Phylogeography and antigenic diversity of low pathogenic avian influenza H13 and H16 viruses

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Abstract

Low pathogenic avian influenza viruses (LPAIVs) are genetically highly variable and have diversified into multiple evolutionary lineages that are primarily associated with wild bird reservoirs. Antigenic variation has been described for mammalian influenza viruses and for highly pathogenic avian influenza viruses that circulate in poultry, but much less is known about antigenic variation of LPAIVs. In this study, we focussed on H13 and H16 LPAIVs that circulate globally in gulls. We investigated the evolutionary history and intercontinental gene flow based on the hemagglutinin (HA) gene and used representative viruses from genetically distinct lineages to determine their antigenic properties by hemagglutination inhibition assays. For H13 at least three distinct genetic clades were evident, while for H16 at least two distinct genetic clades were evident. Twenty and ten events of intercontinental gene flow were identified for H13 and for H16 viruses, respectively. At least two antigenic variants of H13 and at least one antigenic variant of H16 were identified. Amino acid positions in the HA protein that may be involved in the antigenic variation were inferred, and some of the positions were located near the receptor binding site of the HA protein, as they are in the HA protein of mammalian influenza A viruses. These findings suggest independent circulation of H13 and H16 subtypes in gull populations as antigenic patterns do not overlap and contribute to the understanding of the genetic and antigenic variation of LPAIV naturally circulating in wild birds.

Importance

Wild birds play a major role in the epidemiology of low pathogenic avian influenza viruses (LPAIVs) from which these viruses are occasionally transmitted—directly or indirectly—to other species, including domestic animals, wild mammals and humans, where they can cause

subclinical to fatal disease. Despite a multitude of genetic studies, the antigenic variation of LPAIVs in wild birds is poorly understood. Here, we investigated the evolutionary history, intercontinental gene flow, and the antigenic variation among H13 and H16 LPAIVs. The circulation of the subtypes H13 and H16 seems to be maintained by a narrower host range, in particular gulls, than for the majority of LPAIV subtypes and may therefore serve as a model for evolution and epidemiology of H1-H12 LPAIVs in wild birds. The findings suggest that H13 and H16 LPAIVs circulate independently of each other and emphasize the need to investigate within clade antigenic variation of LPAIVs in wild birds.

Keywords: avian viruses, influenza, evolution, epidemiology, ecology, antigenic variation, seabird

Introduction

Wild birds of the orders Anseriformes (mainly ducks, geese and swans) and Charadriiformes (mainly gulls, terns and waders) play a major role in the epidemiology of low pathogenic avian influenza viruses (LPAIVs). LPAIVs are characterized into subtypes based on their surface proteins hemagglutinin (HA, H1-H16) and neuraminidase (NA, N1-N9), e.g. H13N6. Ducks play an important role in the epidemiology of most LPAIV subtypes. However, birds of the order Charadriiformes—in particular gulls— are the major reservoir for subtypes H13 and H16 (Table S1) (1-4). High prevalence of H13 and/or H16 LPAIVs has been observed in juvenile gulls at breeding colony sites (5-7) and in adults during spring and/or fall migration (8, 9). H13 and H16 viruses have a global distribution. Since first detection in 1977, H13 viruses have been detected in North America, South America, Europe, Asia, Africa and Oceania. Since their first detection in 1975, H16 viruses have been detected in North America, South America, Europe and Asia. The spatial isolation of host populations has shaped LPAIV evolution and led to the independent circulation of different virus gene pools between Western and Eastern hemispheres (10). Yet, some pelagic gull populations connect multiple continents through seasonal migration and overlapping distributions and could facilitate rapid and long-distance dispersal of LPAIV genomes (2, 9, 11-14). For instance, great black-backed gulls (*Larus marinus*) migrate between Europe and the east coast of North America, and LPAIVs consisting of both North American as well as Eurasian genes have been isolated from this species (12). Upon intercontinental gene flow, i.e. the movement of genes between the different continents, some LPAIV genes seem to have become established in the population, e.g. H6 (15).

Influenza A viruses (IAV) belong to the family Orthomyxoviridae and are negative sense single-stranded RNA viruses with a segmented genome. The genome consists of eight

segments encoding 12 proteins or more, including the surface proteins HA and NA. The HA protein of IAV is a major determinant for virus binding to cells and subsequent cell entry and for generation of IAV-specific antibodies, and thus subjected to strong selective pressure (16). Indeed, in wild birds—in particular mallards (*Anas platyrhynchos*)—LPAIV infection dynamics seem to be shaped between LPAIV subtypes partially by pre-existing homo- or heterologous antibodies (17). Furthermore, within other host systems, evasion of IAV-specific antibodies by IAVs—so called antigenic variation—has been described for seasonal human IAVs (18, 19), swine IAVs (20-22), equine IAVs (23) and for highly pathogenic avian influenza viruses (HPAIVs) that circulate in poultry (24, 25). Despite numerous studies on the genetic variation of LPAIVs in wild birds, the antigenic variation within LPAIV subtypes that circulate in wild birds is barely investigated (26, 27).

To better understand LPAIV epidemiology in gulls, we investigated the global distribution of H13 and H16 LPAIVs and the antigenic variation of a representative subset of H13 and H16 LPAIVs. Based on the sequencing of HA genes of 84 viruses, and hemagglutination inhibition assays, we showed that intercontinental H13 and H16 gene flow occurred frequently, and that H16 genetic lineages did not form antigenic clusters, suggesting that clade-defining mutations were not in critical epitopes (i.e. part of the antigen that binds to specific antibodies). In contrast, the H13 genetic clades partially corresponded with the antigenic variation of H13 LPAIVs, suggesting part of the clade-defining mutations were in critical epitopes.

