

Short Communication

Exploring the antigenic relatedness of influenza virus haemagglutinins with strain-specific polyclonal antibodies

Blanca García-Barreno,^{1,2} Teresa Delgado,^{1,2} Sonia Benito,^{1,2}
Inmaculada Casas,³ Francisco Pozo³ and José A. Melero^{1,2}

Correspondence
José A. Melero
jmelero@isciii.es

¹Biología Viral, Centro Nacional de Microbiología, Instituto de Salud Carlos III, Majadahonda, 28220 Madrid, Spain

²CIBER de Enfermedades Respiratorias, Centro Nacional de Microbiología, Instituto de Salud Carlos III, Majadahonda, 28220 Madrid, Spain

³Virus Respiratorios & Gripe, Centro Nacional de Microbiología, Instituto de Salud Carlos III, Majadahonda, 28220 Madrid, Spain

Alternative methods to the standard haemagglutination inhibition (HI) and neutralization tests to probe the antigenic properties of the influenza virus haemagglutinin (HA) were developed in this study. Vaccinia virus recombinants expressing reference HAs were used to immunize rabbits from which polyclonal antibodies were obtained. These antibodies were subtype specific but showed limited intra-subtype strain specificity in ELISA. The discriminatory capacity of these antibodies was, however, markedly increased after adsorption to cells infected with heterologous influenza viruses, revealing antigenic differences that were otherwise undistinguishable by standard HI and neutralization tests. Furthermore, the unadsorbed antibodies could be used to select escape mutants of the reference strain, which after sequencing unveiled amino acid changes responsible of the noted antigenic differences. These procedures therefore provide alternative methods for the antigenic characterization of influenza HA and might be useful in studies of HA antigenic evolution.

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Influenza virus is a negative-stranded segmented RNA virus classified in the family *Orthomyxoviridae* comprising three antigenic types: A, B and C. Types A and B cause the annual human epidemics seen in winter months. Although the first human isolate dates back to 1933 (Smith *et al.*, 1933), influenza virus is still a major cause of morbidity and mortality worldwide in humans (Wright *et al.*, 2013). The virion has two major surface glycoproteins inserted into the lipid bilayer, the receptor-binding haemagglutinin (HA) and the receptor-destroying enzyme neuraminidase (NA) (reviewed by Gamblin & Skehel, 2010). Neutralizing antibodies are directed mainly against HA and can prevent serious illness (Couch & Kasel, 1983; Virelizier, 1975). Thus, HA is the main antigenic component of the currently used subunit influenza vaccines.

Sixteen HA antigenic subtypes (H1 to H16) have been identified by serological techniques among influenza viruses infecting different species and two new subtypes (H17 and H18) were recently identified by sequencing of viruses isolated from bats (Tong *et al.*, 2013). However, only viruses of the H1, H2 and H3 subtype have circulated in humans, although sporadic infections with viruses from

other subtypes have been reported (Koopmans *et al.*, 2004; Uyeki & Cox, 2013).

One of the main problems in controlling human influenza infections through vaccination is the high degree of antigenic variation among the HAs of circulating strains, as result of two major antigenic changes: (i) sporadic antigenic shift, which introduces HA subtypes from other species into human viruses through reassortment, causing pandemics among the immunologically naive population; and (ii) antigenic drift, resulting from minor amino acid changes that accumulate unceasingly within subtypes as result of the immune pressure enforced by neutralizing antibodies present in a human population, previously exposed to related viruses (Wright *et al.*, 2013). The analysis of antigenic differences between circulating strains is therefore of the utmost importance for vaccine selection. These differences are usually assessed by haemagglutination inhibition (HI) assays in which post-infection ferret sera are used to inhibit agglutination of red blood cells by homologous or heterologous strains (Hirst, 1943).

We have now developed a novel approach to study antigenic differences between influenza virus HA based on the use of polyclonal antibodies raised in New Zealand rabbits inoculated with vaccinia virus recombinants

One supplementary figure is available with the online version of this paper.

harbouring HA from reference strains, obtained by the method described by Blasco & Moss (1995) and used for immunization as previously described (García-Barreno *et al.*, 2014).

