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Short communication

# Amino acid changes in HA and determinants of pathogenicity associated with influenza virus A H1N1pdm09 during the winter seasons 2015-2016 and 2016-2017 in Mexico

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ABSTRACT

Biennial H1N1pdm09 influenza A virus (IAV) epidemics have been associated with major severity of respiratory disease in Mexico. Atypically and in contrast with what happened in USA, Canada and Europe during 2017, an increase of infections due to the H1N1pdm09 pandemic virus instead of H3N2 was observed. In order to determine the viral contribution to severe acute respiratory disease, we characterized the pathogenicity determinants of IAV in Mexico during the 2015–2016 and 2016–2017 seasons. The RNA segments of 20 IAV samples were sequenced by NGS platform and phylogenetic analysis was conducted. The analysis of the hemagglutinin (HA) sequences established that all virus samples, except one, belong to clade (6B.1). The IAVs presented the substitution S162 N, which introduces a new glycosylation site in the hemagglutinin. We also found the D222 G substitution, which has been associated with a higher tropism towards the lower respiratory tract, and a non-reported insertion of one Ile in NS1 (Ile113). The IAVs from 2016 to 2017 in Mexico belong to the new clade 6B.1. The new glycosylation site in HA (S162 N) is a major change that may affect the efficacy of the current vaccine. We detected in several patients pathogenicity determinants associated with the severity of the respiratory disease.

Biennial presence of pandemic (AH1N1pdm09) IAV has been documented since 2010 to 2016 in the Northern hemisphere, including Mexico (Arellano-Llamas et al., 2017). These biennial emergences of H1N1pdm09 were associated with greater severity of the respiratory disease with a higher number of hospitalizations, and with the use of mechanical ventilation (Wong et al., 2015; Martínez-Briseño et al., 2016). Remarkably, and in contrast to what happened in other countries of North America, Europe and also in Caribbean, Central and South America, there was an increase in infections by AH1N1pdm09 in Mexico during 2017 (PAHO WHO, 2015). Viral determinants could be an important factor involved in this phenomenon. We reported several mutations through the winter seasons 2011–2012 and 2013–2014 in all segments of AH1N1pdm09 (de la Rosa-Zamboni et al., 2012; Arellano-Llamas et al., 2017). One of the most significant changes is K163Q, a substitution fixed in AH1N1pdm09 since 2012 that has reached almost 99% of sequences. Experimental evidence with sera of patients point out that this substitution prevents binding of antibodies in middle-aged humans who have been previously exposed to different H1N1 strains (Linderman et al., 2014). Recently, in late 2015, another change in the adjacent amino acid appeared (S162 N); this change confers a potential gain of glycosylation in HA and in addition with I216 T defines the new clade 6B.1 (Chambers et al., 2016). As a consequence of the worldwide distribution of this variant, the World Health Organization (WHO) suggested the inclusion of the strain Michigan 2015 as a representative of clade 6B.1 (WHO, 2018). In the second wave of pandemic influenza cases during 2010, we observed an elevated incidence of variants

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**Table 1**
Clinical symptoms and ventilatory support of adults and children with acute respiratory infections in Mexico City.

