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Genetic versus antigenic differences among highly pathogenic H5N1 avian influenza A viruses: Consequences for vaccine strain selection

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ABSTRACT

Highly pathogenic H5N1 avian influenza A viruses display a remarkable genetic and antigenic diversity. We examined to what extent genetic distances between several H5N1 viruses from different clades correlate with antigenic differences and vaccine performance. H5-specific antisera were generated, and cross-reactivity and antigenic distances between 12 different viruses were determined. In general, antigenic distances increased proportional to genetic distances although notable exceptions were observed. Antigenic distances correlated better with genetic variation in 27 selected, antigenically-relevant H5 residues, than in the complete HA1 domain. Variation in these selected residues could accurately predict the antigenic distances for a novel H5N8 virus. Protection provided by vaccines against heterologous H5N1 challenge viruses indicated that cross-protection also correlates better with genetic variation in the selected antigenically-relevant residues than in complete HA1. When time is limited, variation at these selected residues may be used to accurately predict antigenic distance and vaccine performance.

1. Introduction

Influenza A viruses (family Orthomyxoviridae) are enveloped, negative-sense RNA viruses with an eight- segmented genome, that are subtyped based upon their immunogenic surface proteins hemagglutinin (HA) and neuraminidase (NA). Aquatic birds are thought to be the primordial reservoir of Influenza A viruses (Alexander, 2000; Munster and Fouchier, 2009; Webster et al., 1992) but, in the past, occasional spill-over to other species has resulted in the establishment of more-or-less species-specific influenza virus strains for, among others, terrestrial birds, pigs, horses, sea mammals, and humans. In poultry, two distinct pathotypes of avian influenza A virus (AIV) can be distinguished, i.e. viruses with high pathogenicity (HPAIV) and viruses with low pathogenicity (LPAIV), characterized by differences in the amino acid sequence preceding the proteolytic cleavage site in the HA protein (Bosch et al., 1981; Garten et al., 1981; Horimoto and Kawaoka, 1994; Stieneke-Grober et al., 1992).

An HPAIV of the H5N1 subtype emerged in Hong Kong in 1997. Direct transmission of this virus from poultry to humans resulted in the infection of 18 people of whom 6 died (Claas et al., 1998; Shortridge et al., 1998). In order to prevent further spread of the virus, Hong Kong's entire poultry population was culled (Sims et al., 2003). Although this 'stamping out' intervention probably resulted in its eradication from terrestrial poultry in that area, the virus lineage kept circulating in aquatic birds and resurfaced in 2003. From that time on it has spread from Southeast Asia through Russia to Europe, the Middle East, Africa and, more recently, North America (Peiris et al., 2007, 2004; Saito et al., 2015; Sims et al., 2005; Sonnberg et al., 2013). The virus has become endemic in poultry and wild birds in many countries, with occasional transmission to humans and a case fatality rate of more than 50% (http://www.who.int/influenza/human_animal_interface/H5N1_cumulative_table_archives/en/). Although there is some indication for human to human transmission (Olsen et al., 2005; Wang et al., 2008), it is generally assumed that the majority of human cases is the result of direct contact with H5N1-infected poultry. To limit human exposure, HPAIV H5N1 may be eliminated from the poultry population by 'stamping out', which is not feasible however in countries where people are dependent on poultry

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farming for their livelihood. Therefore, several countries have implemented vaccination campaigns, albeit generally with limited success due most likely to inadequate vaccination coverage and mismatch between vaccines and outbreak strains (Capua and Cattoli, 2013; Swayne, 2012; van den Berg et al., 2008).

Since their re-emergence in 2003, H5N1 HPAIVs have displayed extensive genetic evolution. Based on the nucleotide sequence of the HA gene, 10 first-order clades (clades 0–9) with more than 30 higher-order clades have been classified by the WHO-OIE-FAO (Smith et al., 2015; WHO/OIE/FAO, 2008, 2009, 2012). Clades 0, 3, 4, 5, 6, 8, 9, and several second- and third-order groups from clade 2 have not been detected since 2008 or earlier. Genetic divergence of the HA genes has resulted in extensive evolution of the amino acid sequence of HA, particularly in the HA1 head domain. Although genetic variation results in antigenic variation, genetic and antigenic distance do not always correlate. The limitation of phylogeny-based prediction of antigenic distance (Donis, 2014) has been demonstrated by the occurrence of inconsistencies in the correlation between the genetic and antigenic distance, as reflected by 'outliers' in antigenic grouping or antigenic cartography (Cattoli et al., 2011b; Ducatez et al., 2011; Kaverin et al., 2007; Koel et al., 2014; Wu et al., 2008), as well as by the lack of protective antibody responses in vaccination-challenge experiments (Abdelwhab et al., 2011; Beato et al., 2013; Cattoli et al., 2011b; Grund et al., 2011; Swayne et al., 2015).