Results

Phylogeographic structure and intercontinental gene flow

Phylogenetic analyses supported that the H13 HA was structured in three major genetic lineages (A-C; Figure 1, S1 and S2). The time to the most recent common ancestor (tMRCA) of the H13 HA gene was dated in 1927 (± 95% HPD (highest posterior density): [1920-1934]). The tMRCA of viruses of clade A (1963 [1958-1966]) was older than the ones of clade B (1975 [1974-1976]) and C (1977 [1976-1978]). Our analyses support that the geographic origin of H13 viruses of clade B and C could be North America and Europe, respectively (posterior probabilities for the geographic origin of the most recent common ancestor [MRCA]: 1 for clade B and 1 for clade C). For clade A, limited historical data of viruses from different locations as well as low posterior probability (0.62) precludes a conclusion on the geographic origin of the MRCA.

Since the first isolation of an H13 IAV from a gull in 1977, 20 potential events of intercontinental gene flow were identified (indicated with 1-20 in Figure 1, S3 and Table 2). Clade A supports the maintenance of H13 in European gulls, with evidence of multiple introductions to North America and Asia (events #3, #5, #6, #7, and #10), and a reverse introduction from North America to Asia (event #8). Clade C was also composed mainly of viruses circulating in Europe, with evidence of multiple introductions to North America (events #12, #15, #19) and Asia (events #13, #16, #17). The introduction of clade C H13 HA in North America (event #19) was followed by an introduction to South America (event #20). Evidence for intercontinental gene flow among North American H13 IAV occurred among eastern and western North American isolates (event #3, #12, #15 and #19). Clade B was composed almost exclusively of viruses circulating in North America, although one gene flow event to South America occurred recently (event #11).

The H16 HA was structured in at least two major genetic lineages (Figure 2, S4 and S5). The MCC tree was structured in three main clades (A-C, Figure S5), while the ML tree provided support for only two main genetic clades (A and B/C merged, Figure S4). The

tMRCA of the H16 HA gene was dated in 1924 [1914-1932]. Clade A included only viruses from Europe and was dated in 1977 [1975-1980]; clade B included only viruses from North America with a time to the tMRCA estimated in 1969 [1967-1971]. Our analyses supported that the geographic origin of clade A and B was Europe and North America, respectively (posterior probabilities for the geographic origin of the MRCA: 0.99 for clade A, 1 for clade B). The tMRCA of clade C was estimated 1965 [1962-1968]. Clade C may have arisen in Europe (posterior probabilities for the geographic origin of the MRCA: 0.87) and consisted of viruses of mixed origin, *i.e*. Europe, Asia and North America.

Since the first isolation of an H16 IAV from a black-legged kittiwake (*Rissa tridactyla*) in 1975, ten intercontinental gene flow events were identified for viruses of clade C (indicated with 1-10 in Figure 2, S6 and Table 3). As for the H13 subtype, strong support for gene flow between Europe and North America was found, in particular from North-Western European countries: Denmark to North-eastern America (Delaware, New Hampshire, Quebec), and Iceland to Newfoundland (events #6 and #10). Evidence for intercontinental gene flow among North American H16 IAV occurred among eastern and western North American isolates (event #3, #6, #8 and #10). In particular, intercontinental gene flow #8 seems to have been maintained in North America after its initial introduction in 2006 [2005-2006], for at least ten years, and may have replaced clade B of H16 HA (Figure 2).

High rates of nucleotide substitution obtained for the H13 HA genetic lineages were consistent with those previously reported for H4, H6 and H7 subtypes circulating in wild ducks (Table 4). However, the nucleotide substitution rate of clade B—that consists exclusively of North American IAV—was lower than mean rates and HPD obtained for the other two H13 clades. The mean d_N/d_S rate obtained for the three H13 genetic clades were comparable to those previously reported for other subtypes and suggests that the HA was under strong purifying selection (Table 4). Nonetheless, a slightly higher d_N/d_S rate obtained

for clade B and C as compared to other lineages suggests that they may be subjected to a more neutral selection. The mean nucleotide substitution and d_N/d_S rates for the H16 gene were also consistent with H13 HA as well as with H4, H6 and H7 subtypes from wild ducks. However, H16 clade C (European mixed)– that consisted of viruses of a geographically more mixed origin – had slightly lower nucleotide substitution rates and higher d_N/d_S rates than clade A (European) and clade B (North American) (Table 4).

Antigenic diversity between H13 and H16 LPAIV

As expected from two different HA subtypes, the H13 and H16 viruses formed two separate antigenic variants. The H13 and H16 viruses were generally well separated, forming groups on opposite sides of the antigenic map (Figure 3, Table 5). A total of nine amino acid positions within/near the receptor binding site of the HA were identified that differed consistently between H13 and H16 viruses (based on alignments of 338 H13 and 192 H16 HA indicated in Table 6), of those, amino acid position 145 was located in the 130-loop, 200 and 208 in the 190-helix and 231 and 233 in the 220-loop of the receptor binding site of the HA (HA numbering based on (28, 29). Of those, amino acid position 233 was listed previously as being involved in differences in receptor-binding site between HAs originating from *Laridae* and *Anatidae* (30). Additionally, the amino acid at position 196 differed between H13 (valine [V]) and H16 (aspartic acid [D]) viruses; this position may contribute to receptor binding specificity as identified previously based on crystal structures of H5 and H13 LPAIV (31). Due to non-specific cross-reactivity, two H13 viruses (i.e. HEGU/AK/458/85 and HEGU/AK/479/85) had unexpected high titers against H16 antisera (Table 5) and were therefore positioned in the center of the map and served to pull H13 and H16 together.

187 ***Antigenic diversity among H13 LPAIV***

188

189 The representative H13 viruses formed at least two different antigenic variants (Figure 3,
190 Table 5). The viruses of H13 clades A and B were genetically distinct (Figure 1) but were
191 antigenically similar (Figure 3), based on the H13 clade A antisera cross-reacting with H13
192 clade B viruses and vice versa. In contrast, H13 clade C viruses reacted poorly—if at all—
193 with antisera that were raised against clade A and B viruses, and, conversely, antisera against
194 clade C viruses rarely reacted with substantial titers with viruses of clade A and B. Thus, H13
195 clade A/B and H13 clade C viruses formed two different antigenic variants. The antigenic
196 diversity of H13 clade A/B combined is about the same as the antigenic diversity of the H13
197 clade C. One H13 clade B virus, i.e. LAGU/DB/1370/86, could not be placed well in the map
198 due to HI titers of 40 or lower (Table 5).