| Table 1 | N = 47 | Percentage |
|---|---|---|
| Fem | 23 | 48.9 |
| Age | | |
| 0/2 | 4 | 8.5 |
| 3 a 5 | 6 | 12.8 |
| 6 a 12 | 4 | 8.5 |
| 13/19 | 1 | 2.1 |
| 20/35 | 11 | 23.4 |
| 36/45 | 10 | 21.3 |
| 46/59 | 11 | 23.4 |
| > =60 | 1 | 2.1 |
| **Admission to Intensive Care Unit (ICU)** | **24** | **51.1** |
| **Death** | **3** | **6.4** |
| **Mechanical ventilation** | **25** | **53.2** |
| Positive culture | 14 | 29.8 |
| **Symptoms** | | |
| Dyspnea | 23 | 48.9 |
| Malaise | 27 | 57.4 |
| Runny nose | 23 | 48.9 |
| Thoracic paine | 10 | 21.3 |
| Diarrhea | 5 | 10.6 |
| Cough | 7 | 14.9 |
| Wheezing | 4 | 8.5 |
| Rales | 7 | 14.9 |
| Cyanosis | 4 | 8.5 |
| Hyporexia | 7 | 14.9 |
| Vomit | 22 | 46.8 |
| Nasal congestion | 21 | 44.7 |
| Odinophagia | 5 | 10.6 |
| Conjunctivitis | 6 | 12.8 |
| **Use of antiviral before admission** | 8 | 17.0 |
| **Comorbidities** | | |
| Total | 13 | 27.7 |
| Diabetes mellitus | 5 | 10.6 |
| Hypertension | 4 | 8.5 |
| Smoking | 7 | 14.9 |
| Asthma | 3 | 6.4 |
| Cancer | 4 | 8.5 |
| Alcoholism | 2 | 4.3 |
| **Body Mass Index** | | |
| > 25 and < 30 | 7 | 14.9 |
| >30 | 21 | 44.7 |
| **Vaccinated against influenza in the last season** | **6** | **12.8** |

carrying substitutions in HA (D222 G/N/A/V) (Vazquez-Perez et al., 2013). These substitutions confer tropism to the lower respiratory tract and are associated with severe and fatal outcomes in patients (Vazquez-Perez et al., 2013; Rykkvin et al., 2013; Goka et al., 2014). Therefore, in this work we present the molecular characterization of IAV collected at two different hospitals in Mexico City: the National Institute of Respiratory Diseases (INER) and the Children's Hospital of Mexico (HIMFG), during the winter season 2016 and 2017. With these data we can infer both the origin and the genetic relationship of IAVs in Mexico with viruses from other countries through phylogeographic analyses, and thus identify the viral pathogenicity determinants causing both changes in immunogenicity and more severe respiratory infections.

For INER a total of 969 individuals with ILI were tested for virus, 108 were influenza positive, 312 were positive for other respiratory viruses and 549 were negative, those numbers for HIMFG were 747, 89, 374 and 284 respectively. For this study, a random sample was taken obtaining a total of 27 samples for the INER and 20 for HIMFG. From the total, 51.1% required admission to intensive care unit (Table 1). Only 6 patients (12.8%) were vaccinated against influenza during the 2015–2016 season We found as risk factors for Intensive Care Unit (ICU) admission the following: age (< 18 yo vs > 18 yo) O.R 4.94, C.I. 95% 1.76–13.8, p = 0.002; male gender O.R 4.55, C.I. 95% 1.33–15.57, p = 0.016; BMI > 25 O.R 36, C.I. 95% 5.79–223.54, p= < 0.001; a positive culture for bacteria O.R 42, C.I. 95% 6.12–287.8, p= < 0.001; and length of the symptoms before medical consultation O.R 2.79, C.I. 95% 1.53–5.08, p = 0.001. Remarkably, none of the vaccinated patients required admission to ICU, while all the patients with body mass index > 31 were admitted to ICU. All patients were positive to AH1N1pdm09 influenza virus; however, only 20 samples were successfully amplified and sequenced in at least 5 segments (Table S1). We observed substitutions in all segments of AH1N1pdm09 from 2016 to 2017 (Table 2); however; the most notable mutations were observed in HA and NS1 proteins. Non-synonymous changes were found in the 2016–2017 Mexican isolates with respect to the prototype isolate H1N1 (A/California/07/2009) (Fig. 1). One of the samples from 2016 (4104), had the greatest divergence (0.021-0027, median 0.025) compared with the other 5 samples from 2016 and the 14 samples from 2017. The sample 4104 had 3 amino acids S84, S162 and I216, distinctive of strains previous to 2015; the rest of the samples had N84, N162 and T216. The change N162 introduced a new glycosylation site in the HA. In this context, Mexican isolate 4104 maintained the eight putative glycosylation sites at HA positions 27, 28, 40, 104, 293, 304, 498 and 557, in contrast to the other strains from 2016 and 2017 that have an extra site of glycosylation N162 (Fig. 1).