While the geographical expansion and long-term endemicity of H5N1 has promoted the genetic (and thus antigenic) evolution of H5N1, vaccination-induced evolution has been postulated to be the main driver of antigenic evolution of AIV in poultry (Cattoli et al., 2011a; Lee et al., 2004; Lee and Suarez, 2005). In addition, natural immunity in avian species that are less susceptible to H5N1 (such as ducks) may also contribute to immune escape. A large number of residues located in the HA1 domain have been identified to be important for antigenic recognition of HA (Cattoli et al., 2011b; Kaverin et al., 2007; Peng et al., 2014; Rudneva et al., 2010, 2008). Antigenic variation of clade 2.1 H5N1 viruses was shown to be determined by a few amino acid changes immediately adjacent to the receptor binding site (Koel et al., 2014), similar to what has been observed for seasonal human influenza A viruses (Koel et al., 2013). Changes in receptor binding avidity via amino acid substitutions in HA followed by compensatory mutations have been proposed to simultaneously alter antigenicity as a kind of 'by-stander' effect (Hensley et al., 2009). Whatever the reason, it is clear that antigenic evolution is a major challenge to vaccine preparedness and that the genetic distance between vaccine strain and challenge strain may not always be a reliable predictor for vaccine efficacy.

It has not yet been well established to what extent genetic and antigenic distances correlate with each other and with vaccine efficacy in chickens for viruses belonging to different HP H5N1 clades. Most of the previous studies on H5N1 antigenic evolution particularly focussed on antigenic variation within single clades (Koel et al., 2014; Zhong et al., 2014) and used ferret sera and/or monoclonal antibodies for antigenic profiling (Ducatez et al., 2011, 2007; Kaverin et al., 2007; Wei et al., 2011; Wu et al., 2008). In the current study we examined the correlation between the vaccine performance in chickens and the HA genetic and antigenic distance for several Eurasian-lineage H5N1 HPAIV strains belonging to more than 10 different clades. To this end, we constructed LP PR8-based 7+1 recombinant viruses, each containing a different HA gene selected from currently circulating H5N1 strains, and generated corresponding recombinant soluble trimeric HA proteins (sHA3) (Cornelissen et al., 2010). By using inactivated virus and sHA3 preparations for the immunization of chickens we generated clade-specific immune sera that only differed in the composition of HA antibodies. The viruses and immune sera were used to determine antigenic distances based on cross-reactivity in hemagglutination-inhibition (HI) and virus-neutralisation (VN) tests. Genetic variation in residues that were previously identified as being important for antigenic recognition of H5 subtype HA (Cattoli et al., 2011b; Duvvuri et al., 2009; Kaverin et al., 2007, 2002; Lee et al., 2004; Peng et al., 2014; Rudneva et al., 2010, 2008) were shown to better correlate with antigenic distances than genetic variation in the complete HA1 domain. Based on these residues we were subsequently able to reliably predict the antigenic distances between the novel clade 2.3.4.4 H5N8 viruses and viruses belonging to other clades. In order to examine the correlation between HA-specific genetic and antigenic distance and in vivo protection in chickens, we also generated clade-specific HP H5N1-based 7+1 challenge viruses that only differed with respect to the origin of their HA genes. These viruses were subsequently used to challenge chickens after vaccination with different sHA3 preparations. This approach allowed us to determine the correlation between genetic and antigenic distance as well as between antigenic distance and survival and virus shedding in vaccination-challenge experiments in chickens.

2. Materials and methods

2.1. Ethics statement

All animal studies were performed in accordance with the Dutch Law on Animal Experimentation (WoD, ID number BWBR0003081) which is the national implementation of European Directive 2010/63/EU. The study protocols were approved by the Institutional Animal Care and Use Committee (IACUC, or DEC in Dutch) and Animal Welfare Body (AWB, or IvD in Dutch) of Utrecht University (permit number DEC#2012. II.08.115), MSD Animal Health (permit number AIC14.021), and Wageningen Bioveterinary Research (permit numbers 2013.060b and 2014068.c). The animal welfare officers of each of these institutions checked the mandatory administration and supervised the proper conduct of procedures and the well-being of the animals that were used.

