
REVIEW

The Power and Limitations of Influenza Virus Hemagglutinin Assays

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Abstract—Influenza virus hemagglutinins (HAs) are surface proteins that bind to sialic acid residues at the host cell surface and ensure further virus internalization. Development of methods for the inhibition of these processes drives progress in the design of new antiviral drugs. The state of the isolated HA (i.e. combining tertiary structure and extent of oligomerization) is defined by multiple factors, like the HA source and purification method, posttranslational modifications, pH, etc. The HA state affects HA functional activity and significantly impacts the results of numerous HA assays. In this review, we analyze the power and limitations of currently used HA assays regarding the state of HA.

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Influenza is one of the most common infectious diseases: 5-10% of adults and 20-30% children are infected with influenza each year. Influenza infection itself lasts only for several days, but the high risk for human health comes from secondary infections that develop in influenza-damaged tissues and from complications that result from preexisting conditions (pregnancy, immunodeficiency) or disorders (diabetes, obesity, cardiovascular disorders). The annual number of influenza-associated deaths is 250,000 to 500,000 [1, 2].

Influenza virus belongs to the Orthomyxoviridae viral family, the most dangerous member of which is influenza A virus. Influenza virus consists of a membrane-enveloped ribonucleoprotein core. Its genome is segmented and contains eight antisense RNAs coding for 11 viral proteins. Viral surface proteins provide virion interaction with the host cell plasma membrane (hemagglutinin, HA), fusion of the virus membrane with the

endosome with subsequent nucleoprotein release from the endosome (HA and M2 protein), and detachment of the newly synthesized virus particles from the host cell surface (neuraminidase, NA) [3-5]. Surface proteins are the major targets for existing antiviral drugs: Arbidol (umifenovir) inhibits HA at the step of the virus membrane fusion with the endosome [6-8]; amantadine and rimantadine inhibit the M2 protein [9, 10]; zanamivir, oseltamivir, and peramivir inhibit NA [9-12]. Rapid microevolution of the influenza virus results in an extremely high variability of its surface proteins, which ensures rapid selection of strains resistant to the action of existing antiviral drugs. Present-day influenza virus strains are already resistant to amantadine and rimantadine [13]. For this reason, active search for new antiviral agents, including HA inhibitors, has to be continued [14, 15]. HA is the most promising target for the new drugs, because it is responsible for the very first stage of viral infection, namely, virus attachment to the host cell. Since HA is not an enzyme, potential HA inhibitors should either inhibit low pH-induced conformational transitions of HA or prevent formation of the complex between HA and cell surface molecules (including oligosaccharides). The main obstacle in the design of new HA inhibitors is that they cannot be characterized by standard enzymological methods and should be tested in a carefully chosen *in vitro* test system. In our review, we discuss the capabilities and limitations of the currently used test systems.

Abbreviations: a.a., amino acid residue; ELISA, enzyme-linked immunosorbent assay; ER, endoplasmic reticulum; GA, Golgi apparatus; HA, hemagglutinin; HA0, hemagglutinin precursor; HA1, hemagglutinin subunit 1; HA2, hemagglutinin subunit 2; HAI, hemagglutination inhibition assay; IEP, immunoelectrophoresis; MAbs, monoclonal antibodies; NA, neuraminidase; RIA, radioimmunoassay; RIP, radioimmunoprecipitation; SA, sialic acid; SRID, single radial immunodiffusion.

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It is not surprising that influenza virus HA has been actively studied — dozens of reviews have been published that search for the correlation between the HA structure and influenza virus virulence. Because HA is a highly variable protein, data on its structure and functions have been accumulated in large dynamic databases, such as the Influenza Research Database (www.fludb.org) and GisAid (www.platform.gisaid.org). Several recent reviews summarize structural properties of HAs from different viral strains [16], effects of posttranslational modifications on the virus virulence [17], HA functional properties (especially, its pH-dependent conformational transitions) [16, 18], and data on the mapping of viral antigenic determinants [19].

The properties of HA outside the virus are affected by multiple factors. The first is the HA primary structure and diverse posttranslational modifications. Multiple virus mutants have been characterized, and the obtained information was used to identify phylogenetic relationships between these viruses (see GisAid database and [20]). The second and the third factors are HA conformation and extent of oligomerization. If the primary structure and posttranslational modifications are determined by the virus strain and the host cell, HA conformation and extent of oligomerization (defined by us as the “HA state”) depend on the method for HA isolation and some external parameters, such as pH, buffer composition, etc.

HA forms functionally active trimers in the virus envelope [19]. Isolated HA can exist either as an active trimer or inactive oligomers composed of a varying number of HA monomers. The number of HA molecules in the oligomer depends greatly on posttranslational modifications, especially, polysaccharide attached to Asn residues in HA [17].

This existence of diverse HA states often causes ambiguities in the estimates of the activity of HA inhibitors or affinity of other HA-interacting molecules, e.g. antibodies (see section “Variety of anti-HA antibodies” of this review). Thus, IC_{50} values for the same inhibitor might differ ten times depending on the HA state.

In this article, we review procedures for HA isolation and methods for the estimation of its functional activity. Special attention is given to the HA states and test systems that could be used for the development of molecules that would recognize these states (e.g. aptamers and antibodies capable of blocking HA interaction with its ligands).