A first example of the results obtained is illustrated in Fig. 1. Purified antibodies from rabbits inoculated with a vaccinia virus recombinant expressing the HA of the A/New Caledonia/20/99 strain (abbreviated, NC99) were tested in ELISA with extracts of cells infected with viruses indicated in Fig. 1(a). The homologous HA and heterologous A/Brisbane/59/07 (abbreviated BR07) HA, both of which belong to the same antigenic pre-pandemic H1N1 subtype, reacted similarly with the α -HA_{NC99} antibodies. In contrast, the HA of the 2009 pandemic A/California/07/09 (CA09) strain was poorly recognized by the same antibodies, i.e. the anti-HA_{NC99} antibodies could discriminate between highly divergent HAs but not between closely

related HAs (for sequence alignment and phylogenetic relationship of the HAs included in Fig. 1., see Fig. S1, available in the online Supplementary Material).

When the α -HA_{NC99} antibodies were adsorbed for 30 min at 20 °C to cells infected for 24 h with NC99 virus, the unadsorbed antibodies lost reactivity with the strains NC99, BR07 and CA09 (Fig. 1b), this was expected for antibodies binding to epitopes of HA_{NC99} expressed at the surface of Madin–Darby canine kidney (MDCK) infected cells. In contrast, adsorption of the α -HA_{NC99} antibodies to cells infected with BR07 abrogated their reactivity with extracts of cells infected with this strain but retained significant reactivity with extracts of cells infected with NC99 (Fig. 1c), i.e. a fraction of the α -HA_{NC99} antibodies are directed against epitopes of the homologous HA which are missing in BR07 HA. Adsorption of the α -HA_{NC99} antibodies to MDCK cells infected with CA09 removed only the small