2.2. Viruses

Recombinant 7+1 PR8-H5 influenza viruses containing low-pathogenic versions of the HA genes from different H5N1 viruses (Table 1) in the genetic background of strain A/Puerto Rico/8/34 H1N1 (PR8) were generated by means of reverse genetics, using PR8 plasmids provided by drs Hoffmann and Webster (St. Jude Children's Research Hospital, Memphis) as described (Hoffmann et al., 2002; Hoffmann and Webster, 2000; Peeters et al., 2012). Ready-to-use plasmids consisting of the different synthetic H5 genes in vector pHW2000 were obtained from GenScript corporation, Piscataway, USA. Each plasmid contained the open reading frame (ORF) of the respective HA gene, flanked by the 3'and 5'untranlated regions of the HA-segment from PR8. The nucleotide sequence encoding the wild-type multi-basic amino-acid sequence was modified to encode the amino acid sequence 323GGTR↓GLF329 (H5-numbering) typical of low-pathogenic AIV. PR8-H5 viruses were inactivated by incubation with 0.02% paraformaldehyde (final concentration) for 16 h at 37 °C.

Recombinant highly pathogenic TT05-H5 influenza viruses containing the wild-type HA genes from the same viruses (Table 1) in the genetic background of strain A/turkey/Turkey/1/2005 H5N1 (TT05) were generated by means of reverse genetics, using TT05 plasmids obtained from dr. Ron Fouchier, Erasmus MC, Rotterdam. Ready-to-use HA plasmids consisting of the different synthetic H5 genes in vector pHW2000 were obtained from GenScript corporation, Piscataway, USA. Each plasmid contained the open reading frame (ORF) of the respective HA gene, flanked by the 3'and 5'untranlated regions of the HA-segment from TT05.

Table 1.
Viruses and HA proteins analysed in this study.

Clade	Abbreviation	Full name	Accession nr	Region of recent activity
1	VN04	A/Vietnam/1194/04	EF541402	Vietnam / Cambodia
1.1	Cam07	A/Cambodia/R0405050/2007	FJ225472	Vietnam / Cambodia
2.1.3.2	Indo05[a]	A/Indonesia/5/05	EF541394	Indonesia
2.1.1	Hunan02	A/duck/Hunan/795/2002	CY028963	Indonesia / China
2.2	MSD05	consensus based on clade 2.2		
2.2	TT05	A/turkey/Turkey/1/2005	EF619980	Turkey / Egypt / Israel
2.2.1	Eg10	A/Egypt/N03072/2010	CY062484	Egypt / Israel
2.2.1.1	Eg08	A/chicken/Egypt/0879-NLQP/2008	GQ184238	Egypt
2.3.2.1	Hubei10	A/Hubei/1/2010	CY098758	Southeast Asia
2.3.2.1	VN11	A/duck/Vietnam/QT801/2011	JN986881	Southeast Asia
2.3.4	Anhui05	A/Anhui/1/2005	HM172104	Vietnam / China
2.3.4.2	VN08	A/Vietnam/HN31432M/2008	HM114617	Vietnam / China
7.1	VN0408	A/chicken/Vietnam/NCVD-04/2008	FJ842482	Vietnam / China

[a] Sera were raised against the Indo05 sHA3protein, but a recombinant virus containing this HA gene could not be generated.

