

## ENSAYO INMUNOENZIMÁTICO PARA EL DIAGNÓSTICO DE INFLUENZA A/ H1N1 DE EPÍTOPES DE PROTEÍNAS INMUNOGÉNICAS

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***Sandra Angélica Rojas-Osornio***

Sección de Estudios de Posgrado e  
Investigación de la Escuela Superior de  
Medicina, Instituto Politécnico Nacional,  
CDMX. Universidad Tecnológica de México,  
Marina-Cuitláhuac, CDMX  
<https://orcid.org/0000-0003-1525-8496>

***Teresita Rocío Cruz-Hernández***

Sección de Estudios de Posgrado e  
Investigación de la Escuela Superior de  
Medicina, Instituto Politécnico Nacional,  
CDMX  
<https://orcid.org/0000-0002-9291-9676>

***Arturo Contis-Montes de Oca***

Sección de Estudios de Posgrado e  
Investigación de la Escuela Superior de  
Medicina, Instituto Politécnico Nacional,  
CDMX  
<https://orcid.org/0000-0001-5273-3167>

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**Resumen:** La pandemia de influenza A/H1N1 de 2009 causó graves consecuencias en la población humana, por ello, la Organización Mundial de la Salud (OMS) lanzó una estrategia de vacunación para proteger a personas de la amenaza que representa esta enfermedad; sin embargo, estudios muestran que la protección no es eficaz. La identificación, selección y diseño de epítopes inmunogénicos del virus puede ser una alternativa para generar mejor protección contra el virus pandémico H1N1. En el presente trabajo se seleccionaron cuatro epítopes diseñados mediante bioinformática a partir de las proteínas más inmunogénicas del virus pandémico aislado en México en 2009 y mediante un ensayo enzimático, se cuantificó la respuesta humoral de individuos vacunados y no vacunados contra este virus. Los resultados muestran que los péptidos diseñados por bioinformática son reconocidos por los anticuerpos de los individuos en estudio por lo que podrían emplearse para diseño de métodos diagnósticos contra diversas cepas de influenza.

**Palabras clave:** Influenza A/H1N1, epítopes, bioinformática, diagnóstico.

## INTRODUCTION

Peptides V, SM, 11 and 14 were designed from hemagglutinin (HA) and neuraminidase (NA) amino acid sequences of influenza A virus isolated in Mexico [A/Mexico/InDRE4487/2009(H1N1)]; they are located in conserved regions of the mentioned proteins, in the vicinity of the binding site to sialic acid (HA1) and stem (HA2) of HA and, in the ectodomain region and stem of NA. The location of the peptides and the respective images are shown in Table 1.

HA promotes virus entry into the cell through the binding of its HA1 region to host cell membrane glycoproteins and glycolipids that contain sialic acid residues. Protein structure studies have shown that the HA1 region is more susceptible to antigenic shift and drift (Cherian S et. Al, 2011 and Hyeon Seok J et al, 2017), and immunogenic studies have found neutralizing antibodies directed at this region (Treanor J et al, 2004; Peter S. Lee et al, 2012 and James R. R. Whittle et al, 2011); while in the HA2 or stem region, high amino acid conservation has been found in different viral subtypes, therefore, antibodies directed to this region are cross-reactive.




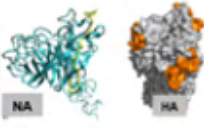
V	SM	Q11	Q14
Vestigial region (HA1) near the sialic acid binding site. HA trimeric structure: peptide V is shown in yellow 	Stem of the HA2. HA trimeric structure: peptide SM is shown in pink 	Ectodomain of NA (red) and stem (HA2 region) of HA (blue) 	C-terminus of NA (yellow) and near the sialic acid (HA1) binding site of HA (orange) 

Table 1. Location of peptides V, SM, Q11, Q14 derived from the HA and NA of the AH1N1 Influenza virus (Taken from the doctoral thesis Quantification of IgA and IgG antibodies against Hemagglutinin and Neuraminidase peptides of the H1N1 pandemic virus in saliva and serum of obese and non-obese individuals” 2019, Rojas-Osornio Sandra Angélica)

Studies of the structure of NA show that the ectodomain acts as the catalytic site of the protein (acylneuraminyl-hydrolase), in such a way that it is an enzyme that catalyzes and hydrolyzes the alpha-ketose bonds between a terminal sialic acid and a D-galactose or Adjacent D-galactosamine. This event promotes the release, spread, and expansion of virions to other cells in the respiratory tract. It also participates in the transport of the virus to other epithelial cells through its union with the mucin present in the respiratory tract. (Gubareva L. V et al, 2000; Wetherall, N. T et al, 2003 and Tamura S. et al, 2004). The C-terminal region has highly conserved amino acid sequences in the NAs of several viral subtypes (H1N1, H3N1, H4N1, H5N1, H6N1, H7N1, H9N1, H10N1, H11N1 and H12N1) (Ghosh A. et al, 2010 and Reyes-Loyola et al, 2013).