199 To gain insight into the molecular basis of the antigenic variation between H13 clade
200 A/B and C, amino acids that differed consistently among the different clades of H13 viruses
201 were indicated (based on the alignment of 338 H13, Table 6). A total of four amino acid
202 positions within/near the receptor binding site of the HA were identified that differed
203 consistently for clade A, B and/or C. Of those, amino acids at positions 149 and 254 differed
204 consistently between clade A/B and C. Viruses belonging to clade C—except a single virus
205 from South America that had a arginine (R) at position 149—had a deletion at position 149
206 (previously identified using a smaller dataset as position 154 (12)), in contrast to viruses of
207 clade A or B that had an aspartic acid (D), glutamic acid (E), asparagine (N) or serine (S) at
208 this position. The correlation between the antigenic distance of H13 representative viruses
209 from A/gull/MD/704/1977 (H13N6) (clade A)—the first detected H13 virus—and the number
210 of HA1 amino acid substitutions from A/gull/MD/704/1977 was 0.87 and was statistically
211 significant ($P < 0.0001$, Pearson correlation).

212

213 ***Antigenic diversity among H16 LPAIV***

214

215 The representative H16 viruses formed at least one antigenic variant (Figure 3 and Table 5).
216 The genetically distinct H16 clades A, B and C did not form separate antigenic clusters in the
217 map, which reflects the raw HI data as there are no patterns for any of the four H16 antisera
218 tested that correspond to the genetic lineages. The antigenic diversity of the H16 viruses is
219 within eight antigenic units, with BHGU/NL/1/07 being on the edge of this antigenic space
220 (i.e. low titers to all sera). The antigenic diversity of H16 clade A/B/C is about the same as the
221 antigenic diversity of the H13 clade A/B combined and similar to the antigenic diversity of
222 the H13 clade C.
223 Though clade A, B and C did not form separate antigenic clusters in our analysis, amino acids
224 that differed consistently among the different clades of H16 viruses were indicated (based on
225 the alignment of 192 H16 HA, Table 6). A total of three amino acid positions within/near the
226 receptor binding site of the HA were identified that differed consistently among the three H16
227 clades and were not associated with antigenic variation. The correlation between the antigenic
228 distance of the representative viruses from A/Black-headed gull/TM/13/76 (H16N3) (clade
229 C)—one of the first detected H16 viruses—and the number of HA1 amino acid substitutions
230 from A/Black-headed gull/TM/13/76 was 0.67 and was statistically significant (P = 0.003,
231 Pearson correlation).

232

233 **Discussion**

234

235 We investigated the evolutionary history and intercontinental gene flow based on the
236 hemagglutinin (HA) gene of H13 and H16 LPAIV and selected representative viruses from

237 genetically distinct lineages to determine their antigenic properties by HI assays. H13 formed
238 at least three distinct genetic clades as suggested previously based on smaller datasets (9, 32-
239 35), while H16 formed at least two distinct genetic clades. Twenty and ten events of
240 intercontinental gene flow were identified for H13 and for H16 viruses, respectively. At least
241 two antigenic variants of H13 and at least one antigenic variant of H16 were identified. The
242 presence of different antigenic variants among viruses of a single LPAIV subtype is in
243 contrast to previous findings based on antigenic characterization of LPAIV H3 (26), and
244 implies that antigenic variation within LPAIV subtypes occurs.

245 The frequency of intercontinental gene flow of the HA gene of H13 and H16 viruses
246 was similar to the HA gene of H6 viruses, but lower than for internal genes (2, 27, 36, 37).
247 Previously, intercontinental gene flow has been described extensively for the H6 HA genes,
248 while no intercontinental gene flow was detected for the H4 and H7 subtypes (15, 38). For the
249 H6 subtype, gene flow has been described ten times with four established genes during a
250 period of 31 years (1975-2006; (15)). Also, evidence for intercontinental gene flow among
251 North American H13 and H16 genes occurred among eastern and western North American
252 LPAIVs in contrast to eastern North American LPAIVs only as reported previously (39).
253 Given the relatively high number of intercontinental flow of IAV internal genes by shorebirds
254 and gulls (2, 27, 36, 37), one may have expected a higher gene flow of gull-associated H13
255 and H16 HA genes, compared to e.g. H6. However, a higher intercontinental gene flow only
256 was apparent with H13 (i.e. 20 events during a period of 35 years). This may suggest i)
257 broader host range, host population size and/or host distribution of H13 than H16, and/or ii)
258 local H13-specific herd-immunity is lower than H16-specific herd immunity and therefore
259 less limiting establishment opportunities in host populations of H13, and/or iii) higher
260 environmental survival of H13 than of H16, and/or iv) introduced H13 HA genes may be less
261 affected by strong subtype-dependant competition with endemic HA genes (e.g. with respect

to linkage to NS1 and NP as these contain most gull-specific features (33)) than introduced H16 genes. Interestingly, no H13 or H16 gene flow was described from Asia to Europe, which is in contrast to e.g. HPAIV H5 viruses that have been introduced from Asia to Europe several times (40, 41). The relatively low frequency of detection of intercontinental gene flow of H13 or H16 genes out of North America and in particular Asia, relative to Europe, may be due to a bias in IAV surveillance and sequencing (i.e. number of available IAV sequences from gulls isolated in Europe is higher than from North America and in particular Asia).

