Evolution of Equine Influenza Virus in Vaccinated Horses

Pablo R. Murcia,[a] Gregory J. Baillie,[b] J. Conrad Stack,[c] Carley Jervis,[d] Debra Elton,[d] Jennifer A. Mumford,[e] Janet Daly,[f] Paul Kellam,[b,g] Bryan T. Grenfell,[h,i] Edward C. Holmes,[i,j] James L. N. Wood[e]

Medical Research Council—University of Glasgow Centre for Virus Research, Institute of Infection, Inflammation and Immunity, College of Medical, Veterinary and Life Sciences, University of Glasgow, Glasgow, United Kingdom[a]; Wellcome Trust Sanger Institute, Wellcome Trust Genome Campus, Hinxton, Cambridge, United Kingdom[b]; Department of Biology, The Pennsylvania State University, University Park, Pennsylvania, USA[c]; Animal Health Trust, Centre for Preventive Medicine, Lanwades Park, Kentford, United Kingdom[d]; Disease Dynamics Unit, Department of Veterinary Medicine, University of Cambridge, Cambridge, United Kingdom[e]; School of Veterinary Medicine and Science, University of Nottingham, Sutton Bonington, United Kingdom[f]; Department of Infection, Division of Infection and Immunity, University College London, London, United Kingdom[g]; Department of Ecology and Evolutionary Biology, Princeton University, Princeton, New Jersey, USA[h]; Fogarty International Center, National Institutes of Health, Bethesda, Maryland, USA[i]; Sydney Emerging Infections and Biosecurity Institute, School of Biological Sciences and Sydney Medical School, The University of Sydney, Sydney, Australia[j]

Influenza A viruses are characterized by their ability to evade host immunity, even in vaccinated individuals. To determine how prior immunity shapes viral diversity *in vivo*, we studied the intra- and interhost evolution of equine influenza virus in vaccinated horses. Although the level and structure of genetic diversity were similar to those in naïve horses, intrahost bottlenecks may be more stringent in vaccinated animals, and mutations shared among horses often fall close to putative antigenic sites.

Equine influenza virus (EIV) of the H3N8 subtype is a significant horse pathogen and an important example of influenza emergence, as it recently transferred into dogs and became established as a novel canine influenza virus (1). EIV is subject to strong immunological pressure (2), and antigenic drift can result in outbreaks of disease even in vaccinated horse populations (3). We have previously studied the intrahost evolution of EIV in naïve horses (4) and during a field outbreak (5). To determine how prior immunity impacts intra- and interhost EIV evolution within a controlled experimental setting, we performed a transmission study using vaccinated horses challenged with a heterologous EIV (6) and examined the intra- and interhost genetic variation of the hemagglutinin 1 (HA1) gene domain at different times postinfection. All animal work was performed under license from the Home Office under the Animals (Scientific Procedures) Act 1986 following full ethical approval.

Welsh Mountain ponies were vaccinated with inactivated whole virus prepared from the Eurasian strain A/equine/Newmarket/2/93. Before the virus challenge experiment, antibody levels in vaccinated horses were measured by single radial hemolysis (SRH) (7) until most declined to <60 mm², a value normally low enough to allow natural infection, including in a previous contact experiment (J. Mumford, unpublished data). A "seeder" (S) horse (naïve to EIV) was experimentally inoculated via nebulized aerosol (20 ml of $\log_{10} 10^{6.3}$/ml of 50% egg infective dose [EID_{50}]) of the American strain A/equine/Newmarket/1/1993. The aim of this step was to readapt the egg-grown inoculum to the horse. Two days later, the seeder horse was housed with a vaccinated horse (V1). V1 was then removed and housed with V2. This procedure was repeated until horse V4, which did not show clinical signs of infection. For this reason, V4 was cohoused with V3 and then moved to a stable with V5. Throughout the study, each horse was cohoused with the next recipient horse for 3 days. Infection of horses was confirmed using an enzyme-linked immunosorbent assay (ELISA)-based test to measure virus excretion (8). SRH was used to detect seroconversion and confirm transmission. Clin-

ical signs were recorded daily, and nasal swabs were collected and processed as previously described (4, 5). Nasal swabs were RNA extracted, and viral copy numbers were determined by qRT-PCR as described previously (4). The hemagglutinin gene (HA1) was amplified by PCR from viral populations of sufficient size with the exception of the sample obtained from horse V4, which was amplified by heminested PCR because it exhibited a low copy number (primers available on request). PCR products were cloned and sequenced using capillary sequencing as described previously (4, 5, 9, 10).