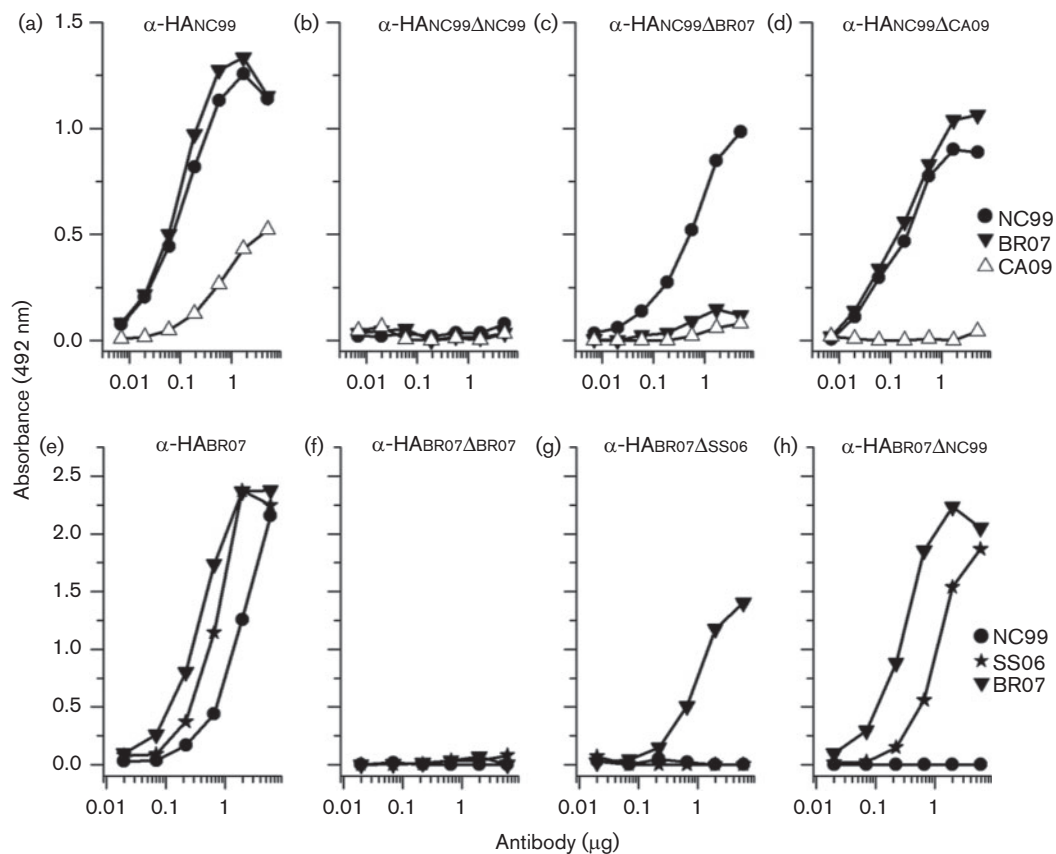


Fig. 1. Reactivity of rabbit anti-HA polyclonal antibodies with extracts of MDCK cells infected with different influenza virus strains. (a) Rabbit α -HA_{NC99} antibodies were purified by protein-A sepharose chromatography (GE Healthcare) and tested in ELISAs as described previously (García-Barreno *et al.*, 2014) with extracts of MDCK cells infected for 24 h with one of the following virus: A/New Caledonia/20/99 (NC99), A/Brisbane/59/07 (BR07) or A/California/07/09 (CA09). (b–d) The purified α -HA_{CA09} antibodies were adsorbed to MDCK cells (1 mg of antibody per 5×10^7 cells) infected with either NC99 (α -HA_{NC99 Δ NC99}), BR07 (α -HA_{NC99 Δ BR07}) or CA09 (α -HA_{NC99 Δ CA09}) viruses and used in ELISAs with the same extracts of (a). (e) Rabbit antibodies specific for the BR09 HA (α -HA_{BR07}) were obtained and tested in ELISA as in (a). (f–h) Purified α -HA_{BR07} antibodies were adsorbed to MDCK cells infected with the viruses indicated in each panel and tested in ELISAs with the indicated cell extracts. The results are representative of at least three independent experiments.

fraction of antibodies that recognized epitopes specific for this strain but the unadsorbed antibodies could not discriminate between the NC99 and BR07 HAs (Fig. 1d). Thus, the adsorption process is able to uncover antigenic differences when the adsorbing and test HAs are highly related (Fig. 1c) but it does not help to discriminate when the adsorbing HA is highly divergent from test HA (Fig. 1d).

Similar results were obtained with antibodies from rabbits immunized with a vaccinia virus recombinant expressing the BR07 HA. This influenza strain represents a more recent isolate of the pre-pandemic H1N1 subtype than A/Solomon Island/3/06 (SS06) and NC99. Again, the purified α -HA_{BR07} antibodies reacted almost identically with extracts of MDCK cells infected with each of the three viruses (Fig. 1e) and lost reactivity after adsorption to cells infected with BR07 (Fig. 1f). Adsorption of α -HA_{BR07} antibodies to cells infected with SS06 uncovered a fraction of antibodies that recognized epitopes exclusive of BR07 (Fig. 1g). Interestingly, adsorption of α -HA_{BR07} antibodies to cells infected with NC99 revealed reactivities with

extracts of cells infected with either of the two other viruses (higher with BR07 than with SS06, Fig. 1h). Thus, another fraction of α -HA_{BR07} antibodies recognized epitopes shared between BR07 and SS06 HAs but absent in NC99. These results suggest that antigenic relatedness between HAs of H1N1 viruses (revealed in ELISA with the rabbit antibodies) decreases as their isolation dates are further apart, this is in agreement with the results of conventional HI tests (WHO, 2009).

To explore further the discriminatory potential of the rabbit α -HA antibodies, we made use of two previously described H1N1 2009–2010 pandemic strains, CA09 and A/Extremadura/6530/10 (EX10). The HAs of these two viruses were undistinguishable by conventional HI tests but reacted differently with four mAbs that recognized overlapping epitopes in the CA09 HA (García-Barreno *et al.*, 2014). Two unique amino acid changes in the EX10 HA were found responsible for the lack of reactivity with the noted mAbs.

Polyclonal α -HA_{CA09} antibodies, previously called α -HACal (García-Barreno *et al.*, 2014), were produced in rabbits as