Antigenic diversity of LPAIV depends partially on the host population size and structure. In this study, both H13 and H16 LPAIV formed at least three or two distinct genetic clades respectively that did not or only partially corresponded with antigenic clusters. The H16 genetic clades did not form antigenic clusters, suggesting that clade-defining mutations were not in critical epitopes. In contrast, the H13 genetic clades partially corresponded with the antigenic variation of H13 LPAIV, suggesting that part of the clade-defining mutations were in critical epitopes. Also, given that the H13 antigenic space is larger than the antigenic space covered by H16 viruses, the host population of H13 may be larger and more widely distributed than the host population of H16 LPAIV, facilitating the circulation of more than one antigenic variant of a single LPAIV subtype. Strong genetic and antigenic divergence between two co-circulating lineages could be the product of a very large host meta-population size and relatively rare cross-species transmission rate (42). Globally, viruses of the H13 subtype seem to be more common than viruses of the H16 subtype (2, 4), which is consistent with the finding that H13 LPAIV consists of multiple antigenic variants. Besides increased host population size and host distribution, prolonged virus survival may shape LPAIV epidemiology and evolution. Antigenic diversity within H13 LPAIV may be shaped by amino acid substitutions near the receptor binding site of the HA protein. In this study, we found evidence that amino acids or deletions at positions 149 and 254 of the HA protein may be

involved in antigenic diversity among H13 strains. In addition, position 149 could be involved in H16 LPAIV antigenic diversity as all H16 viruses had a deletion at this position and H16 clade A, B and C were antigenically similar.

Co-circulating and newly introduced H13 or H16 LPAIV can be either antigenically similar or antigenically different. In the Northern hemisphere, H13 and H16 IAV subtypes circulate most extensively on breeding colonies in hatch-year birds at the end of summer and early fall (5-7). In black-headed gulls (which in Europe are one of the main host for H13 and H16 LPAIV), infection with H13 or H16 result in strong protection against reinfection with the same virus, however susceptibility to infection with the other subtype or with another strain of the same subtype is unknown (43, 44). Our findings support the independent long-term maintenance and co-circulation of at least two genetically distinct lineages of H13 and of H16 in Eurasia. This pattern is similar to the one that has been described for the H3 IAV subtype in ducks in North America (42). Our analysis showed that these genetically distinct co-circulating lineages may belong to the same antigenic variant. Here, we found evidence that genetically distinct co-circulating H13 or H16 LPAIV on a black-headed gull breeding colony site in the Netherlands may be either antigenically different (e.g. H13 clade A virus A/BHGU/NL/7/2009 (H13N2) and H13 clade C virus A/BHGU/NL/20/2009 (H13N2) or antigenically similar (e.g. H16 clade A A/BHGU/NL/10/2009 (H16N3) and A/BHGU/NL/21/2009 (H16N3) and H16 clade C A/BHGU/NL/26/2009 (H16N3). Similar, intercontinental gene flow occurred with HA genes that were antigenically similar to local circulating viruses (i.e. H16 clade C viruses that were genetically closely related to SB/DB/172/06 and SB/DB/195/06 versus local circulating H16 clade B viruses), and HA genes that were antigenically different from local circulating viruses (i.e. H13 clade C viruses, genetically closely related to LAGU/NJ/AI08-0714/08 versus local circulating H13 clade B viruses.

312 Antigenic variation within a LPAIV subtype at the clade level (i.e. H13 clade A/B
313 combined versus H13 clade C) was described here, yet less is known about antigenic variation
314 within genetic clades of H13, H16 or other LPAIV subtypes. For H13, genetic diversity
315 within clades seemed stable—e.g. viruses of clade A, B or C, collected over three decades
316 were antigenically closely related—suggesting no major genetic differences; this is in contrast
317 to the few mutations needed for antigenic change in seasonal human IAV. Similarly, a study
318 on antigenic variation of H3 LPAIV isolated in North America suggested that genetically
319 diverse viruses were antigenically stable (26). Major antigenic changes in seasonal human
320 IAV were due to amino acid substitutions immediately adjacent to the receptor binding site
321 (18); this could potentially also explain antigenic variation between antigenically different
322 viruses of H13 clade A/B combined and clade C (i.e. amino acid positions 149 of the HA).
323 Future work on antigenic variation of LPAIV should include within clade genetic and
324 antigenic variation.
325
326 **Materials and Methods**
327
328 **Viruses.** The HA sequences of H13 (n=64) and H16 (n=20) viruses isolated from wild birds
329 in North America (n=39 and n=5, respectively) and Europe (n=25 and n=15, respectively)
330 between 1976 and 2010 were determined at the University of Minnesota (Saint Paul,
331 Minnesota, USA) and at the Department of Viroscience of the Erasmus Medical Center
332 (Rotterdam, the Netherlands). Details on virus isolates including GenBank accession numbers
333 are summarized in Table S2 and S3; details related to the Sanger sequencing methodology are
334 available upon request. The HA sequences were supplemented with full-length nucleotide
335 sequences of the HA gene of H13 and H16 viruses isolated from wild birds between 1975 and
336 2017 and downloaded from GenBank (https://www.ncbi.nlm.nih.gov). The full dataset

included sequences of H13 (n=519) and H16 (n=276) HA genes and was biased towards virus strains collected since 2000 due to increased surveillance and sequencing since 2000. Of this full dataset, viruses representing the genetically distinct clades were selected (n=44; H13 clade A, B, C and H16 clade A, B, C; see the Results section for clade definition) to investigate the antigenic diversity of H13 and H16 viruses. Of those viruses, viruses that were genetically most divergent were selected (n=10) to generate ferret antisera (Table 1). The antigenic properties of all representative viruses (n=44) were analysed in hemagglutination inhibition (HI) assays using the panel of ten ferret antisera.

