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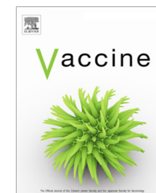

# Directed selection of amino acid changes in the influenza hemagglutinin and neuraminidase affecting protein antigenicity

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ABSTRACT

Influenza virus hemagglutinin (HA) and neuraminidase (NA) proteins elicit protective antibody responses and therefore, are used as targets for vaccination, especially the HA protein. However, these proteins are subject to antigenic drift, decreasing vaccine efficacy, and few to no studies have analyzed antigenic variability of these proteins by growing the viruses under immune pressure provided by human sera. In this work, we show that after growing different influenza virus strains under immune pressure, the selection of amino acid changes in the NA protein is much more limited than the selection in the HA protein, suggesting that the NA protein could remain more conserved under immune pressure. Interestingly, all the mutations in the HA and NA proteins affected protein antigenicity, and many of the selected amino acid changes were located at the same positions found in viruses circulating. These studies could help to inform HA and NA protein residues targeted by antibody responses after virus infection in humans and are very relevant to update the strains used for influenza virus vaccination each year and to improve the currently available vaccines.

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## 1. Introduction

Influenza A (IAV) and B (IBV) viruses are one of the most common pathogens causing respiratory infections in humans. It is estimated that every year, despite comprehensive vaccination programs, the global disease burden from seasonal influenza results in 1 billion infections, 3–5 million cases of severe disease, and between 300,000 and 500,000 deaths [1]. IAVs are classified in subtypes according to the two major surface glycoproteins, the hemagglutinin (HA) and the neuraminidase (NA). In humans, the most common IAV subtypes co-circulating seasonally are H1N1 and H3N2, and two lineages of IBV. Therefore, vaccines include 3 or 4 strains of these subtypes (one IAV H1N1, one IAV H3N2 and one or two IBVs) [2].

The HA protein mediates virus binding and entry into host cells [3]. The HA protein is a trimer and each monomer consists of two different domains: the globular head domain, which contains the receptor-binding site and is the least conserved influenza segment, and the stalk domain [4], which is more conserved and allows the fusion of the viral and endosome membranes once the virus entries into the cell [5]. Influenza virus exposure elicits the induction of neutralizing antibodies able to protect against virus infection through different mechanisms. Neutralizing antibodies to the HA globular head domain can mediate protection through the inhibition of the binding of the HA protein to the host cell receptor [6]. In addition, antibodies binding to the more conserved stalk domain can mediate protection through the inhibition of the fusion of viral and endosomal membranes [7–9].

The NA protein quaternary structure is a homotetramer in which each monomeric subunit forms a stalk domain and a head domain. Each monomer is composed of six topologically identical beta-sheets arranged in a propeller formation [10]. The influenza NA protein has sialidase activity, cleaving terminal sialic acid from glycans on the host surface, facilitating virus release from infected cells [11]. This sialidase activity is important at other stages of the viral life cycle as well. When influenza viruses enter a host, they need to penetrate mucosal barriers in order to the HA protein to reach the sialic acids on the host cell surface [12,13]. In addition, mucosal fluids contain natural defense proteins, such as mucins, that are heavily glycosylated, acting as a decoy for HA binding, a process which neutralizes the influenza virus [14]. Furthermore, viral particles adhere to each other by interactions between HA and sialic acid on glycans on HA or via other glycoproteins in mucus that act as adapters. NA counteracts this aggregation, which

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might consequently enhance virus spread and transmissibility [15]. In addition, neutralizing antibodies to the NA protein can protect against the virus by inhibiting virus release from the cells [16], and anti-NA antibodies inversely correlate with virus shedding and disease symptoms in humans [17–20].

Commercial vaccines are designed to primarily induce an immune response to the hemagglutinin (HA) globular head domain, which is the main correlate of protection [21,22]. Accordingly, it has been shown that current influenza vaccines poorly display NA epitopes and induce very poor responses of NA-reactive B cells and antibodies [16]. In contrast, natural infections induce high levels of NA-reactive B cells and neutralizing antibodies specific for the NA protein [16,20], as well as neutralizing antibodies specific for the HA stalk domain [23,24].

Influenza vaccine efficacy varies among seasons but is never 100% [25,26]. The HA and NA proteins acquire amino acid changes through a process called antigenic drift [27–29]. This process decreases the efficacy of antibody responses induced by previous vaccinations and/or infections and consequently the strains included in the vaccines must be periodically updated in an effort to match the circulating viruses with the vaccine strains [30,31]. Rates of morbidity and mortality increased during influenza seasons in which the circulating virus and the vaccine was "mismatched" [31,32]. While surveillance data help in selecting the vaccine strains, it remains difficult to predict which strains will circulate in the upcoming year. In a previous report, we proposed that growing viruses in the presence of influenza virus immune human sera could assist in predicting the circulating viruses to update the strains used for vaccination [33]. As anticipated, growing viruses in the presence of human sera selected for variants in the HA protein in the same amino acid positions found in circulating viruses, strongly suggesting that this is a valid strategy to predict circulating influenza strains [33].