STRUCTURE AND FUNCTIONS OF HEMAGGLUTININS

Influenza virus HA is an integral membrane protein. It is synthesized as a single precursor polypeptide chain in the endoplasmic reticulum (ER) of the host cell. The precursor molecule undergoes processing that includes (i) trimerization in the ER [21], the vesicular-tubular cluster of the Golgi apparatus (GA), or the *cis*-Golgi network [22,

23] (depending on the virus subtype); (ii) glycosylation [24] and removal of the N-terminal signal peptide [25] in the ER; (iii) palmitoylation of the terminal COOH-group in the early GA cisterns [26]; and (iv) cleavage in the *trans*-Golgi network with the formation of two polypeptide chains connected by a disulfide bond [27, 28]. HAs of the highly pathogenic avian influenza virus H5 and H7 subtypes are cleaved by cellular subtilisin-like proteases [29]. In other virus subtypes HAs are cleaved predominantly by the transmembrane type II serine proteases [30].

The HA molecule in the virus membrane consists of the hydrophilic ectodomain exposed at the membrane outer side, one small transmembrane domain (24–28 a.a.), and one cytoplasmic domain (10–55 a.a.) [31]. Crystal structures for the ectodomains of mature HA [32, 33] (Fig. 1b) and its precursor (HA0) (Fig. 1a) [34] have been solved. The ectodomain consists of distal globular and proximal fibrillar fragments corresponding to HA subunits 1 and 2 (HA1 and HA2), respectively. The surface of the globular fragment bears HA antigenic determinants [35] and serves as a binding site for sialic acids (SAs) of the host cell surface [36]. The fibrillar fragment is formed by six α -helices (two helices from each HA2 molecule); three of them are wound in a left-handed superhelix.

In addition to the supercoiling of the ectodomain fibrillar fragment, HA quaternary structure (Fig. 2) is stabilized by noncovalent interactions between monomer subunits. It was found that stability of the HA trimer in the pandemic A(H1N1)pdm09 strain is determined by amino acids at positions 205 and 402 in the HA1 and HA2 subunits, respectively (Fig. 2) [37]. Another driving force for trimerization is the orientation effect of the membrane-anchored C-terminal sequence of HA2. Thus, both full-size HA [38] and HA with the C-terminal glycosylphosphatidylinositol anchor in place of the transmembrane and cytoplasmic domains [39] form trimers, whereas isolated ectodomain is incapable of spontaneous trimerization [38]. Disulfide bonds between the subunits are another important factor that, together with protein–lipid interactions, affects the structural stability and functional activity of HA molecules. Formation of disulfide bridges between cysteine residues of the transmembrane and cytoplasmic domains is critical for protein trimerization [40]. Interestingly, isolated HA1 molecules, but not ectodomains, can form biologically active trimers [41]. Trimerization in the absence of transmembrane domains is initiated by the interactions between the Ile3–Cys4–Ile5 amino acid triads [42].

The structure and functional activity of HA trimers depend on the conformation of monomeric subunits. It was found that about 10% of HA precursor molecules *in vivo* are not cleaved by proteases and form aberrant inactive trimers and oligomers of unknown structure that include many HA monomers [43].

The first stage of the virus life cycle is its internalization, which is accompanied by a series of conformational changes in the HA molecules. After HA1 binding to SAs

of the host cell membrane proteins, the virus enters the cell by endocytosis. The pH in the endosome decreases to 5.0–5.5 due to the proton channel formation by the M2 protein. Protonation of HA2 results in disruption of non-covalent interactions in the HA globular fragment, formation of a long supercoiled region from the short and long HA2 α -helices, and conversion of the B-loop into a new α -helix (Fig. 1e) [32, 44]. Because of these conformational rearrangements, hydrophobic N-terminal fragments (a.a. 331–353) of HA2 molecules are exposed and

incorporated into the endosomal membrane. The N- and C-termini of the HA2 polypeptide approach each other [45], which results in the fusion of the viral and endosomal membranes and release of the viral nucleoprotein into the host cell cytoplasm [5, 46, 47]. Conformational transitions of the HA molecule can be also induced by temperature shock, thereby confirming the hypothesis of a metastable state of the initial structure [48].

The surface of the HA ectodomain contains multiple sites for N-glycosylation at asparagine residues