Sequences of all segments of AH1N1pdm09 collected in the 2016–2017 influenza season clustered with the 2016–2017 sequences from North America, including strains from USA and Canada, except sample 4104. Influenza viruses from Mexico showed higher homology with the viruses of Lousiana (A/Lousiana/11/2017), Texas (A/Texas/69/2017, A/Texas/03/2017), Connecticut (A/Connecticut/14/2017, A/Connecticut/16/2017) and California 2017 (A/California/30/2017) (0.001). Analysis based on the HA gene showed that Mexican sequences could be divided into 2 groups: Group 1, the sample 4104 in clade 6B (Fig. 2) and Group 2, the rest of the samples from 2016 and 2017 in clade 6B.1. Pathogenesis-related mutations like D222 G in HA (Vazquez-Perez et al., 2013) were detected. Sequences of HA gene indicated that 4 out of 20 hospitalized patients (20%) had polymorphisms at the HA 222 position. More than 90 percent of sequences of the sample 4627 have a glycine residue in the HA 222 position (222 G) and the rest of samples (4435, 17,517 and 7317) have less than 30 percent of glycine residues (Table 2). On the other hand, we found a new insertion at the NS1 protein of three Mexican viruses, an isoleucine at the position 113 (Ile113). To verify the presence of this insertion, we amplified whole NS1 protein and we obtained the nucleotide information using Sanger sequencing. The sequence of NS1 sample 7317 with Ile 113 insertion (A/Mexico/7317/2017(H1N1) was submitted to Genebank, accession number MH893646.

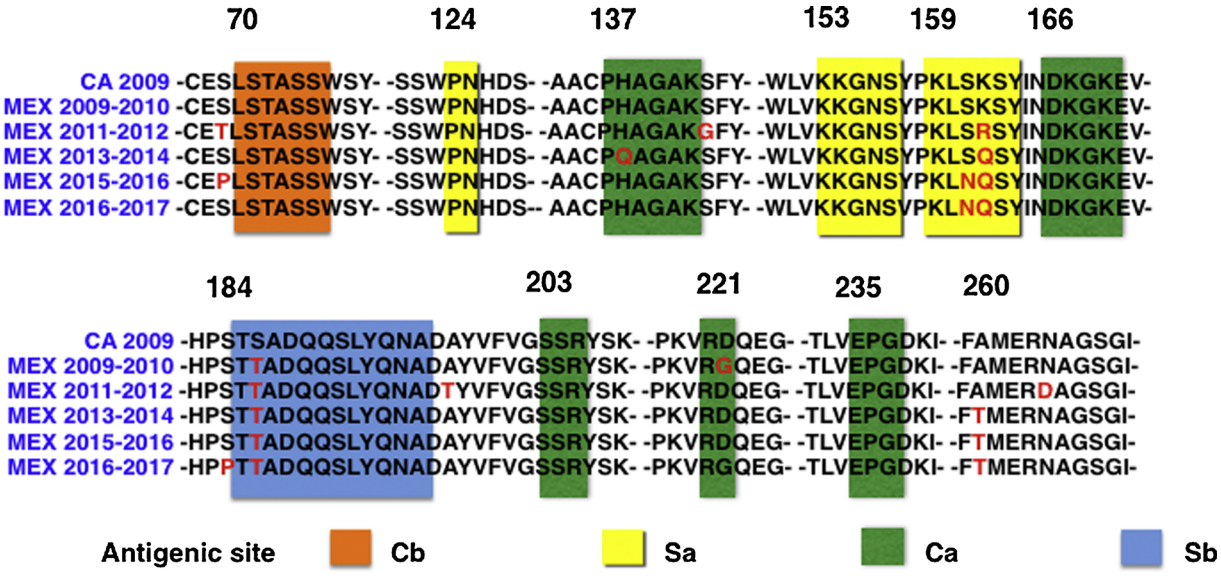
In order to explore the possible impact of the insertion Ile113, three-dimensional models of NS1 protein were predicted. The insertion of Ile113 in NS1_2017B induces an increase in hydrophobic interactions from 4 to 6 compared to NS1_2017A, while two hydrogen-bonds and one ionic interaction are lost (Table S2). On the other hand, in the NS1_2017B structure, a hydrogen-bond interaction between NS1 and CPSF30 is added (Table S3; Fig. 3A and B).

