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Vaccine Impact on Glycosylation Evolution of Influenza A H1N1

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Abstract

Influenza is a viral infection that causes annual epidemics, besides the latent risk of a possible pandemic. Nowadays the best choice to control influenza is vaccination. However vaccine effectiveness oscillates between 30 and 60%, it is inconvenient and must be reformulated every year, because of the high rate of mutability of the influenza virus. On the other hand, vaccinated population has caused the virus to undergo immunological pressure, causing mutations to escape. The principal target of this immunological pressure is due to the presence of glycoproteins on the viral surface, in where there are some well characterized antigenic sites. In the present study, hemagglutinin sequences from Influenza A/H1N1 from the last ten years were analyzed focusing on appearing and disappearing of N-glycosylation sites. As per previous reports, conserved glycosylation sites were identified, mostly on the stalk, other glycosites where found on the globular domain, such as the site 179, which in less than 10 years went from a very low rate, to be present in almost all the sequences, but, apparently this site is still undergoing through a stabilization phase, the main evidence, are the recent mutations that have arisen in the sequon. We discuss the possible repercussions that this could have on the choice of vaccine strains, and the impact of the substrate in where vaccines are developed and how this could be fundamental in their effectiveness.

Keywords

Influenza A H1N1; Hemagglutinin; N-glycosylation; Influenza evolution; Influenza vaccine

Introduction

Influenza is a viral disease caused by influenza A or B virus. Every year, this virus infects and spread throughout the world population causing more than 300,000 respiratory cases. In 2018, a larger number of cases were reported from 2017 [1]. The severity of the illness is variable from a mild auto limited infection to where the patient barely presents the symptoms up to death [2]. Until now, the most effective prophylactic measure is vaccination. However vaccine effectiveness oscillates between 30 and 60% [3-6]. Every year in pursuit to improve protection over this illness, vaccination campaigns take place all over the world and there is continuous research and development of new vaccines. Some of which have already been applied to population [7]. A very important consequence of this is, in general terms, the diversity of antibodies generated on world population in one way or the other have lead influenza virus to undergo a evaluative pressure, but for better understanding of this impact, it is necessary to highlight some of the virus characteristics and part of their biological cycle.

Influenza A virus belongs to Orthomyxoviridae family and has an average size of 100 nm, it is covered by a lipid bilayer taken from the host cell [8], where two important and antigenic glycoprotein's are embedded: hemagglutinin (HA), from which 18 types are known (numbered from H1 to H18); and neuraminidase (NA), with 11 types known (numbered from N1 to N11). HA is the most abundant in the viral surface and has the ability to trigger a major immune system [9]. These glycoproteins are fundamental for some biological process, HA participates in the recognition of the cell receptor, besides it has a participation on biological process, such as fusion of the viral membrane with the membrane of the target cell; on the other hand, NA is a necessary enzyme for the release of newly synthetized virions from the host cell [8].

The survival of the virus is determined by its capacity to transfer from one person to another. However, it is a very common and prevalent disease. Most of the people already have a certain level of protector antibodies acquired from previous infections causing a very drastic selection, among each virus having potential of new host. If we consider influenza virus from a very general point of view like any other species, in order to survive, it must adapt to the changing environment where it survives, it must have high mutation rate,

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capability of producing multiple variants which try to escape from immunological pressure and these will survive, infect and spread. For mutations to be effective, they must be translated to change on the external glycoproteins. One of the most studied changes is N-glycosylation [10-12].

N-glycosylation is a very common post-transductional modification, where a oligosaccharide is attached to a specific motif called sequon, composed by three aminoacids: N – X – S/T, where X could be any except proline [13]. It is important to mention, that for N-glycosylation to occur, there are other factors to be considered, besides the sole presence of the sequon, factors such as the location of the sequon in the structure, and the process of the glycosyltransferases and its function [12].

Another important aspect of the process is regarding the nature of the glycan attached to the sequon which depends on the host or the system used for vaccine production, this means the same sequon from the same strain can bear different types of glycans, depending on the substrate or kind of cell where the virus was replicated [14].

Like mentioned before, HA is the most abundant glycoprotein on the viral surface, and because of this, it is the major target of the immune response; this molecule is formed from a precursor HA0, that is cleavage into two subunits: HA1 and HA2 [8]. On the viral envelope, HA forms a homotrimer, with a globular head formed by HA1; and a stalk, formed by HA2. The globular head displays two important features: The first one is the high mutation rate, compared with the stalk [15]; and the other is the presence of five antigenic sites, Sa and Sb, located near the tip of the HA and the receptor binding site; Ca1 and Ca2 located among the subunits; and Cb, located on the head (S refers to strain-specific, and C to common); therefore it is important to keep up with the mutations on these sites, especially for those vaccine candidate strains [8,16].