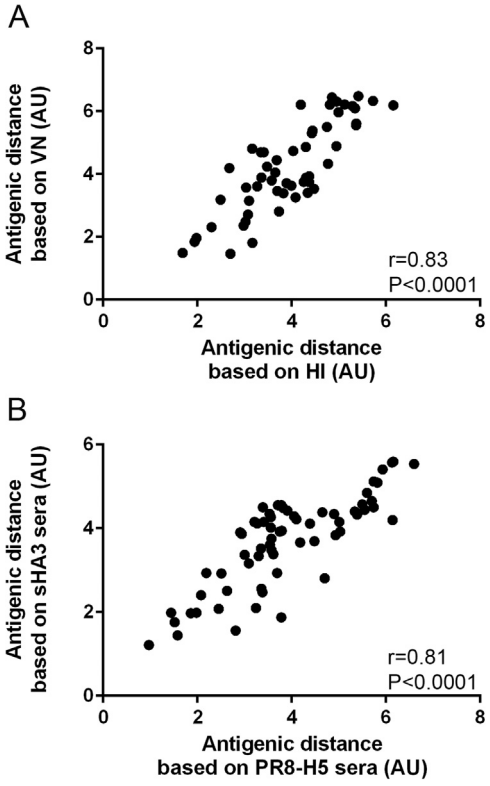

Fig. 1. Correlation between mean antigenic distance data sets. Antigenic distances between different HP H5 proteins were determined using two sets of antisera (raised against PR8-H5 or sHA3 proteins) and two assays (VN or HI) as described previously (Sitaras et al., 2014) and as detailed in the Materials and Methods section for each of the 4 datasets (Supplementary Table 2A-D). A) Scatter plot of mean antigenic distances determined by HI and VN (for both sera sets). B) Scatterplot of mean antigenic distances determined for each sera set (mean of VN and HI). The Pearson correlation coefficients r and the significance of the correlations are indicated.

2.3. Cloning and expression of soluble trimeric HA (sHA3)

sHA3 proteins were expressed in HEK293T cells (ATCC CRL-3216) using pCD5 expression vectors as described previously (Cornelissen et al., 2010; de Vries et al., 2012) or in insect Sf9 cells (ATCC CRL-1711) using the Bac-to-Bac Baculovirus Expression system (Invitrogen). Briefly, H5 encoding cDNAs (corresponding to residues 18–523; H3 numbering) were cloned into pCD5 or pFastBac1 in frame with DNA sequences coding for a signal sequence, an artificial GCN4 isoleucine zipper trimerization motif (RMKQIEDKIEEIESKQKKIENEIARIKK), and Strep-tag II (WSHPQFEK; IBA, Germany). pCD5 expression vectors were transfected into HEK293T cells using polyethyleneimine I and cell culture supernatants were harvested 5–6 days post transfection. pFastbac1 vectors were used to generate recombinant baculoviruses, which were subsequently used for the expression of sHA3 proteins in Sf9 cells according to the instructions of the manufacturer (Invitrogen). sHA3 protein expression and secretion was confirmed by sodium dodecylsulfate (SDS)-polyacrylamide gel electrophoresis (PAGE) followed by Western blotting using a mouse anti-Strep-tag antibody (IBA, Germany). HA proteins expressed in HEK293T cells were purified using Strep-tactin Sepharose beads according to the manufacturer's instructions (IBA, Germany). The concentration of purified protein was determined by SDS-PAGE followed by Coomassie Brilliant Blue staining using different amounts of BSA as a standard. The concentration of sHA3 in Sf9 cell culture supernatants was determined by quantitative Western blot analysis using different amounts of purified protein as a standard.

2.4. Generation of H5 clade-specific chicken sera

H5 clade-specific antisera against each of the PR8-HA viruses were generated by immunizing two 6-week-old white-leghorn chickens each with 0.5 ml of a mixture of inactivated virus and a proprietary oil-in-water adjuvant (nGNE; MSD Animal Health, Boxmeer, The Netherlands) by intramuscular inoculation. H5 clade-specific antisera against sHA3 proteins were generated by immunizing chickens twice at a three weeks interval with 4 μg of purified, HEK293T cell-expressed sHA3 in the presence of nGNE adjuvant. Three weeks after the final immunization the animals were euthanized and blood was collected for the preparation of sera.

2.5. Serological tests and antigenic distance calculations

Influenza virus-specific antibody levels in sera were measured by means of a hemagglutination inhibition (HI) assay using chicken red blood cells and 8 HA units of virus. Virus neutralizing antibody levels in sera were determined by means of a virus microneutralization (VN) test using 100 TCID50 of PR8-H5 virus and MDCK cells (ATCC CCL-34). Both tests were performed as prescribed by the OIE (Office International des Epizooties, 2008) and the HI and VN titres are shown as log2 values of the final dilution that resulted in complete inhibition of hemagglutination or virus neutralization. The tests were performed in duplicate and by two independent persons (Supplementary Table 1). Data sets from HI and VN tests, in which each virus was tested against both sets of antisera (raised against either PR8-H5 or sHA3) were used to calculate antigenic distances between the different H5 clade viruses as described previously (Sitaras et al., 2014). The R-script (The Comprehensive R Archive Network, (Team, 2015)) used to convert HI or VN datasets (in Microsoft Excel format) into antigenic distances and the explanation thereof is shown in Supplementary Fig 1. Antigenic distances are

Table 2.
Mean antigenic distances.