With the insertion of Ile113, the total interaction energy in the dimer increases from -90 to -135 kcal / mol. The total van der Waals interaction decreases from -10 to -6 kcal / mol, while the electrostatic interaction changes from -80 to -129 kcal / mol. The most important electrostatic energy changes are located in residues Met106 and

**Table 2**
Non-synonymous mutations in 2016–2017 Mexican isolates with respect to the prototype isolate A/Michigan/45/2015.

| Strain | Mutations | | | |
|---|---|---|---|---|
| | HA | NA | NS1 | PB2 |
| A/Mexico/4104/2016(H1N1) | V6A;T13A;A65 P;S86 P;N101S;**N179S**;T233I;R240Q; Y246 H;L382Q;N387S | T16S;I117 M;I264 V;K270 N;I288 V;S450G | E55 K;L90I;I123 V;**N205S** | ND |
| A/Mexico/11417/ 2017(H1N1) | R240Q | N171 T;**S200N** | M65 V;S73F | R299 K;T398I;P453 T;K660R |
| A/Mexico/12317/ 2017(H1N1) | R240Q | **S200N** | M65 V;S73F | R299 K;T398I;P453 T;K660R |
| A/Mexico/15017/ 2017(H1N1) | R240Q | **S200N** | M65 V;S73F | R299 K;T398I;P453 T;K660R |
| A/Mexico/15517/ 2017(H1N1) | R240Q;V338I;**H455N**; T491K | NM* | M65 V;V84L | ND |
| A/Mexico/17,517/ 2017(H1N1) | R240Q;V338I | V453G | M65 V;V84L | R299 K;L384 F;T398I;Q447 H;P453T |
| A/Mexico/4435/2016(H1N1) | V64A;**S200P**;R240Q; V289I;N468H | T362I | M65V | R299 K;T398I;P453 T;S643T |
| A/Mexico/4436/2016(H1N1) | V64A;**S200P**;R240Q; V289I;N468H | NM | M65 V;G154R | R299 K;T398I;Q406 P;E407D;P453 T;S643T |
| A/Mexico/4440/2016(H1N1) | R240Q;V338I | W399R;Q408 K;P420 T; V453G | M65V | R299 K;T398I;Q447 H;P453T |
| A/Mexico/4604/2017(H1N1) | V64A;**Y108C**;**K170N**; L178 P;R240Q;V289I; N468H | T362I | M65 V;G154R | ND |
| A/Mexico/4621/2017(H1N1) | D52E;V64A;R240Q;L382Q;**G478E** | NM | M65 V;G154R | ND |
| A/Mexico/4627/2017(H1N1) | V64A;**S200P**;D239X; R240Q;V428A | V34I | M65 V;Y89H | ND |
| A/Mexico/4628/2017(H1N1) | A214E;R240Q | I389 T;I396L | M65 V;ins110I | ND |
| A/Mexico/4687/2017(H1N1) | V64A;S200P;R240Q; V289I;N468H | NM | M65V | R299 K;T398I;P453 T;**K482T**;S643T |
| A/Mexico/4703/2017(H1N1) | R240Q | **V106X**;**P246T**;I389 T;I396L | M65 V;ins110I | ND |
| A/Mexico/7317/2017(H1N1) | R240Q | I389 T;I396L | M65 V;ins110I | R299 K;T398I;P453 T;K660R |
| A/Mexico/8017/2017(H1N1) | V64A;**S200P**;R240Q; V289I;N468H | NM | M65V | Y34 N;R38 K;K48Q;M51 V; R299 K;T398I;P453 T;S643T |
| A/Mexico/8517/2017(H1N1) | V64A;C107R;**S200P**; R240Q;V289I;A302 V; N468H | NM | M65V | V220I;R299 K;T398I;P453 T;S643T |
| A/Mexico/4431/2016(H1N1) | P66S;R240Q;E252D | NM | M65 V;V84I | W49R;R299 K;T398I;P453T |
| A/Mexico/4433/2016(H1N1) | R240Q;**T500**P | NM | M65V | R299 K;T398I;P453T |