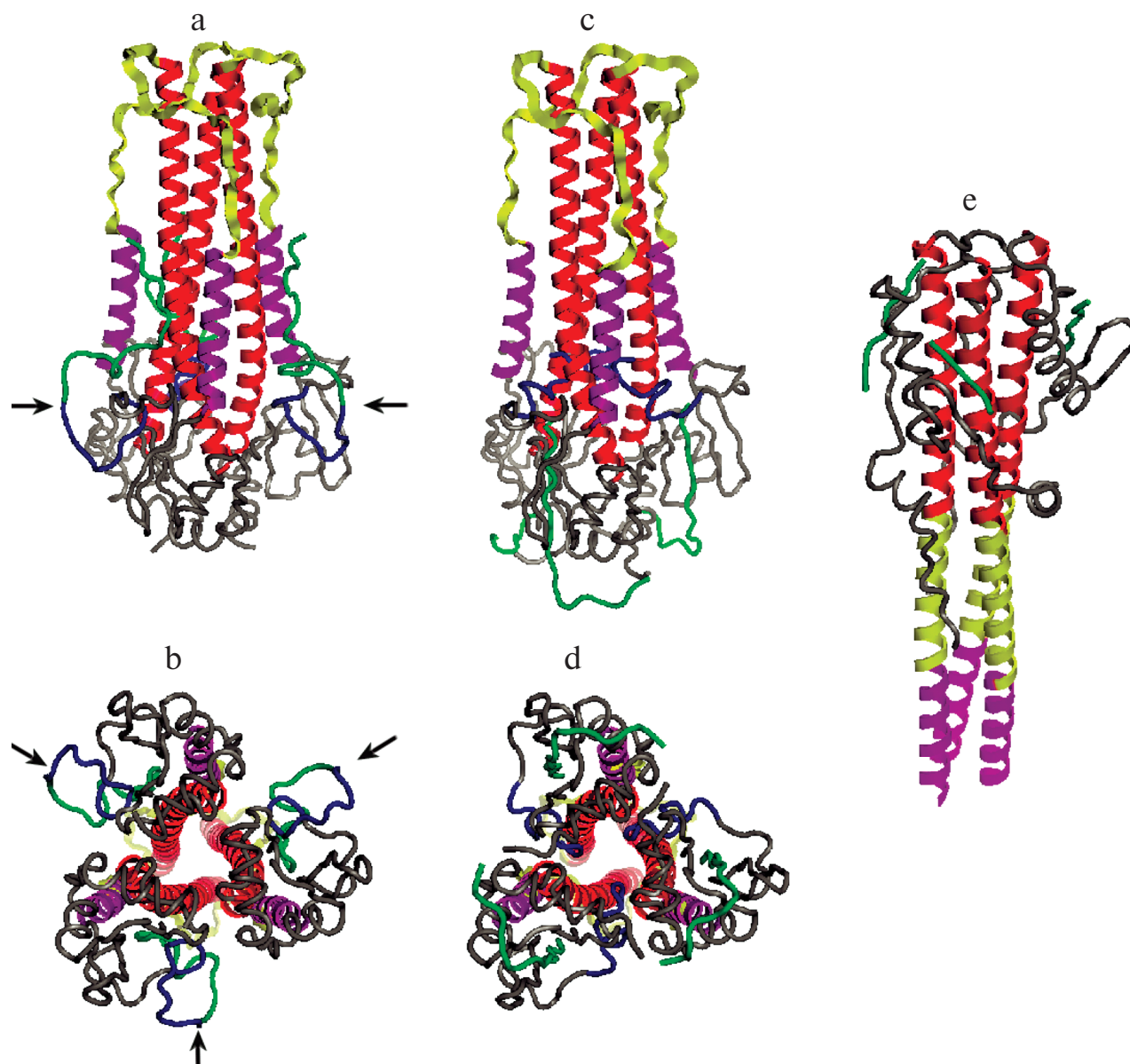


Fig. 1. Conformational changes in the HA ectodomain: a) uncleaved precursor (fragment); b) same as (a), view from the viral membrane side; c) processed HA with local conformational changes (fragment); d) same as (c), view from the viral membrane side; e) HA in the low-pH conformation (fragment). Cleavage sites are indicated with arrows. In the HA2 fibrillar fragment: red, α -helices that form the superhelix; purple, short α -helices; yellow, B-loops that form α -helices during pH-dependent conformational transition; blue and green, HA2 N-terminal (a.a. 331–342) and HA1 C-terminal fragments, respectively, released by proteolysis; gray, rest of the HA2 molecule. HA1 globular fragment and glycosylation sites are not shown.