	VN0408	Anhui05	Cam07	Eg08	Eg10	TT05	Hubei10	Hunan02	MSD05	VN04	VN08	VN11	H5N8
VN0408	0.00	6.14	4.86	3.99	5.67	5.51	5.20	5.65	5.64	5.12	4.58	5.48	4.85
Anhui05	6.14	0.00	3.75	6.16	1.58	1.52	3.36	2.08	0.97	3.30	3.54	3.61	
Cam07	4.86	3.75	0.00	4.91	4.55	4.08	3.85	3.58	4.05	2.75	3.61	4.39	3.36
Eg08	3.99	6.16	4.91	0.00	6.17	5.73	5.72	5.95	6.03	5.49	4.92	5.46	5.68
Eg10	5.67	1.58	4.55	6.17	0.00	2.49	3.12	2.89	2.08	4.09	4.15	3.87	3.63
TT05	5.51	1.52	4.08	5.73	2.49	0.00	3.30	1.89	1.59	4.00	4.00	3.69	2.96
Hubei10	5.20	3.36	3.85	5.72	3.12	3.30	0.00	3.43	2.84	4.06	3.86	2.67	2.92
Hunan02	5.65	2.08	3.58	5.95	2.89	1.89	3.43	0.00	1.97	3.27	3.80	4.05	2.77
MSD05	5.64	0.97	4.05	6.03	2.08	1.59	2.84	1.97	0.00	3.67	3.81	3.44	2.69
VN04	5.12	3.30	2.75	5.49	4.09	4.00	4.06	3.27	3.67	0.00	2.31	4.02	2.74
VN08	4.58	3.54	3.61	4.92	4.15	4.00	3.86	3.80	3.81	2.31	0.00	3.62	3.31
VN11	5.48	3.61	4.39	5.46	3.87	3.69	2.67	4.05	3.44	4.02	3.62	0.00	3.24
H5N8	4.85		3.36	5.68	3.63	2.96	2.92	2.77	2.69	2.74	3.31	3.24	0.00

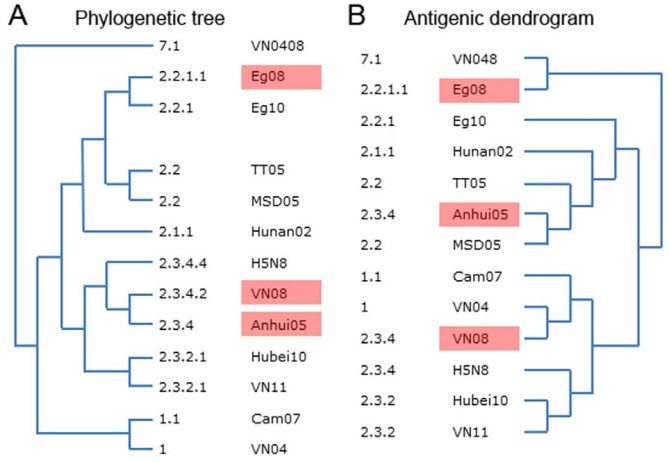

Fig. 2. Hierarchical clustering based on antigenic distances and phylogenetic analysis. A) Phylogenetic tree of the different H5 proteins with similar topology as the extended phylogenetic tree shown in Supplementary Fig 2B. The antigenic distance matrix shown in Table 2 was converted into an antigenic dendrogram as indicated in the Materials and Methods section. H5 proteins the clustering of which deviates between the phylogenetic tree and the antigenic dendrogram are highlighted.

measured in antigenic units (AU) and one unit is equivalent to a two-fold dilution in HI or VN assay data (i.e., 2 AU =4 fold dilution, 3 AU =8 fold dilution). To prevent distortion of antigenic distances we did not use additional methods, like multidimensional scaling to reduce the map of virus locations to a lower dimensional space. The antigenic distance matrix (mean of the 4 data sets) was converted into a dendrogram using the R command plot(hclust(dist(dm))) in which dm is the distance matrix.