Specific antibodies to the two surface glycoproteins, HA and NA, are important as their presence is associated with protective immunity; however, gene rearrangement due to shift and drift produces a change in the viral antigenic cover and, consequently, the neutralizing antibody response against one viral type is not effective for another (Martiniano Bello et al, 2015). This is why periodic and recurrent outbreaks of influenza are generated and, therefore, the influenza vaccine must be prepared and applied each year with the main circulating viral subtypes.

The design of peptides with the use of bioinformatic tools has a great impact today. The V, SM, Q11 and Q14 epitopes designed and used for the present work were selected for their high degree of conservation in various influenza virus strains in order to evaluate the recognition by antibodies in the human population.

## METHODOLOGY

### *Study population and ethical aspects.*

Voluntary individuals between 20 and 40 years of age, men and women, unvaccinated or with the intramuscular application of the vaccine against the influenza A/H1N1 virus, seasonal (containing A/H1N1, A/H3N2, and B strains) or both vaccines were selected. Saliva and serum samples were collected within the first six months after the application of the vaccines.

This work was carried out in accordance with the provisions of the Declaration of Helsinki and all procedures were carried out with the written informed consent of the human volunteers. The study was approved by the Bioethics Committee (CONBIOETICA-09-CEI-012-20160627) and Research (CI-01/25-04-2017) of the Escuela Superior de Medicina, Instituto Politécnico Nacional.

**Peptide synthesis.** The peptides were synthesized by the company Peptide 2.0 Inc. ([www.peptide2.com](http://www.peptide2.com)) according to theoretical predictions made in our work group, using previously described immunoinformatic programs (Reyes-Loyola et al, 2013; Carrillo-Vázquez JP et al, 2015, Peter S et al, 2012 and Martiniano-Bello et al, 2015). The criteria for the selection of the epitopes of the HA and NA proteins of the AH1N1 influenza virus were: pleiotropism (recognized by several MHC haplotypes), high degree of conservation determined by the multiple alignments of the different subtypes and located on the surface of the protein complex. The secondary structures of the proteins shown in Table 1 were determined with the VMD (Visual Molecular Dynamics) program.

**Immunoenzymatic assay.** An indirect type ELISA was implemented in microtiter plates that consisted of: 1) sensitization at 37°C/1 h with the peptides in carbonate buffer pH 9.6; 2) washes 4X with 0.05% Tween20 in phosphate buffer (PBST) after each incubation step;

3) blocking at 37°C/2h with 3% skim milk in carbonate buffer; 4) incubation at 37°C/1 h with saliva (1:2) or serum (1:50) in PBST; 5) incubation at 37°C/1 h with peroxidized anti-human IgA 1:3000 in 2% milk in PBST or peroxidized anti-human IgG 1:5000 in PBST; 6) substrate at room temperature 20 min H<sub>2</sub>O<sub>2</sub>/O-phenylenediamine in citrate-phosphate buffer pH 5.0 and 7) addition of 2.5 M H<sub>2</sub>SO<sub>4</sub> and absorbance reading at λ= 490 nm.

## RESULTS

Figure 1 shows the recognition of peptides by IgA. The unvaccinated and vaccinated groups are recognized by all four peptides; however, it was significantly higher in the unvaccinated for peptide V (p = 0.036). Statistical analysis was performed with the SIGMA plot 2011 program, using the Mann-Whitney U test (n=20).

Peptide V (SSWSYIVETPSSDNGTCYPG) is better recognized in the saliva of unvaccinated individuals; this sequence is present in the vaccine strains and generates effective mucosal protection against the virus; however, the unvaccinated also recognize the peptide, although in lesser amounts, this may be due to a probable previous exposure to these viral strains.

Although peptide V shows high conservation of amino acids in various strains, it should not be forgotten that antibodies directed at this region are neutralizing; however, the affinity of these antibodies could decrease because it is a region that is labile to antigenic shift and drift.

Figure 2 shows the recognition of the peptides by IgG antibodies in serum. In both the unvaccinated and vaccinated groups, all four peptides are recognized; however, recognition was significantly higher in the unvaccinated for the SM peptide (p = 0.009). Statistical analysis was performed with

the SIGMA plot 2011 program, using the t-student test (n=20).