Transmission was successful up to horse V4 (Fig. 1A), and horses shed up to >10^6 viral copies/μl of cDNA (Fig. 1C). All horses except V4 showed mild signs of influenza infection and seroconverted (data not shown). Horse V5 was not infected (it did not seroconvert, and no virus was detected in nasal swabs). Horses V1, V3, and V4 shed less virus than horse V2 (Fig. 1C), the animal with the lowest antibody levels before entering the transmission chain (data not shown) and whose shedding pattern was higher than that observed in the seeder horse.

We generated between 34 and 79 clonal HA1 sequences (a total of 549,927 nucleotides) from 10 daily nasal swabs. HA1 sequences were 903 nucleotides long and included all antigenic sites and the receptor-binding domain. A summary analysis of intrahost variation is shown in Table 1. Overall, we detected 126 mutations, which resulted in a mutation frequency of 2.3×10^{-4} mutations per nucleotide site, similar to the value observed in naïve horses (1.8×10^{-4}) (4). Mutations were distributed throughout the HA1 segment (data not shown), and the ratio of nonsynonymous to synonymous substitutions per site (*dN/dS*) for the whole data set
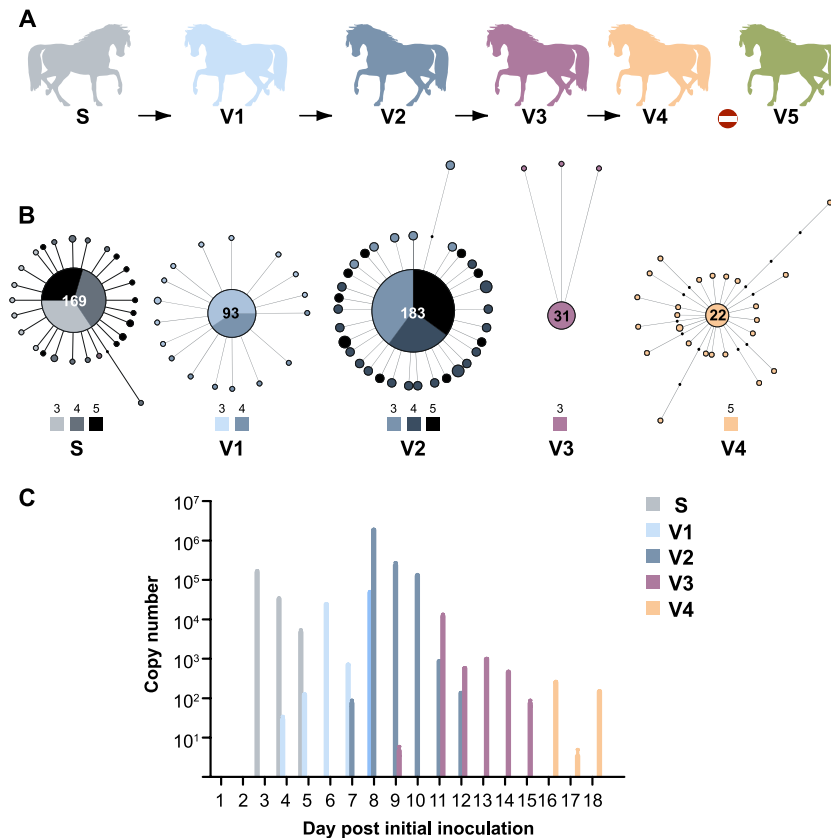
FIG 1 Intra- and interhost genetic variation of EIV in vaccinated horses. (A) Layout of the transmission chain (see main text). S, seeder; V1 to V5, vaccinated horses. The number refers to the order in which animals were introduced into the transmission chain. No virus was detected in horse V5 (shown with a stop sign). (B) Median joining networks derived from the horses included in the study. Each network was inferred by compiling sequences from all available days. The size of each circle is proportional to the frequency of identical sequences in the data set, and the number of sequences that constitute the consensus is indicated. The identification number of each horse is indicated at the bottom of each panel. Colors indicate the day in which the sample was taken relative to the day of contact. (C) Estimation of viral copy numbers from individual swabs by real-time PCR.

(estimated using HyPhy [11]) was 0.84 and, hence, very similar to the value (0.80) observed in naïve horses evaluated in a similar manner (4).