The aim of this study was to determinate the changes on influenza virus that has provoked the acquisition of N-glycosylation, and relate them to the possible effect produced by the continuous use of vaccines on the population in the last 10 years.

Materials and Methods

Sequences

For this study, protein sequences of influenza A H1N1 hemagglutinin were used. All sequences were downloaded from Influenza Virus Database. The criteria for the selection of the sequences were human origin pandemic complete sequences (554 to 566 amino acids), and, not more than two adjacent amino acid deletions. All the different sequences where selected from April 2009 to August 2018. The total sequences used for each year are indicated in Table 1.

Influenza database also contains seasonal influenza sequences, and porcine-like sequences; to avoid the selection of these sequences, multiple alignments were made on Clustal X, followed by phylogenetic trees, using Neighbor-Joining method made on MEGA X; and the reference sequences used for the phylogentic trees were: Vaccinal strains A/Michigan/45/2015 (No. Access: AMA11475.1), A/California/07/2009, (No. Access: YP_009118626.1) and A/Brisbane/59/2007 (No Access: AET50439.1); reference strains A/Puerto Rico/8/1934 (No. Access: ABD77675), A/WSN/1933 (No. Access: ACF54598.1) and A/South Carolina/1/1918 (No. Access: AAD17229); this way, we assure that only pandemic strains were used for this study.

Glycosylation sites

All the results showed in this study consider the numbering of each residue present in hemagglutinin, taking as a reference the numbering H1, starting with signal peptide.

The prediction on N-glycosylation sites was performed in all sequences that previously showed to be derived from the 2009 pandemic, and was made on NetNGlyc 1.0 server [17], taking positive results with potential of 0.5 or higher, probability of a real glycosylation on that site is estimated.

To determine differences in the sequons for site 170, from 2009 to 2018 progressive alignments were made on Clustal X, and the percentage of each sequon was determinated, noting whether glycosylated or not.

Results

Each sequence was analyzed on NetNGlyc 1.0 Server, for the prediction of every N glycosylation site. The results are shown on Figure 1. Eight N-glycosylation sites where predicted: six sites very conserved Asn28, Asn40, Asn104, Asn304, Asn498 and Asn557; one site that disappeared Asn136, and two sites that increased trough this decade Asn179 y Asn293.

The glycosylated sequons are shown on Table 2. All sequons are conserved from 2009 to 2018 except Asn179, that has several changes through mutations.

Site 104, also reported on 1918 influenza virus [18], is located on the side head of HA, under the antigenic site Ca2, and has been stable for a very long time, as part of the HA structure [19], and its importance in the receptor binding union has already been described [20]. The sites 28, 40, 304 and 498 located on the stalk, had already been reported as conserved trough influenza A H1N1 history [12,18,21,22], and has been found that these sites are fundamental for correct protein folding and hemagglutinin stability during synthesis [23-25].

Even though site 557 (with NGS sequon) has a very high incidence in N-glycosylation predictions, some research groups have analyzed the H1N1 variant [12]; the reference virus Caledonia/20/1999 [21]; and even A/Puerto Rico/8/1934 [22]; they all agree that sequon NGS does not consists of any kind of glycan, besides, this residue is part of the transmembranal domain that anchors to the membrane of the host cells [8].

On the other hand, site 293 (with NTT sequon) appears to be a relatively new site, because it started to appear steadily from 2009 to date, and has been a much conserved site, even on the sequences it is not predicted with any glycosylation. However, some researchers have analyzed this site through glycan characterization, and they agree that this site has a place where glycosylation process takes place [12,26].

She YM in 2017 [12] described two more glycosylations detected on candidates for a vaccine derived from A/California/07/2009 virus. The first one is site 136, with the sequon NTS; however, this glycosylation just appeared from 2009 to 2013 in less than 0.4% of the sequences, to finally be substituted by mutation N136K, which already had been dominant in the sequences. The other site mentioned is 490, but this site does not present a formal N-glycosylation sequon, just the motif NTC, for this reason was not predicted as a N-glycosylation site.