Genetic analyses. The nucleotide sequences of the coding region of H13 and H16 HA were aligned with the program CLC 8.0 (CLC bio, Aarhus, Denmark). Neighbor-Joining trees were then generated, with 1000 bootstraps, in order to assess the overall genetic structure of the H13 (n=519) and H16 (n=276) HA sequences. To lower the bias in species and geography (e.g. black-headed gulls (*Chroicocephalus ridibundus*) from the Netherlands and glaucous-winged gulls (*Larus glaucescens*) from Alaska), duplicate sequences (i.e. identical sequences of the same host species, location and date) were identified with Mothur 1.39.5 (45) and removed, resulting in final alignments of H13 (n=338) and H16 (n=192) HA. To identify the genetic structure of H13 and H16 virus subtypes Maximum-likelihood trees with 1000 bootstraps were generated with the software PhyML 3.1 (46). The general time reversible (GTR) evolutionary model, an estimation of the proportion of invariable sites (I) and of the nucleotide heterogeneity of substitution rate (α) was used as selected by Model Generator 0.85 (47). To investigate the evolutionary history of H13 and H16 virus subtypes Bayesian Markov Chain Monte Carlo coalescent analyses were performed. The temporal structure of the dataset was assessed with the program TempEst 1.5.3 (48). Both datasets showed a positive correlation between genetic divergence and sampling time and appear to be suitable

for phylogenetic molecular clock analyses. Time to the most recent common ancestors (MRCA) as well as geographic ancestral states (i.e. continent), and their associated posterior probabilities were obtained based on the method described by Lemey et al. with the program BEAST 1.10.1 (49, 50). A strict molecular clock model was selected as relaxed clock models (uncorrelated exponential and uncorrelated lognormal) resulted in low effective sample sizes (ESS < 200) in spite of high chain length (>200 million states). In all simulations a Bayesian skyline coalescent tree prior (51) was selected. The Shapiro-Rambaut-Drummond-2006 nucleotide substitution model was selected (52), and has been used in population dynamic studies of other IAV subtypes (15, 38, 42, 53). Overall, a similar methodology was used as in previous studies on IAV evolutionary dynamics of subtypes H4, H6 and H7 (15, 38, 54). Analyses were performed with two independent chain lengths of 100 million generations sampled every 1000 iterations; the first 10% of trees were discarded as burn-in. Substitutions rates based on independent analyses of the major H13 and H16 clades were obtained using the program BEAST 1.10.1. Nonsynonymous substitutions (d_N) and synonymous substitutions (d_S) rates were obtained using the single likelihood ancestor counting method implemented in HyPhy (55). Computations were performed with the Datamonkey webserver (56, 57).

Antisera. Post-infection antisera were prepared upon nasal inoculation of ferrets (> 1 year of age, male, two ferrets per virus) with virus (cultured on embryonated chicken eggs, per ferret 10^6 - 10^7 median egg infectious dose (EID_{50})/100 µl) and blood collection by exsanguination 14 days later. An overview of antisera used in this study is provided in Table 1. Antisera were pre-treated overnight at 37ºC with receptor-destroying enzyme (*Vibrio cholerae* neuraminidase), followed by inactivation for 1 hr at 56ºC before use in HI assays.

Antigenic analyses. HI assays were performed according to standard procedures (58). The HI

titer is expressed as the reciprocal value of the highest serum dilution that completely inhibited hemagglutination. To investigate antigenic variation among and within H13 and H16 viruses, antigenic cartography methods were used as described previously (19). Briefly, antigenic cartography is a method to analyse and visualize HI assay data. The titers in an HI table can be thought of as specifying target distances between antigens and antisera. In an antigenic map, the distance between antigen point A and antiserum point S corresponds to the difference between the $\log_2$ value of the maximum observed titer to antiserum S from any antigen and the titer of antigen A to antiserum S. Modified multidimensional scaling methods are used to arrange the antiserum and antigen points in an antigenic map to best satisfy the target distances specified by the HI data (18). Because antigens are tested against multiple antisera, and antisera are tested against multiple antigens, many measurements can be used to determine the position of the antigens and antisera in an antigenic map, thus improving the resolution of the HI data.

Ethics statement. This study was approved by the independent animal experimentation ethical review committee Stichting DEC consult (Erasmus MC permit 122-98-01, 122-08-04 and 15-340-03) and was performed under animal biosafety level 2 (ABSL-2) conditions. Animal welfare was monitored daily, and all animal handling was performed under light anesthesia (ketamine) to minimize animal discomfort.

Data availability. Sequences are available in GenBank under accession numbers KF612922 to KF612965, KR087564, KR087572, KR087577 to KR087595, KR087597 to KR087601, KR087604 to KR087615, and MK027211 and MK027212.

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Figure legends

Figure 1. Maximum clade credibility (MCC) trees for influenza A virus H13 hemagglutinin subtype (n= 338). Branches were colored according to most probable geographic origin (red: North America; orange: South America; dark blue: Europe; light blue: Asia; green: Oceania; gray: not identified). Black node bars represent the 95% highest posterior densities for times of the common ancestors. Numbers highlight intercontinental gene flow events as detailed in Table 2 and Figure S3. Virus strain names and posterior probabilities are detailed in Figure

S2.

Figure 2. Maximum clade credibility (MCC) trees for influenza A virus H16 hemagglutinin subtype (n=192). Branches were colored according to most probable geographic origin (red: North America; orange: South America; dark blue: Europe; light blue: Asia; green: Oceania; gray: not identified). Black node bars represent the 95% highest posterior densities for times of the common ancestors. Numbers highlight intercontinental gene flow events as detailed in Table 3 and Figure S6. Virus strain names and posterior probabilities are presented in Figure S5.

Figure 3. Antigenic map of H13 and H16 influenza A viruses (n=44). Different subtypes and genetic clades are indicated with colors (yellow: H13 clade A; orange: H13 clade B; red: H13 clade C; blue: H16 clade A; purple: H16 clade B; green: H16 clade C). White circles indicate the antisera. Respective virus strains are abbreviated; the full name can be found in Table 5. Asterices indicates antigens BHGU/NL/20/09, BHGU/SE/1/06, BHGU/SE/1/03, GBBG/AK/1421/79, BHGU/NL/1/07, HEGU/NY/AI00-532/00 and LAGU/NJ/AI08-0714/08 that had only two numerical HI titers to the tested sera and hence their placement in the map is not robust. In this map the distance between the points represents antigenic distance as measured by the hemagglutination inhibition (HI) assay in which the distances between antigens and antisera are inversely related to the log2 HI titer. Each square in the grid of the antigenic map equals a two-fold difference in the HI assay.