To estimate changes in genetic diversity of within-host viral

TABLE 1 Analysis of intrahost variation of EIV in vaccinated horses

Horse	Day	No. of sequences	No. of mutations	Mean pairwise distance	Mean dN/dS	No. of stop codons	No. of mutations in Ag sites[a]
S	3	65	7	0.00023	0.38	0	2
	4	71	11	0.00034	0.62	0	1
	5	63	13	0.00045	0.49	0	0
V1	4	66	11	0.00037	0.51	1	0
	6	45	7	0.00034	NA[b]	0	2
V2	3	79	7	0.00019	0.72	0	1
	4	65	19	0.00063	0.61	1	2
	5	74	10	0.0003	0.38	2	2
V3	3	34	3	0.00019	0.57	0	0
V4	5	47	38	0.00179	1.75	0	5

[a] Ag, antigenic.
[b] NA, not applicable.

populations, we compiled sequences from individual horses and aligned them using Se-Al (http://tree.bio.ed.ac.uk/software/seal/). We calculated the mean pairwise distance (Table 1) using PAUP* (Sinauer Associates, version 4.0b10) and inferred median joining networks using Network 4.6.00 (http://www.fluxus-engineering.com/sharenet.htm) to visualize the structure of the mutational spectra. All data sets resulted in star-like phylogenies (Fig. 1B) in which most sequences were identical to each other and had no evidence of evolution through secondary branching events, and mutant clones usually differed at one nucleotide with respect to the consensus sequence. Such phylogenetic structure is similar to that observed in naïve animals (4). We further divided all haplotypes by (i) host, (ii) sampling day, and (iii) viral population (host and day) and measured the overall differentiation in the data set as described previously (12) using D_{est} (13). We observed small but significant levels of subpopulation differentiation when samples were divided by viral population (0.0010; $P = 0.026$) but not by sampling day (0.0004; $P = 0.128$) and almost-significant values when divided by horse (0.0010; $P = 0.057$). Accordingly, we infer that interhost transmission plays a role in shaping patterns of intrahost diversity of EIV by allowing the generation of "private" mutations. In addition, some variants (14%) displayed nonsynonymous mutations at putative antigenic and

TABLE 2 EIV mutations present in multiple horses

Mutation[a]	Motif[b]	Horses[c] (day)
A413G (E123G)	<u>AgA</u>	S (5), V4 (5)
T497A (L151Q)	<u>AgA</u>	S (3), V1 (6)
A614G (Q190R)	AgB	V1 (6), V2 (3)
T752C (I236T)	<u>AgD</u>	S (4), V2 (3)
A869G (D275G)	AgC	S (4), V4 (5)

[a] Amino acid numbering is based on mature HA3.
[b] Underlined motifs are located within nine amino acid residues of their respective antigenic site.
[c] Horses are indicated as in Fig. 1.

glycosylation sites as well as at the receptor-binding domain (data not shown) such that they were able to exhibit altered antigenicity and/or host range, although the observation that they were present only as singleton mutations suggests that positive selection had little effect on their frequency. Notably, no mutations were present on multiple days from the same horse, suggesting the presence of strong intrahost population bottlenecks between sampling points. Despite this, we did detect common mutations in different animals, and it is striking that all of these common mutations were nonsynonymous and fell either at putative antigenic sites (14) or very close to them (within a range of nine amino acids) (Table 2).

We next examined the impact of prior immunity on within-host viral populations by determining the variation of the HA1 gene of EIV in vaccinated horses. The levels of variation (mean pairwise distances and mutation frequency), the topology of the inferred trees, and the *dN/dS* ratio that we observed are similar to those previously reported in naïve horses (4), in vaccinated horses naturally infected during an outbreak of EIV (5), and in a previous study on the evolution of a swine influenza virus (SIV) in naïve and vaccinated pigs (10). Hence, the rapid production and turnover of mutations are the dominant signals in intrahost populations of influenza virus, regardless of the genetic background of the virus and the immune status of its host. It may be that the effect of prior immunity (presumably principally effected through circulating antibody) is simply to dampen the rates of replication in a relatively nonspecific manner prior to the initiation of an adaptive immune response at the site of infection. Any time lag depends on various factors, such as the size of the resident memory cell population, the match between viruses during past and current infections, the time elapsed between infections, and the local inflammatory response (15), which, in turn, may be differentially modulated depending on the virus strain.

However, while mutational dynamics appear to be similar between vaccinated and naïve animals, our study did reveal two factors that might reflect the impact of prior immunity on within-host viral populations (although the small number of animals precludes definitive conclusions). First, within-host bottlenecks seem to be more stringent in vaccinated animals, as we did not observe any mutations that persisted between sampling times, in contrast to previous studies of naïve animals (4, 10). Although it is possible that such mutations were present but not detected due to insufficient sequencing depth, this evolutionary pattern is similar to what was previously observed in SIV in vaccinated pigs (10) and suggests that viral clearance within the host might be more efficient in vaccinated animals. Second, all of the mutations that are shared between horses were located at or very near putative anti-

genic sites, although it is unclear whether they are convergent, representing similar selection pressures among horses, or are transmitted among them as minor subpopulations. Further studies with a larger number of animals and deeper coverage (i.e., ultradeep sequencing) should be performed to confirm this finding. Overall, our results highlight the significance of studying influenza infections in natural hosts with prior immunity, as they can shed light on the underpinning mechanisms that govern genetic (and likely antigenic) variation and thus provide insight on strain selection for the development of more-effective vaccines.