Non-synonymous mutations with respect to the prototype isolate A/Michigan/45/2015 are reported. Each polymorphism was searched in a set of database sequences retrieved from Flusurver,(Arriola et al., 2017). * No mutation founded, ˆSequences not obtained. Bold mutations occurs at a site known to be involved in drug-binding or alters host-cell specificity.




| | 47 | 84 | 97 | 216 | 272 | 283 | 321 | 374 | 451 | 520 |
|---|---|---|---|---|---|---|---|---|---|---|
| 2009-2010 | V | S | D | I | V | K | V/I | E/K | S | V |
| 2011-2012 | V | S | D | I | V | K | V | K | N | A |
| 2013-2014 | V | S | N | I | V | E | V/I | K | N | V |
| 2015-2016 | V/A | S/N | N | I/T | V/I | E | V/I | K | N/H | V |
| 2016-2017 | V/A | N | N | T | I/V | E | V/I | K | N/H | V |

**Fig. 1.** Amino acid substitutions in the HA antigenic sites of the 2016–2017 influenza A H1N1 Mexican isolates. A) Amino acid sequences are shown for the HA antigenic region of the twenty sequenced 2016–2017 Mexican isolates (GenBank accession numbers, Table S1), the reference California 2009 isolate (CA2009, accession number CY054707) and Mexican isolate from MX2009 to 2014. Amino acid positions are numbered without considering the HA signal peptide. Amino acid substitutions in the Mexican isolates are marked in red. Antigenic sites are shaded: Sa – yellow, Sb – blue, Ca – green, Cb – orange. Substitutions S162 N and K163Q are shown to be within antigenic sites. B) Others amino acid substitutions observed from 2009 to 2017 in Mexican isolates.

Asp125; the most important van der Waals energy changes are located in residues Arg108 and Asp 125 (Table S3).

Pandemic influenza virus is still a cause of severe respiratory illness in Mexico. Biennial patterns of infection, detected since 2010, have also broken out in the winter season 2016–2017, where an increase of infections associated with hospitalizations and severity was observed in the entire country. Our data show that absence of vaccination, obesity and adult age are risk factors for severity, as was discussed before (Arriola et al., 2017). In this context, we continue analyzing viral sequences to explore viral determinants implicated with infections in Mexico during 2017. According to previous reports (Chambers et al., 2016; Linderman et al., 2014) one of the most important changes in the last years is located in HA. The substitution S162 N introduced a new glycosylation site in the immunogenic motif-Sa, very likely affecting the recognition of the antibodies elicited by the vaccination. In our case, all samples with the exception of one from 2016, have this new site of glycosylation. Probably, the increase of acute respiratory infection cases in Mexico could be due to these factors: low rates of vaccination and low protection. Vaccines used in the winter seasons 2015–2016 and 2016–2017 in Mexico had the California 2009 strain. On the other hand, other determinants of pathogenicity that confer tropism to epithelial cells of the low respiratory tract, such as the variants of position 222 in HA, have been associated with severity and mortality around the world. In 2016 and 2017 20% of the sequences presented variants at the position 222. Another relevant change was observed in the NS1 protein of three samples during the 2017 winter season, as one Ile113 insertion was detected in nasopharyngeal and endotracheal samples of patients with severe respiratory outcomes. NS1 is a multifunctional and elastic protein that can bear multiple substitutions not affecting its functions (Heaton et al., 2013). One of NS1 most well-known functions is the antagonist effect on innate immune response elicited by interferon (Kochs et al., 2007). Other interactions with cellular factors have been reported, including the cleavage and polyadenylation specificity factor 30 protein (CPSF30), with binding sites located between amino-acids 103–106 and 144–188 in NS1 (Nemeroff et al., 1998). NS1 residues F103 and M106 are indispensable for the NS1/CPSF30 interaction (Nemeroff et al., 1998; Hale et al., 2010), while additional residues (K108, D125, D189) may increase affinity. NS1_2017A, NS1_2017B and A/California/07/2009(H1N1)/2009 sequences are all classified into the A3 NS1 group (Sevilla-Reyes et al., 2013), which may display weak interactions with CPSF30 (16). According to our results, the presence of the Ile113 residue increases the