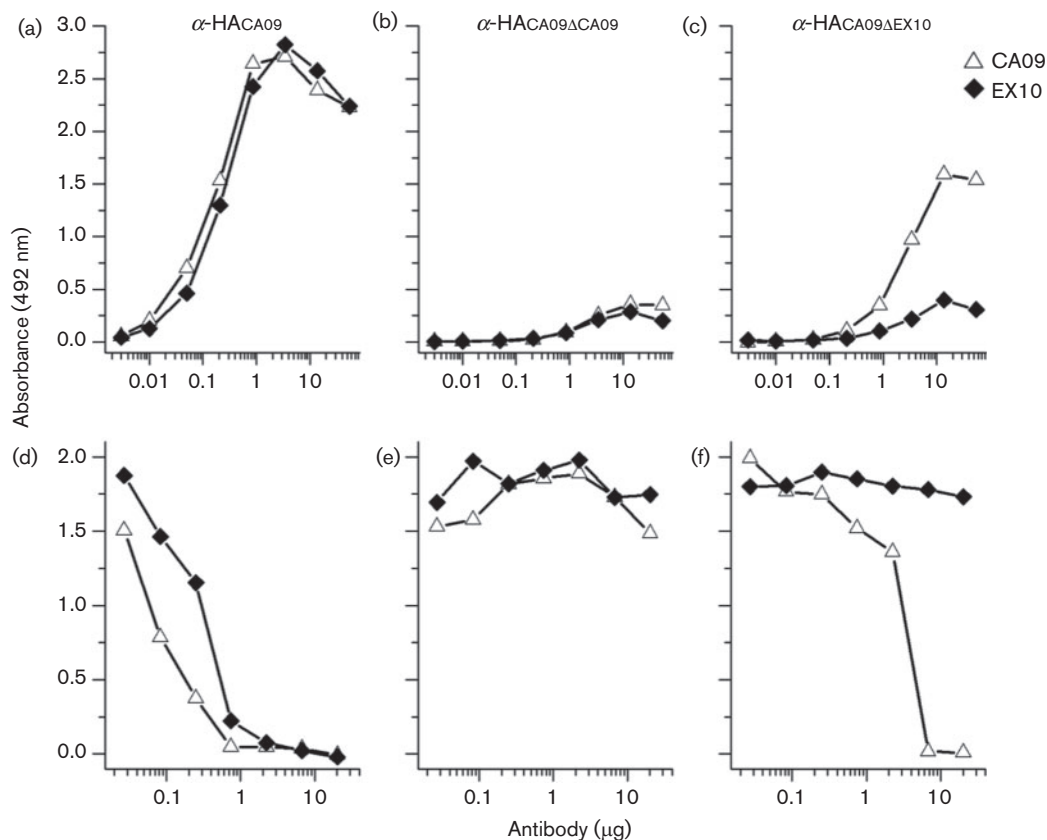


Fig. 2. ELISA binding and virus neutralization with rabbit α -HA_{CA09} antibodies. (a–c) Rabbit α -HA_{CA09} antibodies were tested in ELISA with extracts of MDCK infected with the indicated viruses. The antibodies were used before (a) or after adsorption (b) and (c) to MDCK cells infected with the noted viruses. (d–f) The same antibodies of the upper panels were used in a microneutralization assay as described previously (García-Barreno *et al.*, 2014). The amount of viral antigen produced 72 h post-infection (ordinates - vertical axis) was estimated by ELISA with a pool of α -NP and α -M antibodies as described previously (Sánchez-Fauquier *et al.*, 1987). The results are representative of at least three independent experiments.

before. These antibodies reacted to the same extent with the CA09 and EX10 HAs present in infected cell extracts (Fig. 2a). Adsorption of the α -HA_{CA09} antibodies to cells infected with CA09 abrogated the reactivity with extracts of cells infected with either CA09 or EX10 (Fig. 2b). In contrast, adsorption of α -HA_{CA09} antibodies to cells infected with EX10 abrogated their reactivity with EX10 HA but still retained significant reactivity with CA09 HA (Fig. 2c), highlighting the importance of the adsorption step to discriminate between highly related HAs.

The neutralizing capacity of the α -HA_{CA09} antibodies before and after adsorption to infected cells was evaluated in a microneutralization assay (García-Barreno *et al.*, 2014). The infectivity of CA09 and EX10 viruses was efficiently inhibited by the purified α -HA_{CA09} antibodies, reflected in reduced virus antigen production, as measured by ELISA (Fig. 2d). In full agreement with the ELISA binding results, the ability of α -HA_{CA09} antibodies to neutralize CA09 and

EX10 was completely lost after adsorption to MDCK cells infected with CA09 (Fig. 2e). In contrast, adsorption to MDCK cells infected with EX10 eliminated neutralization of this virus by α -HA_{CA09} antibodies but reduced only partially their capacity to neutralize CA09 (Fig. 2f), i.e. some α -HA_{CA09} antibodies that recognized epitopes missing in EX10 were still capable of binding to CA09 HA and neutralize CA09 infectivity.