Because the HA head domain is highly variable and easily tolerates the incorporation of amino acid changes [34], the potentiation of immune responses directed to other viral antigens such as the HA stalk domain and the NA is being pursued [7,15,35]. However, few studies have analyzed antigenic drift in the HA stalk domain [36] or the NA protein using immune pressure provided by human sera, an approach that mimics the immune pressure provided by human populations. In this paper, we compared the selection of amino acid changes in the HA ad NA proteins by growing influenza viruses in the presence of human sera [33,36]. We found that, using the same human sera, the accumulation of amino acid changes in the HA protein is higher in the HA head domain than in the HA stalk domain or the NA protein. All the amino acid changes in the HA affected protein antigenicity, as previously shown [33,36]. In addition, and importantly, we selected two amino acid changes in the NA protein which also affected the antigenicity of the protein, measured by the binding of monoclonal antibodies (mAbs). These data are highly informative to update the strains used for vaccination each year and for elucidating the most important antigenic sites in the HA and NA proteins targeted after influenza virus infection in humans. Furthermore, these data suggest that the NA protein could be more resistant to immune pressure than the HA protein, at least when the viruses are grown under immune pressure provided by human sera.

## 2. Materials and methods

### 2.1. Study design and human subjects

Human subjects were enrolled as part of either an 'acute influenza' surveillance protocol (subjects 54FUR021, 54FUR023, 54FUR038, 54FUR040, 54FUR047, 54FUR055, 54FUR058, 54FUR065, 54FUR087, 54FUR090, and 54FUR091, named in the manuscript with the last two numbers), or from a prospective 'family flu' surveillance study of families with at least one child in the household <4 years of age (subjects FAM195, FAM196, FAM 297, FAM298 and FAM 300, named in the manuscript with the last three numbers). In both cohorts, subjects reporting influenza-like illness (fever, cough, rhinitis) were asked to visit the University of Rochester Vaccine Research Unit for sampling by nasal wash and nasopharyngeal swab (combined). Sera from subjects enrolled in the acute influenza surveillance protocol were obtained at the acute visit (2–3 days post-infection), and around 28 days later. For the 'family flu' surveillance protocol, sera from subjects were obtained before the start of the flu season (during the fall), and approximately 28 days after the acute illness visit. For this study sera from subjects 21, 23, 38, 40, and 47, infected during the 2010/2011 season and sera from subjects 55, 58, 65, 87, 90, and 91, infected during the 2012/2013 season, were collected at the acute visit (2–3 days after infection). Some of these individuals received influenza vaccination at the beginning of the season (H3 component A/Perth/16/2009 and A/Victoria/361/2011, for seasons 2010/11 and 2012/13, respectively; see Supplementary Table 4 in [33]). Sera from subjects FAM195, FAM196, FAM297, FAM298, and FAM300 (named as 195, 196, 203, 256, 297, 298, and 300) collected at 2010 fall, were used. Subjects 195, and 196 were infected with pH1N1 viruses in November 2009. Subjects 297, 298 and 300 were not infected (data not shown). Certain individuals had received influenza vaccination specific for pH1N1 (A/California/04/2009) virus, during 2009 or 2010 (see supplementary Table 3 in [36]). Each study was approved by the University of Rochester Human Research Subjects Review Board (protocol numbers 09-0034 and 07-0046). Informed written individual or parental consent was obtained for each participant.

### 2.2. Virus passages

Viruses A/Perth/16/2009 (from International Reagent Resources, FR-370), A/Victoria/361/2011 (from BEIResources, NR-44022) and A/California/04/2009/E3 [37] (MOI 0.1) were incubated during 1 h at room temperature with different dilutions of sera from patients. As controls, viruses were also passaged in the absence of human sera. Then, sera-virus mixtures were used to infect MDCK cells (24-well plate format). When cytopathic effect (CPE) was evident (approximately 24 h post-infection, hpi, in the absence of sera/mAb, and at 48–72 hpi in the presence of sera/mAb), total RNA was collected from the wells incubated with the highest Ab/sera amounts, a retrotranscriptase (RT)-PCR was performed, and the PCR products were sequenced, using the protocol described below. Supernatants were collected and passaged in the presence of sera/mAbs up to 16 times.

### 2.3. HA and NA sequencing

RNAs were obtained from infected cell culture extracts using the RNeasy mini kit (Qiagen) according to the manufacturer's instructions. Reverse transcription reactions were performed for 2 h at 37°C using the high-capacity cDNA reverse transcription kit (Life Technologies, Carlsbad, CA). The cDNAs were amplified by using the Pfx polymerase (Life Technologies). The primers used for HA gene amplification and sequencing were previously described [33,36]. For NA gene A/California/04/2009 strain amplification, we used the primers NAcal-1-VS (5′-ATGAATCCAAAC CAAAAGATAATAACC-3′) and NAcal-1410-RS (5′-TTACTTGT CAATGGTAAATGGCAAC-3′), for NA amplification of A/Perth/16/2009 and A/Victoria/361/2011 strains we used the primers NAvict-VS (5′-GCAGGAGTAAAGATGAATCC-3′) and NAvict-RS (5′-CTAAAATTGCGAAAGCTTATATAGGC-3′). The same forward and

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