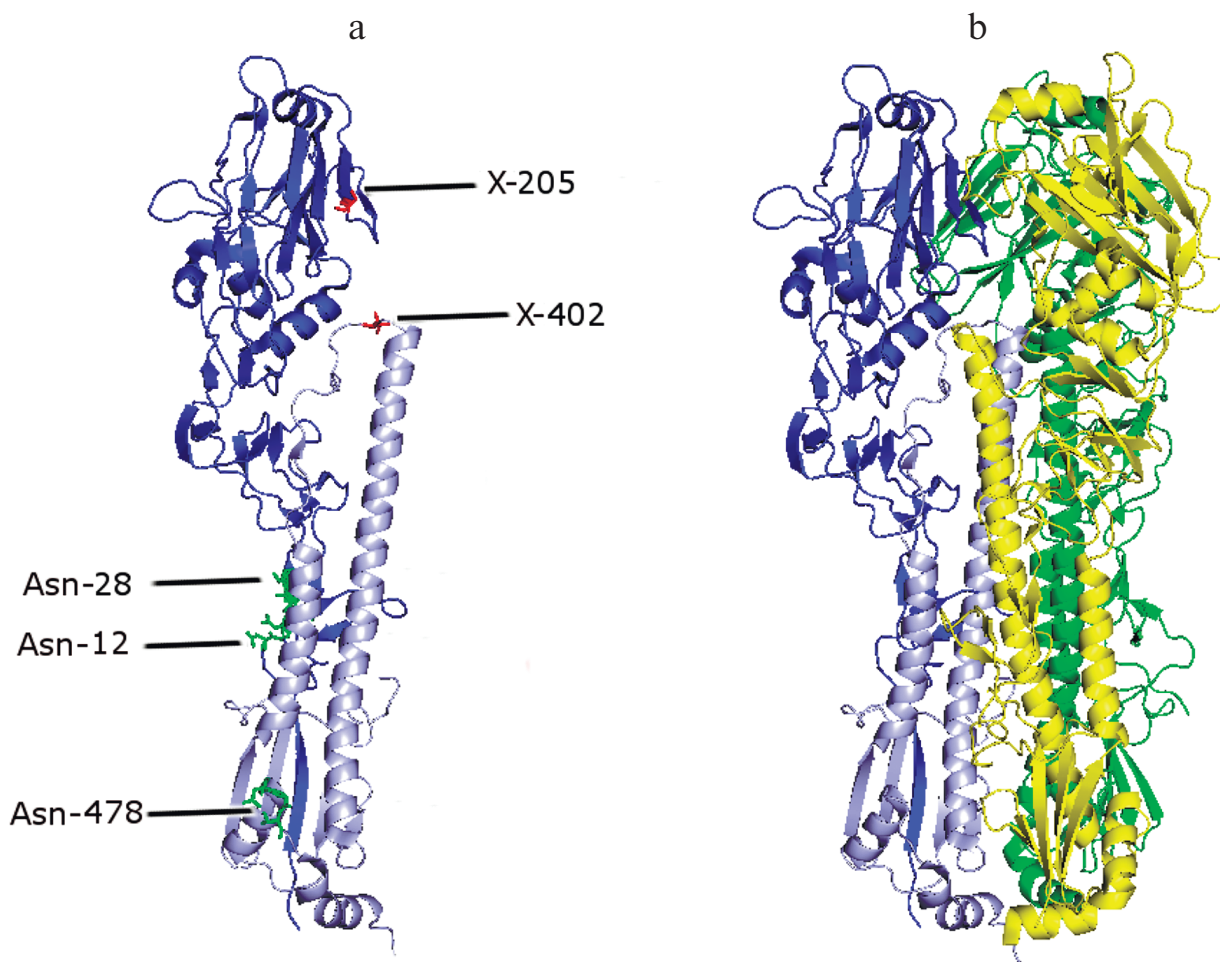


Fig. 2. a) Influenza virus HA monomer (blue, HA1; light blue, HA2). Residues 205 and 402 that determine monomer predisposition for trimerization are shown in red; conserved glycosylation sites (Asn–X–Thr), where the Asn residues are Asn12, Asn28, and Asn478, are shown in green. b) Influenza virus HA trimer (individual monomers are colored differently).

(Asn–X–Thr/Ser, where X is any amino acid except Pro) [49]. Two of these residues (Asn12 for HA1 and Asn478 for HA2; numeration as in the H7 subtype HA2) are strictly conserved in all known subtypes, and Asn28 is conserved in all subtypes except H4, H8, H9, and H12 (Fig. 2) [50]. The number and chemical nature of modifying carbohydrate moieties depend on the host cells [51]. Carbohydrate moieties play an important role in HA processing and intracellular transport [24]; they also screen viral antigenic determinants at the cell surface, which suppresses immune response to the viral infection [17].

ISOLATION OF HAs

Each viral particle contains 400 to 1000 HA molecules at its surface [51, 52]. This number is sufficient for direct isolation of HA from virions, and corresponding methods were developed in 1950s–1970s. To isolate HA, virus membranes are disintegrated with organic solvents

or mild ionic detergents [53–55]. The major drawback of these procedures is that they often result in partial denaturation and aggregation of the HA; on the other hand, they preserve the glycosylation profile of the HA molecules [55].

Recombinant HA is now commercially available. However, the use of prokaryotic expression systems is hindered by (i) the need for separate expression of HA1 and HA2 subunits, (ii) the need for HA refolding after its purification from inclusion bodies [56], (iii) decreased expression levels because of partial degradation of hydrophobic regions, such as the signal peptide, HA2 N-terminal fragment, and transmembrane domain [57], and (iv) the absence of posttranslational modifications, above all, glycosylation. The latter problem could be solved by expressing HA in transgenic *E. coli* cells containing the system for protein *N*-glycosylation from *Campylobacter jejuni* [58].

The HA expressed in eukaryotic expression systems is more suitable for functional activity studies. Recombi-

nant HA is most commonly produced in cultured insect cells [59–61]. HAs expressed in a baculoviral expression system (i) are glycosylated and (ii) exist as trimers and/or their associates, as directly demonstrated by hemagglutination tests (see section “Purifications of HAs” of this review) or indirectly proven in exclusion chromatography studies. Thus, recombinant HA from the influenza A/New Caledonia/20/99 virus was found to be glycosylated at all six sites predicted based on the analysis of its amino acid sequence [59].

The major differences between insect cells and typical influenza virus host cells are the low activity of terminal glycosyl transferases [62], high activity of *N*-acetyl- β -glucosaminidases [63, 64], and the absence of terminal sialylation of *N*-glycans [65] in insect cells. As a result, HAs, obtained in baculoviral expression systems, have different composition of modifying carbohydrate moieties and contain *N*-glycans that are shorter and less branched than in the native virus. HA with more native glycosylation profile could be synthesized using baculoviral expression vectors [66–68] or insect cell lines [69, 70] that bear genes for glycosyl transferases and proteins involved in SA synthesis and transport under control of a strong promoter [71]. So far, carbohydrate modifications typical for mammalian hosts could be reproduced in baculoviral expression systems only partially.

HAs could be synthesized directly in mammalian cells as individual proteins [72] or components of virus-like particles containing a full [73] or reduced [74, 75] set of recombinant viral proteins. It is preferable to use cell cultures that resemble most the natural host cells, because this would preserve posttranslational modifications and the glycosylation profile of the native virus and ensure cleavage of the HA0 precursor [61].

Therefore, the method for HA isolation determines the chemical structure of HA protein (including posttranslational modifications) and its state.

HEMAGGLUTINATION TEST

Influenza virions can agglutinate erythrocytes with the formation of a viscous gel. The agglutination occurs

through the binding of virion-embedded HA to sialylated surface proteins of several erythrocytes at once.