We have previously identified an antigenic site in the globular head of the CA09 HA where the unique amino acid changes of EX10 were located (García-Barreno *et al.*, 2014). To test if the polyclonal α -HA_{CA09} antibodies could select escape mutants with changes in the noted antigenic site, serial dilutions of CA09 virus were plaqued with or without an excess of the α -HA_{CA09} antibodies, depleted of those binding to EX10 HA (α -HA_{CA09 Δ EX10}). The virus titre was reduced about five logs by the antibodies (Fig. 3a, CA09 row), in agreement with the microneutralization

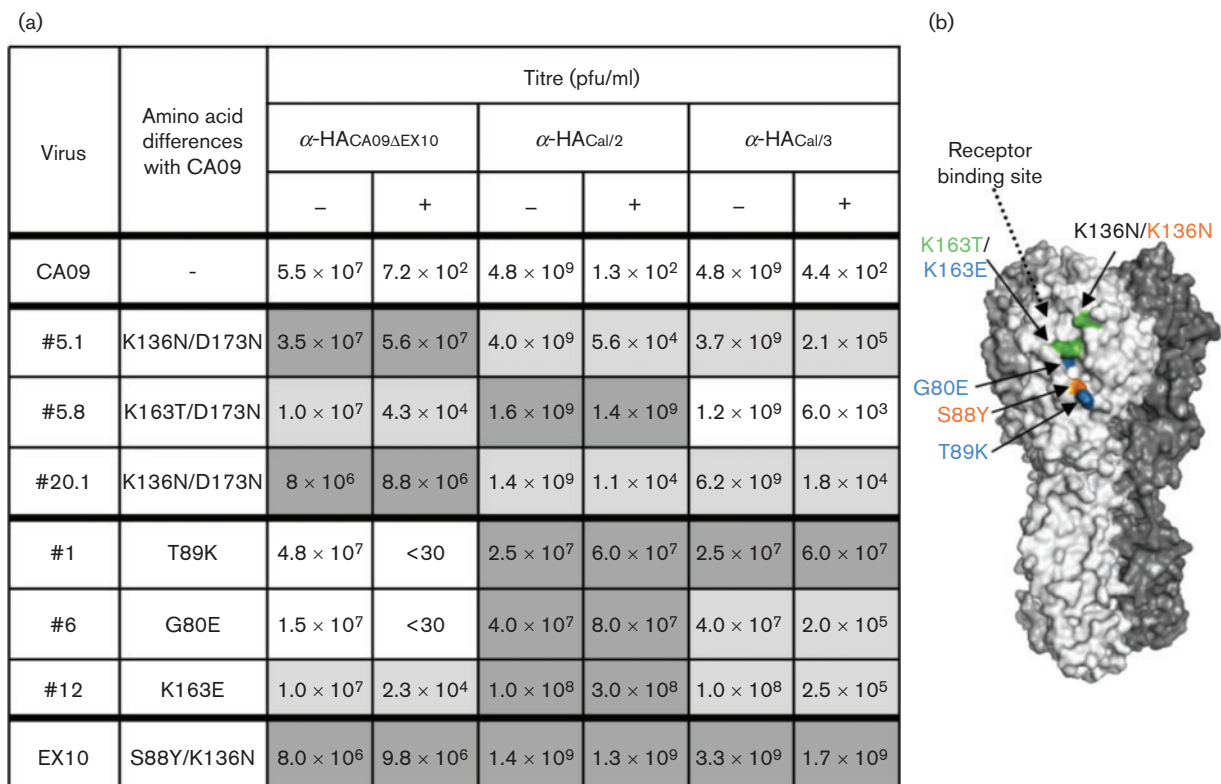


Fig. 3. Selection and characterization of escape mutants. (a) Viruses were titrated in plaque assays either with or without the denoted antibodies. The viruses #5.1, #5.8 and #20.1 were recovered from individual plaques of CA09 grown in the presence of α -HA_{CA09 Δ EX10} antibodies. All other viruses and mAbs were previously described (García-Barreno *et al.*, 2014). mAbs α -HA_{Cal/2} and α -HA_{Cal/3} derived from two independent hybridomas but recognize overlapping epitopes in the CA09 HA. Viruses #1, #6 and #12 are escape mutants of CA09 selected with mAb α -HA_{Cal/2} (García-Barreno *et al.*, 2014). Note that titre differences of the same virus plaque without antibodies are due to different stocks used in successive experiments. Dark grey boxes indicate antibody resistance, light grey boxes partial resistance and white boxes no resistance to antibodies. (b) Three-dimensional structure of the HA trimer (Zhang *et al.*, 2010). The three subunits are highlighted in different shades of grey. Green, changes in mutants resistant to α -HA_{CA09 Δ EX10} antibodies. Blue, changes in mutants resistant to mAb α -HA_{Cal/2}. Orange, changes unique in EX10 in comparison with CA09.