2.6. *Vaccination-challenge experiments in chickens*

Vaccinations were conducted at the animal facilities of MSD Animal Health, Boxmeer, The Netherlands, whereas challenge inoculations were performed in the high-containment facilities of the Central Veterinary Institute (CVI), Lelystad, The Netherlands. At the age of 3 weeks (d21) and 6 weeks (d42) 3 groups of chickens (24 animals per group) were vaccinated by intramuscular (i.m.) injection of 0.5 ml antigen consisting of Sf9 cell culture supernatants containing 4 μg sHA3-MSD, sHA3-VN11 or sHA3-Eg08, respectively, with nGNE as adjuvant. As a control, one group of 20 animals was mock vaccinated twice with PBS/nGNE. Blood was taken on the day of the second vaccination (d42) and 3 weeks after the second vaccination (d63). Three weeks after the second vaccination (d63), the animals of each group were divided into 4 groups of 6 animals (5 animals for the non-

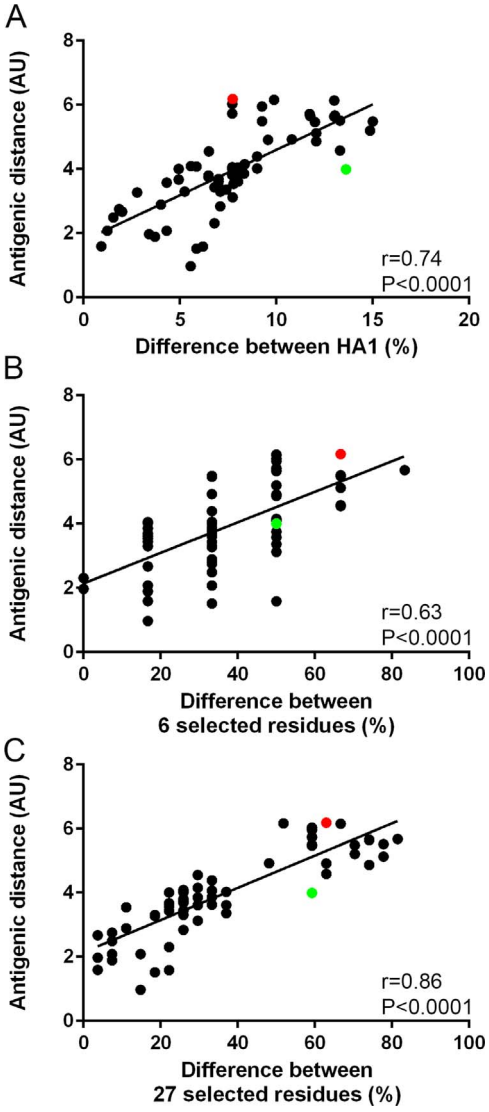

Fig. 3. Correlation between antigenic distance and genetic differences. Scatterplots of genetic differences between H5 protein pairs (indicated in % different amino acids) for the complete HA1 domains (A), for 6 selected residues surrounding the receptor binding site (based on (Koel et al., 2014)) (B), and for 27 selected residues in HA1 (C) and mean antigenic distances (shown in Table 2). The Pearson correlation coefficients r and the significance of the correlations are indicated. Red and green dots correspond to Eg08-Eg10 and Eg08-VN0408 pairs, respectively. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