Tables

Table 1. Representative viruses selected to generate ferret antisera used to map the antigenic diversity of H13 and H16 influenza A viruses

Subtype	Clade	Virus strain name
H13	A	A/Gull/Maryland/704/1977 (H13N6)
	A	A/Black-headed gull/Netherlands/2/2007 (H13N6)
	B	A/Ring-billed gull/Georgia/AI00-2658/2000 (H13N6)
	B	A/Gull/Minnesota/1352/1981 (H13N6)
	C	A/Laughing gull/ New Jersey/AI08-0714/ 2008 (H13N9)
	C	A/Great black-headed gull/Astrakhan/1420/1979 (H13N2)
H16	A	A/Black-headed gull/Sweden/2/1999 (H16N3)
	B	A/Herring gull/New York/AI00-532/2000 (H16N3)
	C	A/Black-headed gull/Turkmenistan/13/1976 (H16N3)
	C	A/Black-headed gull/Sweden/5/1999 (H16N3)

644

645 **Table 2.** Intercontinental gene flow events for influenza A virus H13 hemagglutinin. MRCA:
646 Most Recent Common Ancestor. HPD: Higher Posterior Density. Event # corresponds to the
647 numbers indicated in Figure 1 and S3

648

H13 Clade	**Event #**	**Time of the MRCA ± 95% HPD**	**Geographic origin of the MRCA (posterior)**	**Location of introduction**
A	1	1963 [1958-1966]	North America (0.62)	Oceania

	2	1974 [1972-1975]	North America (0.73)	Europe
	3	1988 [1987-1989]	Europe (1)	North America
	4	1990 [1988-1991]	Europe (0.82)	South America
	5	1996 [1995-1997]	Europe (0.75)	Asia
	6	2003 [2003-2004]	Europe (1)	Asia
	7	2005 [2004-2005]	Asia (0.48)	North America
	8	2009 (2009-2010]	North America (0.9)	Asia
	9	2006 [2006-2007]	Europe (0.96)	Asia
	10	2011 [2010-2011]	Europe (1)	Asia
B	11	2013 [2012-2014]	North America (0.96)	South America
C	12	1987 [1985-1988]	Europe (0.99)	North America

13	2002 [2002-2003]	Europe (1)	Asia
14	2005 [2004-2005]	Asia (0.55)	North America
15	2010 [2009-2010]	Europe (1)	North America
16	2004 [2003-2005]	Europe (0.97)	Asia
17	2013 [2013-2014]	Europe (0.99)	Asia
18	2014 [2013-2014]	North America (0.39)	Asia
19	2011 [2010-2011]	Europe (0.99)	North America
20	2012 [2011-2012)	North America (0.94)	South America

649

650

651 **Table 3.** Intercontinental gene flow events for influenza A virus H16 hemagglutinin. MRCA:
652 Most Recent Common Ancestor. HPD: Higher Posterior Density. Event # corresponds to the
653 numbers indicated in Figure 2 and S6

H16	Event	Time of the	Geographic origin	Location of introduction

Clade	#	MRCA ± 95% HPD	of the MRCA (posterior)	
C	1	1971 [1968-1972]	Europe (0.97)	Asia
	2	1976 [1976-1976]	Asia (0.71)	Europe
	3	1976 [1972-1980]	Europe (0.86)	North America
	4	1999 [1999-1999]	Europe (1)	Asia
	5	2003 [2002-2004]	Europe (1)	Asia
	6	1999 [1998-2000]	Europe (0.99)	North America
	7	2008 [2007-2009]	Europe (0.99)	Asia
	8	2006 [2005-2006]	Europe (0.97)	North America
	9	2006 [2006-2007]	North America (0.55)	South America
	10	2008 [2007-2009]	Europe (0.63)	North America

654

655 **Table 4.** Molecular evolution of the HA gene of influenza A virus subtypes H13 and H16

Genetic lineage	N[1]	Time period[2]	Substitution rate[3]		d_N/d_S
			Mean	95% HPD	Mean
H13	338	40	3.8	3.6-4.1	0.13
H13 - A	54	39	3.8	2.3-4.9	0.09
H13 - B	76	39	0.8	0.6-1.0	0.18
H13 - C	208	37	5.5	5.0-6.0	0.16
H16	192	41	3.1	2.8-3.4	0.09
H16 - A	56	33	4.5	3.9-5.2	0.10
H16 - B	19	35	4.6	3.9-5.2	0.06
H16 - C	117	40	1.5	1.2-1.8	0.11

656 [1] number of nucleotide sequences included in the analysis; [2] in years; [3] per 10^{-3} substitution / site /
657 year; HPD: highest posterior density.
658

659 **Table 5.** Hemagglutinin inhibition data of H13 and H16 influenza A viruses (n=44)