The SM peptide (KGAINSTSLPFQNIHPIPKYVKSTKLRLATC) is best recognized in serum from unvaccinated individuals; Depending on their location (HA stem), the antibodies generated could be cross-reactive due to their high degree of conservation in different HA subtypes.

## FINAL COMMENTS

The four peptides analyzed in this study are recognized by the IgA and IgG antibodies of the samples of the individuals under study, which indicates that there are antibodies against the influenza virus, both in mucous membranes and at the systemic level. We also observed that individuals who are not vaccinated have antibody titers against these peptides, this could be explained by previous exposure to the virus, since it is highly contagious, and we observed recognition in individuals even without apparent immunization with the virus.

The analysis of the antigenicity of the peptides in our study by optimizing this assay in saliva and serum from unvaccinated and vaccinated individuals has an impact on the development of diagnostic tests for influenza A.

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## CONFLICTS OF INTEREST

The author declares no competing financial interests.

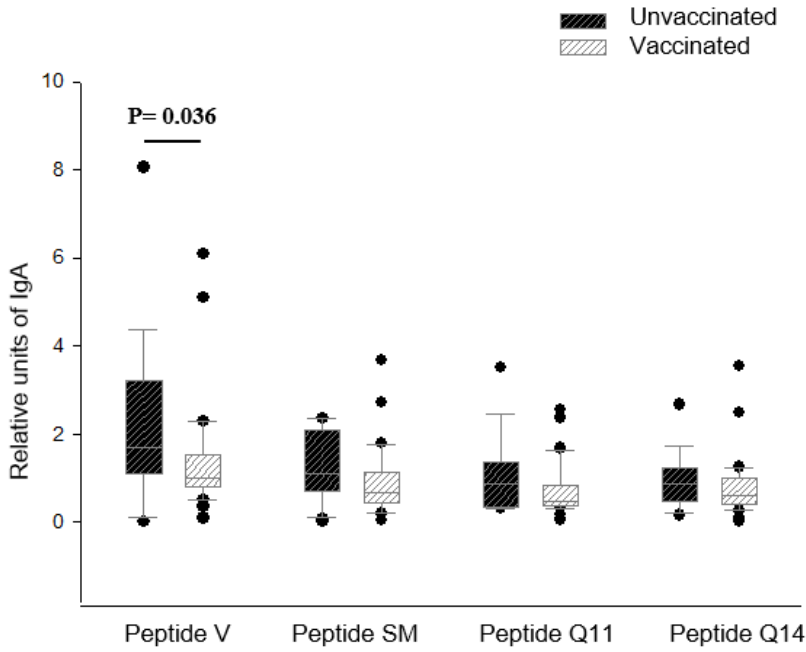


Figure 1. IgA antibody titer in saliva (1:2) of unvaccinated and vaccinated individuals against influenza A virus

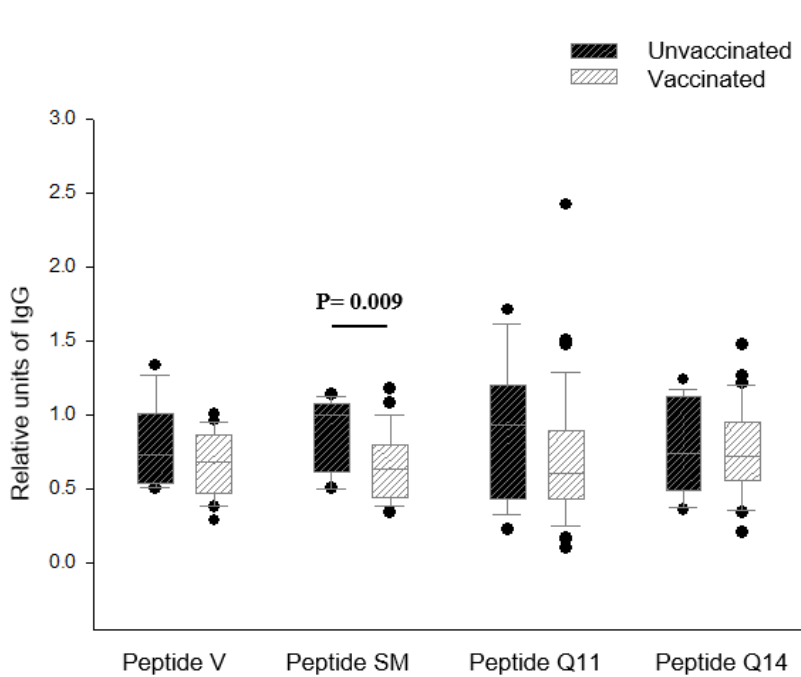


Figure 2. IgG antibody titer in serum (1:50) of unvaccinated and vaccinated individuals against influenza A virus

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