Year	Different sequences	Total Sequences
2009	2025	6579
2010	854	1587
2011	610	1107
2012	338	559
2013	500	1093
2014	307	710
2015	174	463
2016	496	2063
2017	73	258
2018	229	707
Total (2009-2018)	5533	15126

Table 1: Sequences of hemagglutinin from influenza A/H1N1 pdm09

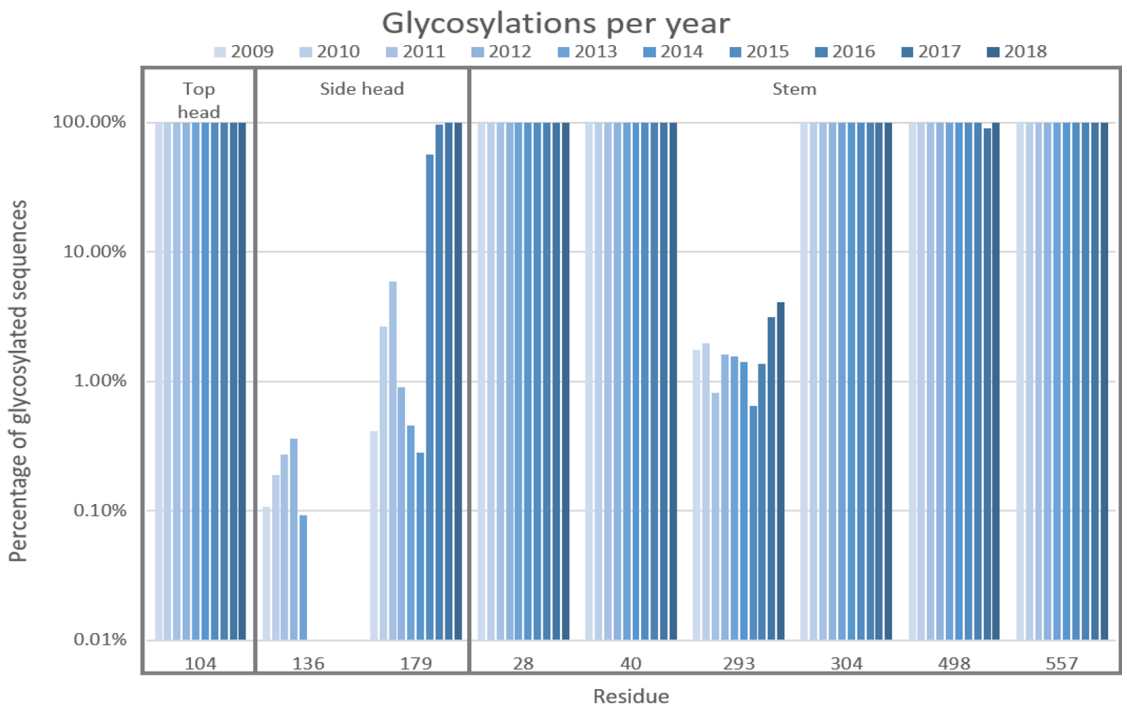

Figure 1: Glycosylations detected on influenza A H1N1 hemagglutinin-Each bar shows the percentage of sequences in which a possibility of N-glycosylation was detected, *via* NetNGlyc 1.0 Server. The numbering in the residues is based on H1 numbering, including signal peptide

The appearing or disappearing of N-glycosylation, and their influence over immunologic and physiochemical parameter on hemagglutinin trimer has been widely described. It has been demonstrated that the removal or addition of a glycosylation in 142 and 177 sites, change the resistance or susceptibility of antibodies. When this glycosylations where induced on Influenza A strains of 1918 and 2009, these did not affect the trimer structure [18]; also, it has been demonstrated that the addition or removal of these glycosylation sites have a different effect, depending of the site, this was seen on seasonal strains, when the site 142 was removed, the virus presented a minor neutralization rate, than when site 71 was removed [26].

Site 179 is relatively new, starting to appear constant in 2009 to 2014, with an oscillating rate of 0.28% to 5.86%, until 2015, where more of half of the analyzed sequences presented this site, and to keep growing in future years up to be dominant in more than the 99% of the sequences.

This glycosylation is ubicated in the antigenic site Sa and is speculated to be some kind of "shield" to this site from immune response. However, is interesting that this sequon has been in constant change: the few sequences with glycosylation on this site found in 2009 presented the NKS sequon. On the other hand, the vaccinal strain A/Michigan/45/2015, a representative sequence from that year presented NQS sequon, while the sequences from 2018 almost all present the NQT sequon.

Discussion

Glycosylation on HA can be "conserved" and "transitory". The first one is given by glycosylation sites that are conserved through long periods of time, even decades, and possibly have very specific functions, such as stability of some important domains of the molecule [20]. The second kind usually appears on the fast evolving domain of the HA, the globular head, and possibly as a way to escape immune pressure given by the host population [13,22].