Suptype				H13						H16			
Clade				A	A	B	B	C	C	A	B	C	C
	Virus name	Subtype	Virus abbreviation	BHGU/NL/2/07	GULL/ML/704/77	GULL/MN/1352/81	RBGU/GE/AI00-2658/00	GBBG/AK/1420/79	LAGU/NJ/AI08-714/08	BHGU/SE/2/99	HEGU/NY/AI0-532/00	BHGU/SE/5/99	BHGU/TM/13/76
H13 / A	A/Black-headed gull/Netherlands/2/07	H13N6	BHGU/NL/2/07	**320**	280	80	<10	20	<10	<10	<10	<10	25
	A/Black-headed gull/Netherlands/4/07	H13N6	BHGU/NL/4/07	1280	400	320	<10	35	<10	<10	<10	10	40
	A/Black-headed gull/Netherlands/7/09	H13N2	BHGU/NL/7/09	10	160	<10	<10	<10	<10	10	<10	<10	15
	A/Black-headed gull/Sweden/10/05	H13N6	BHGU/SE/10/05	240	320	40	<10	10	<10	<10	<10	<10	15
	A/Great-black headed gull/Sweden/1/03	H13N6	GBBG/SE/1/03	80	240	20	<10	<10	<10	<10	<10	<10	<10
	A/gull/ML/704/77	H13N6	GULL/ML/704/77	40	**240**	20	<10	<20	<10	<10	<10	<10	<10
H13 / B	A/gull/MN/1352/81	H13N6	GULL/MN/1352/81	120	160	320	<10	20	<10	<10	<10	<10	<10
	A/gull/NJ/34/92	H13N6	GULL/NJ/34/92	80	240	80	<10	240	<10	<10	<10	<10	<10
	A/Herring gull/DB/13/90	H13N2	HEGU/DB/13/90	40	140	140	10	25	<10	<10	<10	<10	<10
	A/Laughing gull/DB/1370/86	H13N2	LAGU/DB/1370/86	10	40	<10	10	40	<10	<10	<10	<10	<10
	A/ring-billed gull/GE/AI00-2658/00	H13N6	RBGU/GE/AI00-2658/00	10	60	40	**640**	15	<10	<10	<10	<10	<10
	A/ring-billed gull/MN/AI10-1708/10	H13N6	RBGU/MN/AI10-1708/10	80	200	120	10	10	<10	<10	<10	<10	<10
H13 / C	A/Black-headed gull/Netherlands/1/00	H13N8	BHGU/NL/1/00	35	<10	<10	<10	1280	120	<10	30	<10	30
	A/Black-headed gull/Netherlands/20/09	H13N2	BHGU/NL/20/09	<10	<10	<10	<10	280	<10	<10	<10	<10	35
	A/Black-headed gull/Netherlands/4/08	H13N8	BHGU/NL/4/08	<10	<10	<10	<10	140	80	<10	<10	<10	25
	A/Black-headed gull/Sweden/1/03	H13N8	BHGU/SE/1/03	<10	<10	<10	<10	560	40	<10	<10	<10	<10

	A/Black-headed gull/Sweden/1/06	H13N8	BHGU/SE/1/06	<10	<10	<10	<10	120	<10	<10	<10	<10	<10
	A/Black-headed gull/Sweden/1/99	H13N6	BHGU/SE/1/99	10	<10	10	30	160	<10	<10	<10	<10	10
	A/Black-headed gull/Sweden/2/03	H13N8	BHGU/SE/2/03	<10	<10	<10	<10	200	50	<10	<10	<10	10
	A/Great-black headed gull/AK/1420/79	H13N2	GBBG/AK/1420/79	10	35	10	<10	**2720**	160	10	<10	35	25
	A/Great-black headed gull/AK/1421/79	H13N2	GBBG/AK/1421/79	<10	<10	<10	<10	140	80	<10	<10	<10	<10
	A/Great-black headed gull/AK/591/82	H13N2	GBBG/AK/591/82	<10	40	<10	<10	480	100	<10	<10	40	80
	A/Great-black headed gull/GJ/76/83	H13N2	GBBG/GJ/76/83	<10	<10	<10	<10	320	80	<10	<10	<10	30
	A/Herring gull/AK/458/85	H13N6	HEGU/AK/458/85	30	20	<10	<10	1920	480	70	<10	80	80
	A/Herring gull/AK/479/85	H13N6	HEGU/AK/479/85	140	35	10	<10	1920	640	280	120	280	120
	A/Laughing gull/NJ/AI08-714/08	H13N9	LAGU/NJ/AI08-714/08	<10	<10	<10	<10	320	**560**	<10	<10	<10	<10
H16 / A	A/Black-headed gull/Netherlands/5/07	H16N3	BHGU/NL/5/07	35	25	<10	<10	140	<10	960	160	320	640
	A/Black-headed gull/Netherlands/1/07	H16N3	BHGU/NL/1/07	<10	<10	<10	<10	<10	<10	80	<10	<10	40
	A/Black-headed gull/Netherlands/10/09	H16N3	BHGU/NL/10/09	20	80	<10	<10	280	15	1280	160	640	640
	A/Black-headed gull/Netherlands/21/09	H16N3	BHGU/NL/21/09	70	200	20	<10	240	<10	480	<10	240	280
	A/Black-headed gull/Netherlands/3/07	H16N3	BHGU/NL/3/07	100	90	20	<10	100	<10	120	140	60	120
	A/Black-headed gull/Sweden/2/99	H16N3	BHGU/SE/2/99	10	<10	<10	<10	10	<10	**960**	80	35	380
	A/Black-headed gull/Sweden/8/05	H16N3	BHGU/SE/8/05	<10	<10	<10	<10	10	<10	1280	<10	30	140
H16 / B	A/Herring gull/DB/2617/87	H16N3	HEGU/DB/2617/87	<10	<10	<10	<10	<10	<10	<10	120	20	1600
	A/Herring gull/NY/AI0-532/00	H16N3	HEGU/NY/AI0-532/00	<10	<10	<10	<10	<10	<10	<10	**320**	<10	320
	A/Laughing gull/DB/2839/87	H16N3	LAGU/DB/2839/87	<10	<10	<10	<10	<10	<10	160	80	20	1920
H16 / C	A/Black-headed gull/Netherlands/26/09	H16N3	BHGU/NL/26/09	10	25	<10	<10	20	<10	30	80	20	1280
	A/Black-headed gull/Sweden/5/99	H16N3	BHGU/SE/5/99	10	<10	<10	<10	70	<10	560	30	**1600**	400
	A/Black-headed gull/TM/13/76	H16N3	BHGU/TM/13/76	25	30	<10	<10	27,5	<10	50	320	100	**4800**
	A/environment/Sweden/2/05	H16N3	ENV/SE/2/05	20	30	10	<10	140	30	960	320	1280	640
	A/Little tern/Sweden/1/05	H16N3	LITE/SE/1/05	<10	15	<10	<10	15	<10	10	30	20	1280
	A/shorebird/DB/172/05	H16N3	SB/DB/172/05	<10	<10	<10	<10	30	<10	240	60	200	1280
	A/shorebird/DB/195/06	H16N3	SB/DB/195/06	<10	<10	<10	<10	<10	<10	<10	30	20	560
	A/Slender-billed gull/AK/28/76	H16N3	SBGU/AK/28/76	20	140	10	<10	50	<10	80	160	100	1280