results (Fig. 2f). Virus stocks were recovered from several plaques grown in the presence of the antibodies and their resistance to neutralization tested by plaque titration. Two of these viruses, named #5.1 and #20.1 were completely resistant to neutralization, i.e. no reduction of virus titre was seen with the antibodies (Fig. 3a). The third virus, #5.8 was only partially resistant to the polyclonal antibodies (Fig. 3a); in this case, the virus titre was reduced less than three logs, compared with five logs observed for the wt (compare rows CA09 and #5.8). The sequence of the HA gene revealed that the three mutants have an amino acid substitution D173N which is commonly found in viruses related to CA09 and even in viruses recovered from individual plaques that turned out not to be resistant to the antibodies. Thus, it is unlikely that D173N contributes to resistance to neutralization through antibodies, although it may contribute to virus fitness, but this has not been explored further. Mutants #5.1 and #20.1 contained an additional amino acid change (K136N) and the mutant #5.8 contained a K163T substitution. The location of these changes in the HA three-dimensional structure (Fig. 3b) showed a clear overlap with the site where both the amino acid changes unique to EX10 and the mutations selected in viruses that escaped neutralization by mAb α -HA_{Cal/2} were previously mapped (García-Barreno *et al.*, 2014).

To gain a more complete picture of the effect of mutations on resistance to neutralization by polyclonal and monoclonal antibodies, plaque titrations were extended to viruses and antibodies used in our previous study (García-Barreno *et al.*, 2014). Two of the escape mutants selected with the murine mAb α -HA_{Cal/2} (#1 and #6, Fig. 3a) were fully neutralized by the α -HA_{CA09 Δ EX10} antibodies, whereas the third mutant (#12) was partially resistant. Interestingly, this mutant has an amino acid substitution (K163E) at the same position as mutant #5.8 (K163T), selected with the polyclonal antibodies.

Mutants #5.1 and #20.1 shared the amino acid change K136N with EX10 virus and the three viruses were fully resistant to the α -HA_{CA09 Δ EX10} antibodies. However, #5.1 and #20.1 viruses were partially neutralized by the α -HA_{Cal/2} and α -HA_{Cal/3} mAbs, whereas EX10 was fully resistant to these antibodies, in agreement with the previously described synergistic effect of S88Y and K136N changes in abrogating reactivity with the noted mAbs. In summary, although the mutations selected with the α -HA_{CA09 Δ EX10} antibodies and the α -HA_{Cal/2} mAb are closely located in the HA molecule, and seemingly are part of the same antigenic site, individual mutations have subtle different effects upon neutralization by those antibodies. It is worth stressing that the antigenic site characterized in this study is relevant for the human antibody response, since human post-infection sera competed for binding of α -HA_{Cal/2} to CA09 HA (García-Barreno *et al.*, 2014).

Recently, Smith *et al.* (2013) and Koel *et al.* (2004) performed an in-depth analysis of the antigenic and genetic changes that drove human influenza HA evolution,

particularly of the H3 subtype but also of the H1 subtype and influenza type B. Their main conclusion is that viruses could be grouped into antigenic clusters (rather than forming a continuous antigenic lineage) based on reactivity with post-infection ferret sera in the HI test. These clusters correlate with genetic lineages of a phylogenetic tree built with HA sequences. Sequence differences between H3 antigenic clusters normally involved several amino acid substitutions (Smith *et al.*, 2004) in agreement with a later study by Shih *et al.* (2007). However, when the effect of individual amino acid differences on HA antigenicity was tested, only a limited subset of changes adjacent to the receptor binding site were found as the main contributors to antigenic differences between clusters (Koel *et al.*, 2013).

Although the number of H1 viruses analysed by Koel *et al.* (2013) was much more limited than the number of H3 viruses, they found that the substitution K140E (residue 157 by our numbering) was sufficient to induce the antigenic transition from NC99 to SS06-like viruses. Intriguingly, residue 157 is relatively close in the HA structure to the K163T and K136N mutations selected in our study with the α -HA_{CA09 Δ EX10} antibodies. Conceding that our data are still very limited, it would be interesting to expand these data to other influenza viruses and subtypes to judge the real potential of our approach for studies of HA antigenic evolution.

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