The number of agglutinated erythrocytes is proportional to the HA content and could be used for estimating the functional activity of the protein itself and of inhibitors of complex formation between HA and SA. The classical procedure uses 0.5–1.0% suspension of erythrocytes mixed and incubated with the virus suspension, with negative control containing erythrocytes only, and positive control containing erythrocytes and virions [76]. The virus titer is determined using serial dilution of virion suspension; the functional activity of the SA–HA complex formation inhibitors is estimated by adding different inhibitor concentrations to the reaction mixture. The apparent inhibition constant (K_i^{HI}) is defined as the lowest inhibitor concentration at which no erythrocyte agglutination is observed [77]. For polyvalent HA inhibitors (e.g. SA-modified synthetic polymers), K_i^{HI} is calculated based on the total concentration of binding sites [78–80].

Hemagglutination inhibition assay (HAI) has been successfully used for estimating inhibition constants for SA synthetic analogs [81, 82], polyvalent SA synthetic analogs [78–80], liposomes with surface-exposed SA residues [83, 84], and some SA-binding proteins, e.g. α_2 -macroglobulin [85]. The obtained values correlated well with the dissociation constants of the corresponding complexes determined by ^1H NMR analysis [77] and data of enzyme immunoassay (ELISA) [79].

The hemagglutination test can be performed not only for influenza virions, but for isolated HA molecules as well if these molecules are in the form of trimers to provide the formation of a multiple-contact network. Thus, the HA ectodomain that exists in solely monomeric form does not agglutinate erythrocytes, while oligomerization-prone HA1 (a.a. 1–330) does [86]. Removal of the HA1 N-terminal fragment (a.a. 1–8) that contains the oligomerization signal Ile–Cys–Ile results in complete loss of the HA1 activity, while removal of the C-terminal portion (a.a. 321–330), on the contrary, stabilizes the trimer and facilitates hemagglutination. The larger HA1 fragment (a.a. 1–104) is also capable of oligomerization but does not agglutinate erythrocytes because of the absence of the SA-binding site (Table 1) [42].

Table 1. Correlation between HA1 oligomerization state and activity in the hemagglutination test

HA fragment	Subtype	SA-binding site	Expression system	Composition	Relative activity, %	Source
1–330*	H5	+	<i>E. coli</i>	monomer/oligomer	100	[42]
1–320*	H5	+	<i>E. coli</i>	monomer/oligomer	4	[42]
1–104*	H5	–	<i>E. coli</i>	monomer/oligomer	0	[42]
9–330*	H5	+	<i>E. coli</i>	monomer	0	[42]
1–480	H1	+	<i>E. coli</i>	monomer	0	[86]

* In HA1.

Nonglycosylated recombinant HA is active in the hemagglutination test; therefore, neither location nor the presence of glycoside moieties in the HA molecule affect its ability to bind SA and agglutinate erythrocytes.

The species specificity of HAs should be taken into consideration when performing the hemagglutination test, because mutations in HA might affect the specificity of SA binding. Thus, HAs of influenza subtypes H1, H2, and H3 interact with the Sia2-6Gal fragment typical for human and swine epithelial cells; HAs of influenza subtypes H5 and H7 bind the Sia2-3Gal fragment found in equine and avian epithelial cells [4, 87]. Therefore, erythrocytes of a particular biological species should be used in tests with specific virus strains. The difference in the constants of HA binding to different SA fragments is often only within an order of magnitude [88], which allows ignoring the species specificity, although the sensitivity of the method is decreased.

IMMUNOCHEMICAL TESTS

Variety of anti-HA antibodies. Antigenic determinants differ in influenza virus strains. For example, the

variety of surface epitopes (as well as corresponding antibodies) could be seen in influenza virus subtype H3. The HA1 globular fragment in this virus contains four antigenic determinants (Fig. 3, a-d), as determined using an array of monoclonal antibodies (MAbs) [35, 89]. Two of the epitopes (B and especially D) are present only in the trimer, thereby making it possible to use antibodies against these epitopes for studying HA quaternary structure, e.g. by following the dynamics of HA posttranslational trimerization *in vivo* [43, 90]. The affinities of the anti-D epitope antibodies differ more than ten times depending on the state of HA oligomerization [91, 92].

Structure and localization of the HA antigenic determinants in virus subtype H1 differ from those in subtype H3. Thus, HAs from subtype H1 have at their surface two specific (Sa and Sb) and three cross-reactive (Ca₁, Ca₂, and Cb) epitopes that are present in different strains [93, 94]. All these epitopes, except Sb, exist only in HA trimers [95].

Antigenic determinants in virus subtypes H1 and H3 partially overlap: the B site corresponds to the Sa site and a small fragment of the Sb site; the D site corresponds to the Ca₁ site. At the same time, the A site has a considerably smaller surface area than the Ca₂ site located in the