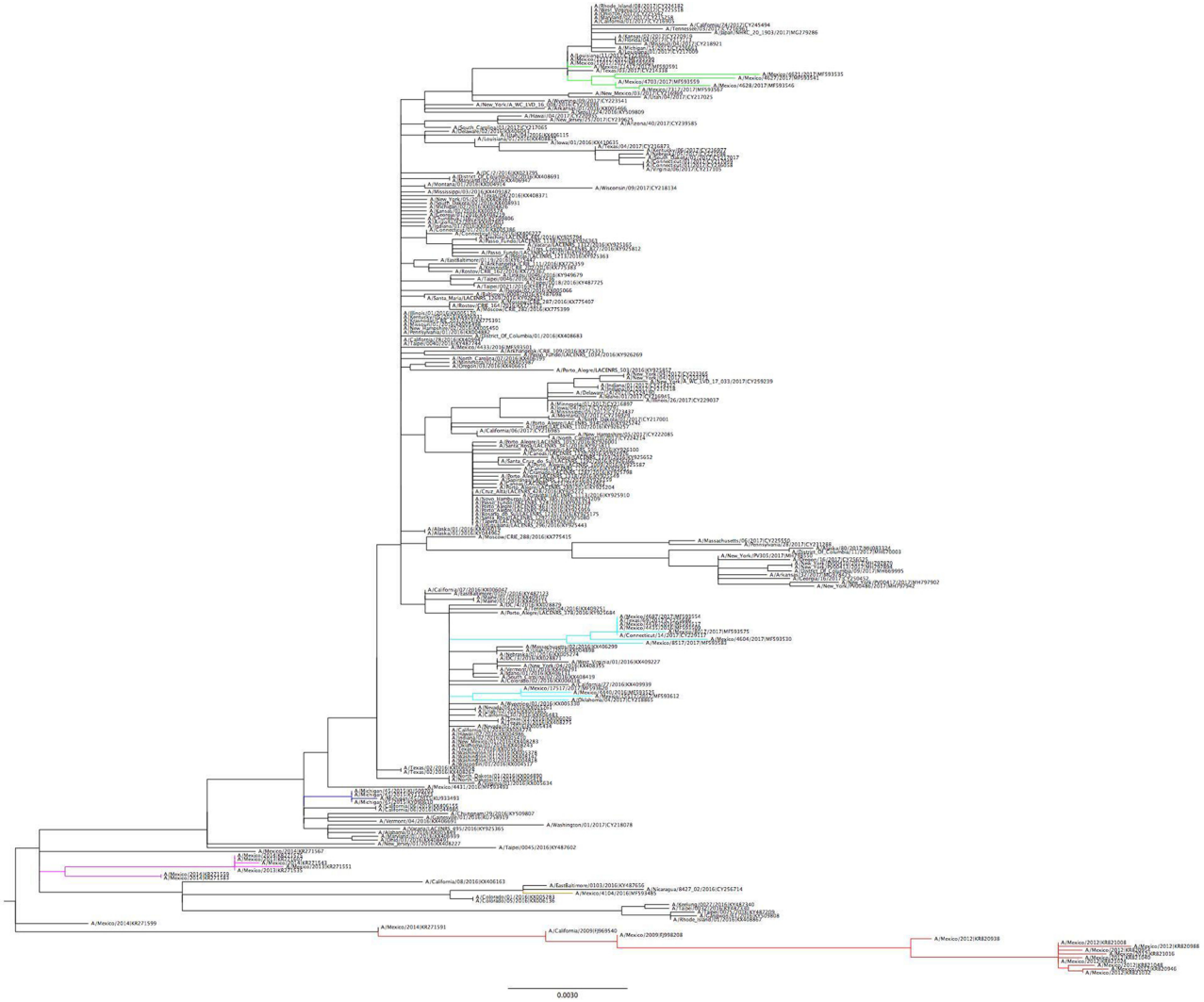



**Fig. 2.** Maximum likelihood (ML) phylogenetic trees for influenza HA segment. ML trees from 256 HA A(H1N1)pdm09 sequences were produced with 1000 bootstrap replicates. Phylogenetic tree included 6 Mexican sequences from 2016, 14 from 2017, 10 from 2013–2014 season, 17 from 2012 and 1 from 2009. Vaccine strains California 2009 and Michigan 2015 are also included. Colors for seasons: RED, California and Mexico 2009–2012; MAGENTA, Mexico 2013–2014. BLUE; Michigan 2015. NS tree: CYAN; Mexican sequences from 2016 to 2017, 17,517, 15517, 4435, 4436, 4440, 4604, 4687, 8017 and 8517. BRIGHT GREEN, Mexican sequences from 2016 to 2017, 11417, 12317, 15017, 4621, 4627, 4628, 4703 and 7317. ORANGE; Mexican sequence from 2016, 4104.

number of intramolecular hydrophobic interactions in NS1/CPSF30 by two. These interactions (Met106/Met124 and Ile112/Leu115) are located near the ends of the structure of NS1. Additionally, the ionic interaction that was present in Asp125/Lys219 in NS1_2017A/CPSF30 is lost with the insertion of Ile113, which also contributes in NS1_2017B to increase the total interaction energy with F2F3/CPSF30. This could possibly point out to a stronger inhibition of the immune response by the 2017B influenza strain during the infection process. On the other hand, the NS1/CPSF30 hydrophobic interactions are significantly weakened by the presence of Ile113. Functional and biological relevance remains to be experimentally elucidated. We can hypothesize that the presence of Ile113 will produce a stronger interaction between NS1 and CPSF30, leading to a more effective suppression of the immune response and, consequently, to higher virulence.

Molecular determinants of pathogenicity and amino-acids changes related with decrease or loss of vaccine immunogenicity are important issues that need a permanent surveillance in influenza severe respiratory disease. The increase of cases of AH1N1pdm09 in Mexico during 2017 could be due to predominance of the new clade 6B.1 and low coverage of vaccination. Severity of respiratory disease could be explained in some cases by the presence of determinants of pathogenicity in HA and NS1 proteins.

## Ethical approval

The Science and Bioethics Committee of the INER revised and approved the protocol (B-0615) and the consent procedure.