660

661 **Table 6.** Amino acid differences within/near the receptor binding site of the HA protein

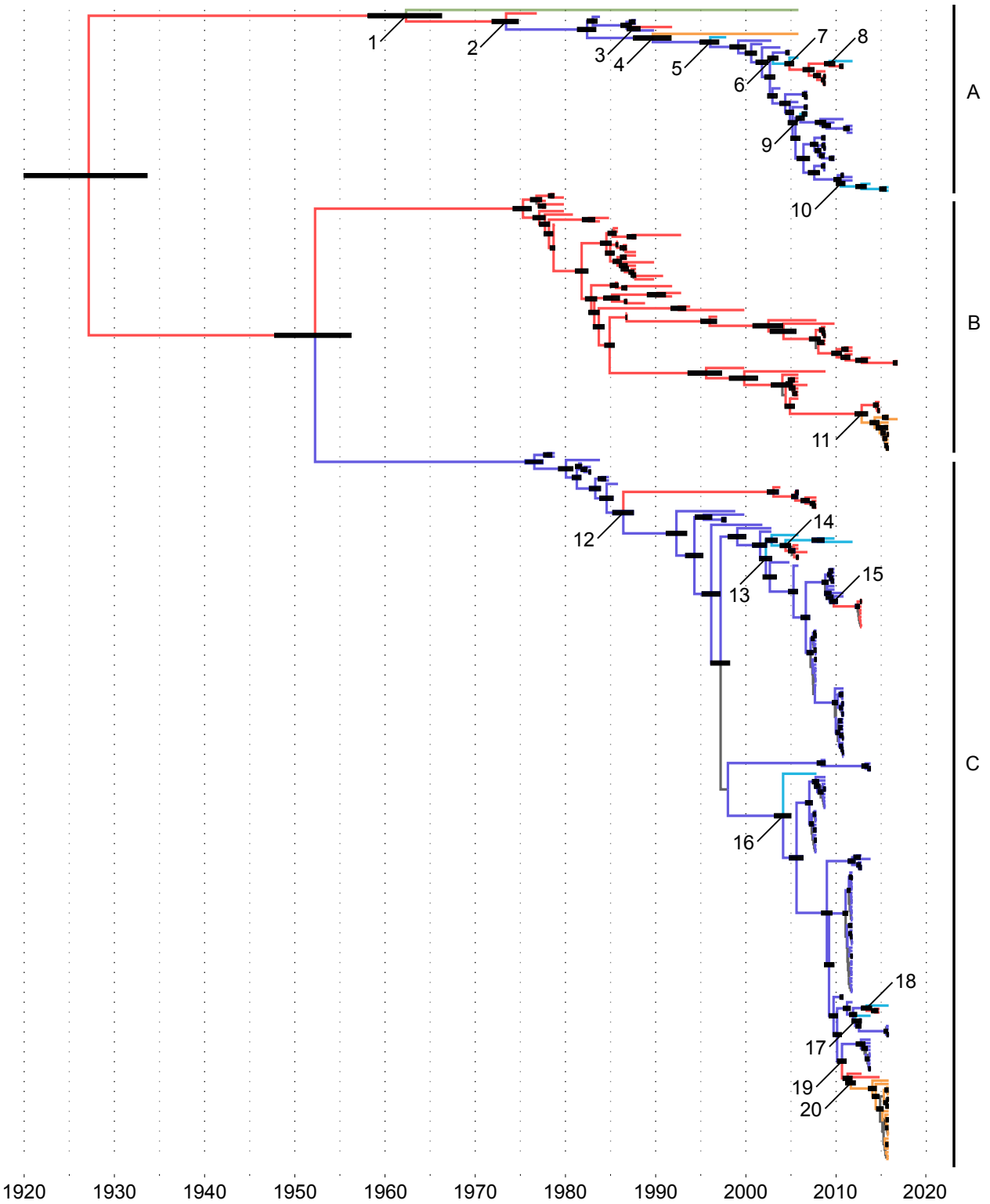
662 among H13 and H16 subtypes and clades, based on the HA gene of H13 (n=338) and H16

663 (n=192) LPAIVs, including the 130-loop (position 136-147 according to Burke & Smith

664 2014), 190-helix (200-208) and 220-loop (230-240). DEL, deletion of amino acid.

Amino acid position Clade	**139**	**142**	**145**	**149**	**166**	**176**	**177**	**196**	**198**	**200**	**208**	**217**	**218**	**224**	**231**	**233**
H13 A	D	A,T,S	A	D,E,N,S	K,Q	K	T	V,L	V	E	S,G	K	S,L	K	P	Y
H13 B	D	A,T,S	A	D,N,S	K,R	G,R	T	V,I	T,A	E	S,G	S,R,N,H	S,L	K,N	P,L	Y, Q
H13 C	D	V,A	A	DEL,R	K,R,S	G,R	T,A,V	V,I	T,A,E	E	D,N,S	S,R,G	S,T	N,T,K	P	Y
H16 A	E	T	S	DEL	L	G	E	D	E	T	K	K	E	E	I	D
H16 B	D	V	S	DEL	DEL	G	D	D	E,?	T,V	K	K,E	E	E	I	D,E,N
H16 C	D	V,A	S	DEL	K,DEL	G	E,D	D	E	T	K	K	E	E	I,V	D, N

665



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