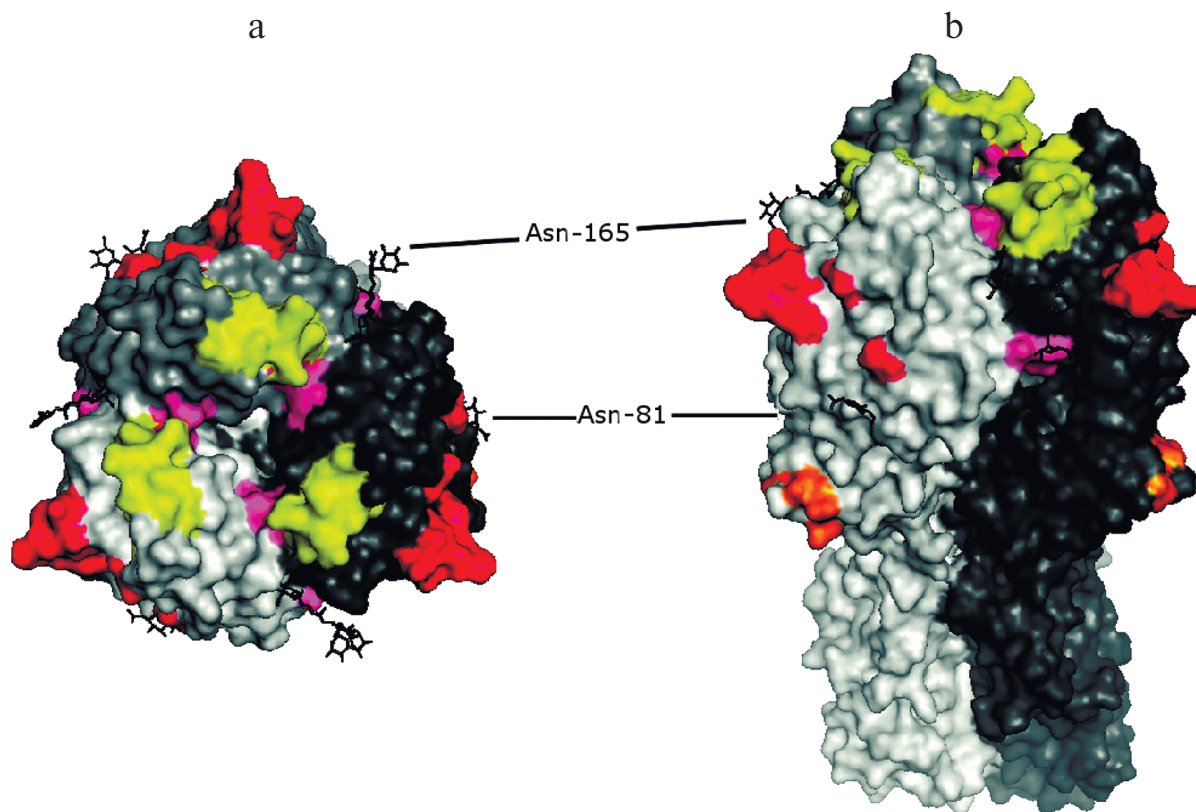


Fig. 3. Antigenic structure of the HA ectodomain from the influenza virus H3 subtype (A/Hong Kong) at pH 7.0: a) top view; b) side view. Monomers in the trimer are shown in variations of gray color; carbohydrate modification are outlined in black. HA1 epitopes A, B, C, and D are colored red, yellow, orange, and pink, respectively. Only glycosylation at Asn81 and Asn165 is shown, which masks the regions corresponding to the Ca and Sa epitopes, respectively, in subtype H1 strains.

Table 2. Anti-HA antibodies and their specificity against different HA states

Antibody	Subunit (epitope)	Affinity ratio		Method	Source
		trimer/monomer	pH 7.0/pH 5.0		
1	2	3	4	5	6
Monoclonal antibodies against HA subtype H3					
N1	1 (B)	29.0 [†] $\infty^{*§}$	29.0 [†] 15.4	RIP	[39] [43]
N2	1 (B)	$\infty^{*†}$ $\infty^{*§}$	$\infty^{*†}$ 19.8	RIP	[39] [43]
12/1	1 (B/D)	$\infty^{*§}$	34	RIP	[43, 97]
A1	2 (?)	0.15 [§]	$4.8 \cdot 10^{-3}$	RIP	[43]
A2	1 (?)	0.17 [§]	$1.5 \cdot 10^{-3}$	RIP	[43]
88/2	1 (?)	$6.9 \cdot 10^{-3§}$ — —	$2.2 \cdot 10^{-2}$ $7.3 \cdot 10^{-2}$ $<1.8 \cdot 10^{-3}$	RIP RIA ИГА	[43, 97] [101] [101]
A2	1 (C)	— 1.3 1.6	1.3 1.3 —	HAI RIA RIA	[97] [101] [91]
A20	1 (A)	— — — 3.3 2.4	1 1.3 1.1 — —	HAI HAI RIA RIA RIA	[97] [101] [101] [91] [92]
A21	1 (D)	— — — 9.3 5.9	$>1.0 \cdot 10^3$ $7.3 \cdot 10^3$ 11 — —	HAI HAI RIA RIA RIA	[97] [101] [101] [91] [92]
BB8	2 (353-368; site I)	— —	1.0 [§] 0.3	RIA	[103]
CF2	2 (331-365; site I)	— —	0.6 [§] 0.4	RIA	[103]
ИIF4	2 (455-505; site II)	— —	1.0 [§] 0.5	RIA	[103]
CB8	2 (368-442; site III)	— —	0.5 [§] 0.5	RIA	[103]
FE1	2 (455-505; site IV)	— —	1.8 [§] 0.5	RIA	[103]
Monoclonal antibodies against HA subtype H1					
Y8-10C2 H17-L2 H28-E23	1 (Sa) 1 (Ca) 1 (Sb)	— — —	$4.4 \cdot 10^{-2}$ 11.9 1	RIA	[95]
Polyclonal antibodies against polypeptides [‡]					
11	1 (105-139)	0.6 [†]	0.6 [†]	RIA	[102]

Table 2 (Contd.)

1	2	3	4	5	6
17	1 (174-197)	0.15 [†]	0.15 [†]		
19	1 (201-227)	0.25 [†]	0.25 [†]		
23	1 (266-302)	0.1 [†]	0.1 [†]		
24	2 (331-359)	1.8·10 ^{-2†}	1.8·10 ^{-2†}		
25	2 (345-359)	1.1 [†]	1.1 [†]		

* The lower value does not exceed the background.