Nucleotide sequence accession numbers. The clonal HA1 sequences generated from the daily nasal swabs were deposited in GenBank under accession numbers KC295823 to KC296431.

REFERENCES

1. **Crawford PC, Dubovi EJ, Castleman WL, Stephenson I, Gibbs EP, Chen L, Smith C, Hill RC, Ferro P, Pompey J, Bright RA, Medina MJ, Johnson CM, Olsen CW, Cox NJ, Klimov AI, Katz JM, Donis RO.** 2005. Transmission of equine influenza virus to dogs. Science **310**:482–485.
2. **Murcia PR, Wood JL, Holmes EC.** 2011. Genome-scale evolution and phylodynamics of equine H3N8 influenza A virus. J. Virol. **85**:5312–5322.
3. **Newton JR, Daly JM, Spencer L, Mumford JA.** 2006. Description of the outbreak of equine influenza (H3N8) in the United Kingdom in 2003, during which recently vaccinated horses in Newmarket developed respiratory disease. Vet. Rec. **158**:185–192.
4. **Murcia PR, Baillie GJ, Daly J, Elton D, Jervis C, Mumford JA, Newton R, Parrish CR, Hoelzer K, Dougan G, Parkhill J, Lennard N, Ormond D, Moule S, Whitwham A, McCauley JW, McKinley TJ, Holmes EC, Grenfell BT, Wood JL.** 2010. Intra- and interhost evolutionary dynamics of equine influenza virus. J. Virol. **84**:6943–6954.
5. **Hughes J, Allen RC, Baguelin M, Hampson K, Baillie GJ, Elton D, Newton JR, Kellam P, Wood JL, Holmes EC, Murcia PR.** 2012. Transmission of equine influenza virus during an outbreak is characterized by frequent mixed infections and loose transmission bottlenecks. PLoS Pathog. **8**:e1003081. doi:10.1371/journal.ppat.1003081.
6. **Daly JM, Yates PJ, Newton JR, Park A, Henley W, Wood JL, Davis-Poynter N, Mumford JA.** 2004. Evidence supporting the inclusion of strains from each of the two co-circulating lineages of H3N8 equine influenza virus in vaccines. Vaccine **22**:4101–4109.
7. **Wood JM, Mumford J, Folkers C, Scott AM, Schild GC.** 1983. Studies with inactivated equine influenza vaccine. 1. Serological responses of ponies to graded doses of vaccine. J. Hyg. (Lond.) **90**:371–384.
8. **Cook RF, Sinclair R, Mumford JA.** 1988. Detection of influenza nucleoprotein antigen in nasal secretions from horses infected with A/equine influenza (H3N8) viruses. J. Virol. Methods **20**:1–12.
9. **Hoelzer K, Murcia PR, Baillie GJ, Wood JL, Metzger SM, Osterrieder N, Dubovi EJ, Holmes EC, Parrish CR.** 2010. Intrahost evolutionary dynamics of canine influenza virus in naive and partially immune dogs. J. Virol. **84**:5329–5335.
10. **Murcia PR, Hughes J, Battista P, Lloyd L, Baillie GJ, Ramirez-Gonzalez RH, Ormond D, Oliver K, Elton D, Mumford JA, Caccamo M, Kellam P, Grenfell BT, Holmes EC, Wood JL.** 2012. Evolution of an Eurasian

avian-like influenza virus in naive and vaccinated pigs. PLoS Pathog. **8**:e1002730. doi:10.1371/journal.ppat.1002730.

11. **Pond SL, Frost SD, Muse SV.** 2005. HyPhy: hypothesis testing using phylogenies. Bioinformatics **21**:676–679.

12. **Stack JC, Murcia PR, Grenfell BT, Wood JL, Holmes EC.** 2013. Inferring the inter-host transmission of influenza A virus using patterns of intra-host genetic variation. Proc. Biol. Sci. **280**:20122173.

13. **Jost L.** 2008. G(ST) and its relatives do not measure differentiation. Mol. Ecol. **17**:4015–4026.

14. **Wiley DC, Skehel JJ.** 1987. The structure and function of the hemagglu-tinin membrane glycoprotein of influenza virus. Annu. Rev. Biochem. **56**:365–394.

15. **Kohlmeier JE, Woodland DL.** 2009. Immunity to respiratory viruses. Annu. Rev. Immunol. **27**:61–82.