential that public health laboratories all around the world undertook detailed surveillance to monitor the spread and impact of the pandemic (H1N1) 2009 virus, as well as to predict potential changes in its virulence (11). Rapid availability of results has enabled laboratories worldwide to better support clinicians in decision making regarding whether to initiate or to continue antiviral therapy for high-risk patients.

Real time RT-PCR has become the method of choice for the laboratory diagnosis of influenza virus infections due to its increased diagnostic sensitivity, specificity, time to result, and sample throughput in comparison to traditional methods like virus culture. Several rRT-PCR protocols for the detection of the novel *Influenza A H1N1 virus* have been reported. In this study, the performance of the Real Time Ready Influenza A/H1N1 Detection Set-Roche was evaluated using samples collected regionally during the 2009 H1N1 epidemic in Argentina. The results showed that sensitivity and specificity of the evaluated assay were 74.5 % and 97.1 %, respectively. Because both of the methods (CDC and Roche) are based on gene amplification, which is the most sensitive method to detect pathogens, it is surprising that the sen-sitivity of the Roche assay yielded less than 80 %. This could be due to different reasons: the fact that it is based on the specific amplification of only two viral genes, whereas the CDC protocol consists of the amplification of three genes; the possibility that the specific primers of each assay may have a different sequence and/or that the criteria of interpretation inherent to each method are

different. As a matter of fact, the low sensitivity of the evaluated method to detect 17 samples from patients infected with the novel pandemic strain and 14 samples from individuals infected with influenza A, according to the results yielded by the CDC protocol**,** could be attributed to such reasons. However, in 8 of the 17 samples in which the *H1* gene was not detected by the Roche assay, the influenza A gene was correctly detected.

Finally, the moderate concordance observed among the two methods could be due to the lower sensitivity of the commercial assay to detect H1N1 positive samples with low viral load (data not shown) or specimens that were positive for the *Inf A* gene alone (absence of the *H1* gene). Since sensitivity may vary due to different factors, further investigation (*e.g.* viral culture) is needed to evaluate the possible explanations for the discordant results obtained.

A new version of the kit (November 2009) (6) is now available, and a recent evaluation of its performance showed good sensitivity compared to other molecular assays (13). Indeed, it would be interesting to further evaluate the performance characteristics of this later released version on locally obtained specimens.

This study allows to highlight the importance of evaluating sensitivity and specificity of the new commercial kits as soon as they become available. During sanitary emergencies, the availability of alternative diagnostic tests should be warranted. Moreover, as seasonal and zoonotic influenza viruses continue to drift and shift, their performance on regionally obtained samples, must be continuously assessed.

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