§ As determined for the HA precursor (HA0).

† As determined at pH 7.0 and 5.0 for the HA ectodomain in trimeric and monomeric forms, respectively.

‡ Against HA fragments (numbers of amino acid residues are shown in parentheses).

same region of the protein surface; the C site and the Cb site do not overlap. The latter fact could be explained by the difference in the glycosylation profiles of these sites: the surface area corresponding to most of the subtype H1 Cb site is *N*-glycosylated at Asn81 in some subtype H3 strains (e.g. A/Hong Kong/1/68), whereas a fragment of the HA surface corresponding to the C site in subtype H3 is *N*-glycosylated at Asn271 in some subtype H1 strains (e.g. A/PR/8/34) [94]. Similarly, glycosylation of the Asn165 residue in HA of the A/Hong Kong/1/68 strain masks the Sa site [94, 96].

Antibodies against various regions of the HA molecule could be used not only for discrimination between monomers and trimers, but also to monitor HA conformational transitions. Several articles have been published on changes in HA antigenic properties induced by pH decrease [97-100]. It was found that acidification transforms the structure of epitopes in the globular domain and leads to exposure of HA molecule regions that are screened at pH 7.0 (see section "Structure and functions of hemagglutinins" of this review). When exposed, these regions become available for binding corresponding antibodies [101]. Thus, lowering pH to 5.0 increases MAb affinity to the HA2 N-terminal fragment (a.a. 331-329) by two orders of magnitude [102]. Experiments with an array of anti-HA2 MAbs identified four epitopes whose availability increases several-fold as a result of the pH-dependent conformational transition (Table 2) [103].

As mentioned above, glycosylation might reduce accessibility of HA epitopes. Due to conformational flexibility [104] and structural variability [105], *N*-glycans can sterically block the HA surface within a 10-15 Å radius from the glycosylation site. If the screened regions overlap with antigenic determinants, the probability of antigen recognition by the antibody considerably decreases. Thus, mutation Asp63Asn and resulting glycosylation of the Asn residue decrease the affinity of the MAb for HA of the Hong Kong influenza virus (H3N2) [106]. Another example of such screening is the earlier mentioned masking of C and Ca/Sb epitopes in the A/PR/8/34 and A/Hong Kong/1/68 virus strains, respec-

tively. Targeted abolishment of glycosylation sites, on the contrary, results in epitope unmasking in globular and fibrillar HA fragments [107].

However, there are some exceptions [108]. It is known that particular antibodies can bind in the vicinity of glycosylated Asn residues [109] and, in many cases, *N*-glycans act as mediators of this binding [110, 111]. Generally, *N*-glycans by themselves do not exhibit high antigenic activity; however, certain antibodies have been described that interact exclusively with a glycosylated form of the avian influenza virus HA and, therefore, could be used as secondary antibodies in the HA determination by the ELISA (see section "Enzyme immunoassay") [112].

Table 2 shows antibodies that could be used for selective detection of various HA forms. The specificity of these antibodies was determined from their relative affinity to various HA conformations in the hemagglutination inhibition assay (see section "Hemagglutination test"), radioimmunoprecipitation (RIP), or radioimmunoassay (RIA).

The RIP method includes introduction of a radioactive label in the antigen molecule, precipitation of the antigen-antibody complex, and estimation of the amount of bound antibodies from the decrease in radioactivity of the unbound antigen [113]. Labeled HA is usually purified from a cell culture infected with the virus in the presence of L-[³⁵S]-methionine [43, 97]. Solid-support RIA is based on the competition between labeled and non-labeled antibodies for binding with an immobilized antigen [92, 114]. RIA in a solution involves formation of complexes between labeled HA molecules and antibodies, immobilization of these complexes on protein A-Sepharose, and determination of radioactivity of the bound HA fraction [115]. Labeled HA or antibodies for RIA are usually obtained by radioiodination at tyrosine residues in Na¹²⁵I solution using chloramine T [116].

Identification of HA in a specific conformation/state requires solving two major problems: choosing antibodies with required specificity, and selecting the optimal immunochemical test. Further, in this article we consider

possible solutions to these problems with specific references to the single radial immunodiffusion (SRID), immunoelectrophoresis (IEP), and enzyme-linked immunosorbent assay (ELISA) methods.

Single radial immunodiffusion (SRID) of Mancini.

Single radial immunodiffusion (SRID) was developed in 1965 for quantitative analysis of antigens in vaccines and estimation of their activity with corresponding antibodies [117]. The method is based on the diffusion of viral antigen (HA, in our case) in an agarose gel containing antibodies at a known concentration. The area of diffusion is directly proportional to the amount of HA in the analyzed sample and inversely proportional to the working antibody concentration. Virus preparation with known HA concentration is used as a standard. SRID is now commonly used for testing anti-influenza vaccines [118].

HA trimers at the virus surface tend to associate with each other and other viral proteins [119]. Formed associates of varying molecular masses diffuse in the gel at different velocities, which results in deviation from direct proportionality between the area of diffusion and antigen concentration. To ensure accurate quantification, the HA should be in its trimeric form, which can be achieved by treating viral particles with nonionic [118, 120] or zwitterionic [121] detergents.

It is preferable to use polyclonal serum in SRID, since it provides sharper edges of the HA diffusion zone than MABs [41, 59, 61, 122-126]. This considerably limits application of SRID for studying HA ligands, since this requires the use of antibodies able to compete with ligands for the binding of a particular HA epitope.