Although site 179 appeared as part of the evolution and viral adaptation, this event has two sides: the fact that allows the virus to escape from the immunity, but on the other side, depending on the nature of the attached glycans, this could made the viral particle more sensitive to the neutralizing action of collectins, such as SP-D, proteins that are part of innate immunity, found naturally on respiratory tract of humans [26]. Finally, the acquisition of these glycosylation, evidences that the behavior of the influenza virus on the post pandemic period (2009 to date) is very similar to the evolutive behavior influenza A H1N1 presented in 1918. In both cases, most of mutations lead to the acquisition of N-glycosylation sites in the globular domain, such as 179. It is unlikely that these mutations are the result of randomness because, in both cases the mutation, occurred in the same antigenic region Sa. Even more intriguing, is the high mutation rate that sequon 179 presents. When pandemic sequences were analyzed, we could notice 3 mutations: from 2009 to 2013 sequon, 179 was NKS; from 2013 to 2016, sequon was NQS; and from 2017 to 2018, sequon was NQT. We consider this as an irrefutable proof of the capacity of the site to generate protection against host immunological pressure, allowing the spread of the virus, nowadays seasonal, among the human population.

The above is based on several works, that had considered as one of the main purpose of glycosylation on viral glycoproteins, is to evade immune system, by hiding with oligosaccharides the important recognition sites from neutralizing antibodies [26,27], therefore we could speculate that site 179 would reduce immunological pressure over the adjacent amino acids. However, site 181, part of the mentioned sequon, in the last two years consolidated a new mutation, S181T; in such way that in 2017 and 2018, most of the sequences in site 179-181 had this change. Without a doubt, the three amino acids that conform the sequon 179 of influenza A H1N1pdm09, are part of the most evolved region in the last 10 years, going from SKS to NQT.

After 2009 pandemics, the first vaccinal strain was A/California/07/2009 and did not had glycosylation on site 179 (SKS), with which we could suppose that the induced humoral response could induce the production of antibodies, directed to Sa, causing this way enough immunological pressure over the region, to the point of selecting the virus with a glycosylation on site 179. Recently,

Residue	Sequon
28	NTS
40	NVT
104	NGT
136	NTS*→(KTS)
179	NKS/NQS/NQT
293	NTT
304	NTS
498	NGT
557	NGS

*Disappeared sequons; (N136K): Non-glycosylated sequon

Table 2: Residues and the sequon that might be glycosylated

WHO made the recommendation for the use of a new H1N1 strain: A/Michigan/45/2015, which already presents glycosylation on site 179 (NQS), expecting a better protection, based on the similarity of the vaccinal strain and circulating strains [28]. Despite the antigen change, glycosylation on keep present on circulating strains, but like mentioned before, during 2017 to 2018 a new mutation consolidated: S181T. It is hard to explain what could have caused this change, possibly the immunological pressure caused by the constant vaccination with a glycosylated strain, generated virus with some variants in site 181, and in presence of antibodies generated by A/Michigan/45/2015 (sequon NQS), were able to select the new mutation (sequon NQT).

Talking about protection given by the new antigen, no decrease has been observed in the positive cases per year [1], this is caused because the use of an antigen isolated in 2015 is used in season 2017. However, it should not be ruled out that a possible cause could be the kind of substrate where the vaccines were produced, since being produced in chicken embryo or nonhuman cells rather than human cells, could cause a different kind of glycans. This difference in the glycosylation patters definitely could cause a very important role in the final antigen structure of vaccinal strain, causing the production of neutralizing antibodies against a structurally different antigen, than the circulating among human population.

Several efforts have been made to demonstrate the antigenic capability and conferred protection by vaccines produced in several platforms, some works have shown that a “naked” hemagglutinin (with no glycosylation) is highly antigenic and could be an adequate option [29]. However, other works have proposed that high-mannose glycans are indispensable [30], thus they have developed an insect cell platform, capable of producing selectively this kind of glycan, but the evaluation of those antigens are usually done with virus sub cultivated on chicken embryos or non-human cells which again, implies that the challenge is being carried out on a virus structurally different from the virus that is transmitted by a patient.

Conclusion

N-glycosylation present in influenza glycoproteins, is without a doubt an evolutive process which should be considered in the development of new vaccines, not only the evaluation of necessary sequences, but also it is important to considerer the impact of the viral substrate over the final antigen structure, and the capability of generate antibodies capable of neutralizing virus transmitted by an infected person to another.

It is still necessary further characterization of the kind of glycans present in wild influenza virus, in order to compare the similarity that share with the glycans observed on vaccinal antigens, produced in different viral platforms, it is essential to focus efforts on answering these questions, and finally, with this, the perspective of changing the production of influenza vaccines could open, particularly by considering the use of a substrate capable to generate more accurate glycans to the ones in circulating strains, and with this the production of antigens structurally similar to the ones are transmitted during contagion.

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