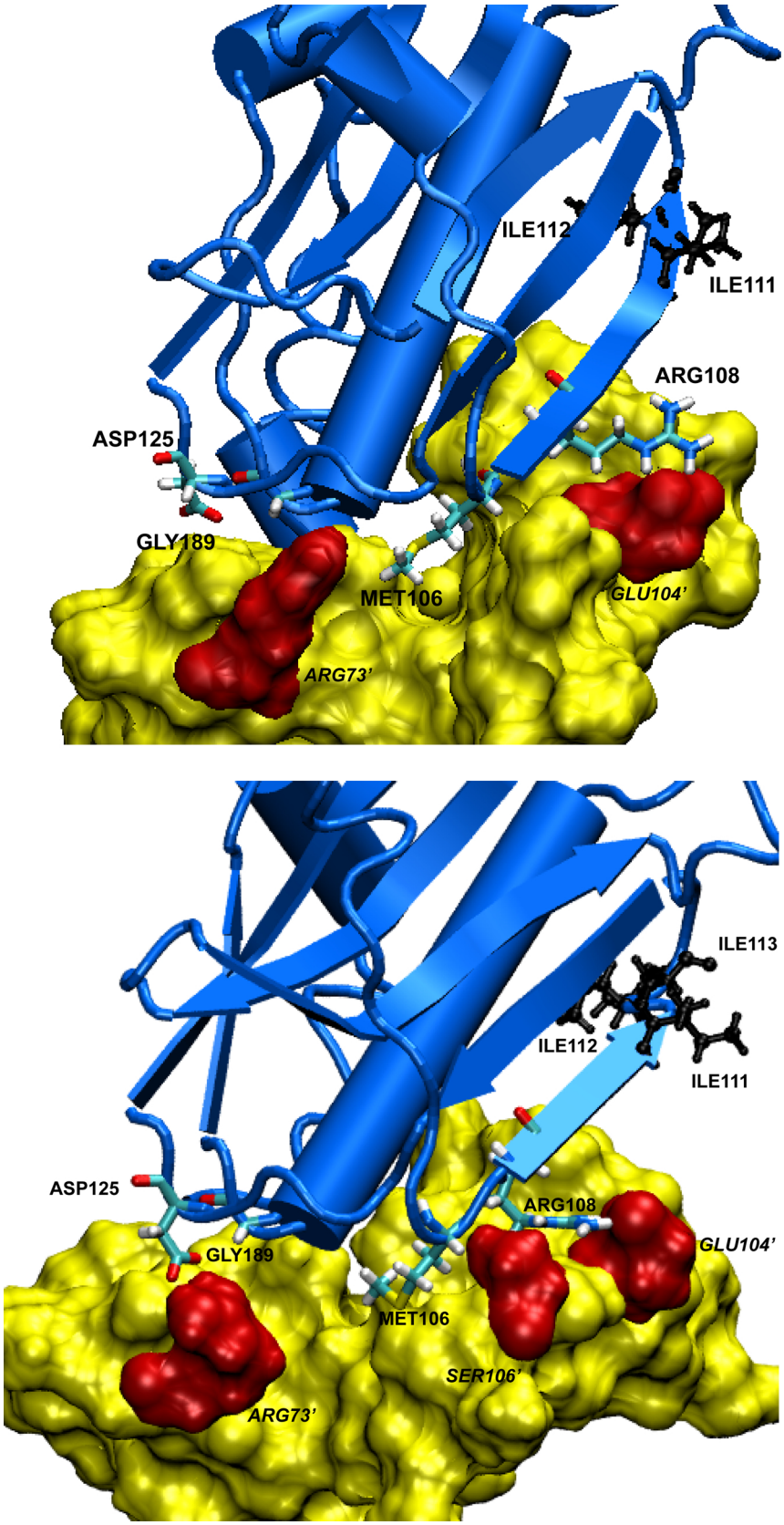



**Fig. 3.** Structural representation of NS1_2017A (A) and NS1_2017B (B) in complex with the F2F3 fragment of CPSF30. The monomeric NS1 is shown in cartoon format (blue), while the CPSF30-F2F3 fragment is shown in a surface representation (yellow). Residues that participate in the interaction are represented in spheres and sticks format. A) NS1_2017A complexed to F2F3/CPSF30. Residues Met106, Arg108, Asp125 and Gly189 interact with Arg73′ and Glu104′ (red) from CPSF30. Residues Ile 111 and Ile112 point away from the interaction surface. B) NS1_2017B complexed to F2F3/CPSF30, which newly interacts with residue Ser106′ (red) from CPSF30. Ile113 has no direct interaction with the contact surface. This figure was made with the Visual Molecular Dynamics (VMD) program (http://www.ks.uiuc.edu), developed with NIH support by the Theoretical and Computational Biophysics group at the Beckman Institute, University of Illinois at Urbana-Champaign.

## Declaration of Competing Interest

All authors declare that they do not have any competing interests.

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