As mentioned above, HA molecules undergo conformational transitions as pH decreases from 7.0 to 5.0. It was found that the low-pH HA form could not be evaluated by SRID (Fig. 1b), despite that fact that the used polyclonal antibodies recognize both conformations. This phenomenon could be explained by removal of the low-pH from the SRID assay because of its selective adsorption on the nitrocellulose filter during gel blotting [127-129]. The ability to distinguish selectively native HA conformation is an important advantage of the SRID method.

Immunoelectrophoresis. The immunoelectrophoresis (IEP) procedure is like SRID except that HA migrates in the agarose gel under applied voltage [130]. The parameter measured in IEP is the length of the precipitation zone, which is the length of the HA run in the gel (vs. diameter of precipitation zone in SRID). Similarly to SRID, the efficiency of HA assay by IEP depends on the protein state, i.e. extent of oligomerization and conformation of antigenic determinants.

Enzyme-linked immunosorbent assay (ELISA).

Enzyme-linked immunosorbent assay (ELISA) is a universal method that can be used for quantitative analysis of HA and investigation of HA ligands. The method includes HA immobilization, its interaction with a

labeled antibody or an inhibitor, and label visualization. The most commonly used procedure is so-called sandwich ELISA that is 2-5-fold more sensitive than the standard protocol that includes antigen immobilization directly on a solid support [131]. The sandwich method requires the presence of at least two epitopes on the antigen molecule surface. The influenza virion contains several hundreds of surface HA molecules. Sandwich ELISA can be performed with [132] or without secondary antibodies [126, 133]. The main advantage of the indirect method is that once-generated secondary antibodies can be used in many experiments with HA from the same viral strain [132].

Antibodies used in ELISA might recognize particular states of HA. Both polyclonal serum and MABs generated by standard hybridoma technologies can be used in ELISA [134]. MABs have been obtained to various HA molecule epitopes (see section "Variety of anti-HA antibodies") that can be used for assaying HA in its specific conformational states.

To determine total content of HA (all states included), it is essential to use a combination of MABs that recognize native and denatured HA to ensure HA identification irrespective of its conformation [132].

The use of MABs in virus typing has one characteristic distinction from other methods. It is known that influenza virus strains experience antigenic drift; this also concerns HA antigens. This results in periodical seasonal mutations at least every 2-8 years [135-138]. The relative conservatism of HA antigenic determinants within a subtype allows use of the same MAB in ELISA for several years only. Thus, antibodies against HA from the A/Hiroshima/52/05 (H3N2) strain recognize all H3N2 strains isolated between 2000 and 2008, but cannot be used for the identification of earlier strains of this virus [134].

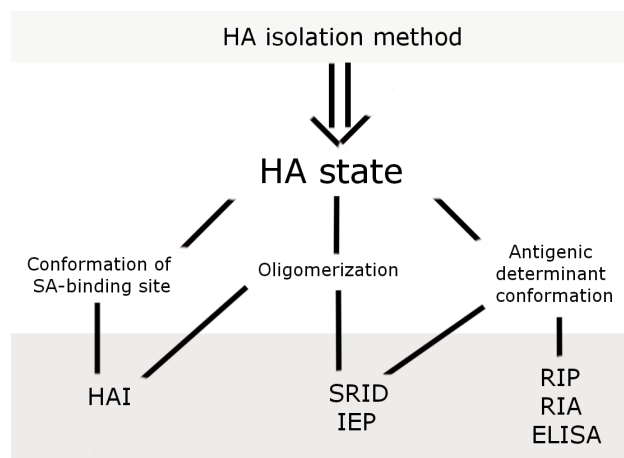


Fig. 4. Relationships between methods for HA isolation, HA states, and possibility of estimating HA state by hemagglutination- and antibody-based methods.

As one of the two influenza virus surface antigens, HA is an essential component of anti-influenza vaccines [139, 140]. It also plays an important role in influenza diagnostics [141] and serves as a target protein in the development of anti-influenza medications [14, 142–144]. Functional and conformational changes that accompany HA processing and functioning during infection are mechanisms that ensure high polymorphism of the virus. This polymorphism complicates selective purification and identification of HA in its particular states, and therefore, significantly hinders development of inhibitors against these states. Inhibitors against specific HA states would not only directly suppress HA functioning, but also prevent some functionally important conformational transitions, which might considerably broaden the spectrum of potential antiviral agents. For example, Arbidol binds in the cavity between HA1 and HA2 and stabilizes HA in a conformation that is typical for HA before membrane fusion (Fig. 1, a and b). It inhibits the pH-dependent conformational transition of HA, thereby preventing correct virus internalization [8].

We summarize the capabilities and limitations of the hemagglutination-based tests and various immunological methods for HA assay in Fig. 4.

Existing procedures for studying influenza virus antigens allow selective determination of HA in virtually any state. Using hemagglutination-based tests, it is possible to investigate oligomeric HA and to design inhibitors against this form. The development of a broad array of MAbs against various HA epitopes made it possible to analyze not only HA oligomeric composition, but also more precise details of the HA structure. For example, ELISA can be used for distinguishing between HA trimers and monomers, between HA in the conformations typical for pH 5.0 and 7.0, and in some cases, between glycosylated and nonglycosylated HA forms, as well as for the development of inhibitors that would compete with MAbs for binding with specific epitopes. Since MAbs are not applicable in SRID and IEP, these two methods do not allow direct study of HA inhibition; however, they are widely used for determination of native HA prefusion conformations.

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