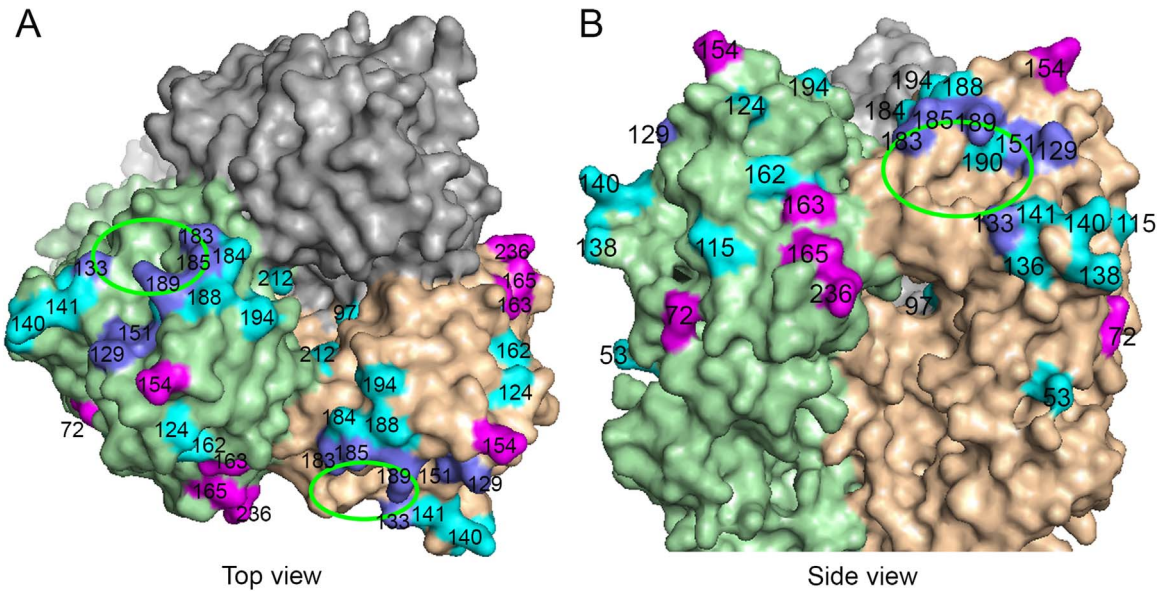

Fig. 4. 3D structure of HA. Top (A) and side (B) views of the structure of HA protein of A/Vietnam/1194/04 (VN04) generated with PyMol software are shown (PDB 2IBX). Each HA monomer is coloured differently. 6 residues identified previously to be important for the antigenic recognition of clade 2.1 H5 proteins (Koel et al., 2014) are coloured dark blue. Putative N-glycosylation sites in HP H5 proteins are coloured purple. Other residues that were previously reported to be important for antigenic recognition (Cattoli et al., 2011b; Duvvuri et al., 2009; Kaverin et al., 2007, 2002; Lee et al., 2004) and that are not strictly conserved between the HP H5 proteins analysed are coloured light blue. The position of the receptor binding site is indicated by the green oval. The numbering corresponds with the numbering shown in Supplementary Fig 3 and Supplementary Table 4. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

vaccinated controls) and inoculated with 10^5 TCID50 challenge virus (TT05, TT05-H5(VN11), TT05-H5(Eg08) and TT05-HA(VN0408), respectively) by the combined intranasal (i.n.) and intratracheal (i.t.) route (0.1 ml each).

2.7. Quantitation of virus excretion

Swabs were taken from the choana and cloaca of all birds at days 1, 2, 3, 5, 7 and 10 after challenge. RNA was extracted from swabs and viral RNA was quantitated by matrix-gene real-time reverse transcriptase PCR as described (Fouchier et al., 2000; Spackman et al., 2002). PCR cycle threshold values were converted to TCID50 values using a standard curve consisting of serial dilutions of RNA isolated from a carefully titrated stock of TT05.

2.8. Phylogenetic and statistical analyses

All full-length and unique HP H5 sequences were downloaded from the Genbank and GISAID databases. H5 protein trees were constructed by using the PHYLIP Neighbor Joining algorithm using the F84 distance matrix. This tree was used as a guide-tree to select H5 sequences representing all main branches of the tree. The selected H5 proteins were used to construct a summary-tree of similar topology as the guide-tree. The reliability of phylogenetic inference at each branch node was estimated by the bootstrap method with 1000 replications; evolutionary analysis was conducted using Molecular Evolutionary Genetics Analysis (MEGA) software version 5 (Tamura et al., 2011). Graphpad Prism software was used to calculate the Pearson r correlation coefficient and the corresponding p values to determine the correlation between variables.

3. Results

3.1. Correlation between genetic and antigenic distance

In order to examine the correlation between the genetic and antigenic distances of currently circulating H5N1 viruses of different clades, we first performed a detailed phylogenetic analysis (Supplementary Fig 2). In agreement with previous analyses (Neumann et al., 2010; Skeik and Jabr, 2008; Wei et al., 2012; WHO/OIE/FAO, 2012), the phylogenetic tree demonstrates the enormous diversification of H5N1 into different first order clades as well as numerous new subclades of the second-, third-, and fourth-order, mostly within clades 1 and 2. Based on this analysis, we selected a number of representative viruses from each of the most prominent H5N1 clades (Supplementary Fig 2 and Table 1). The HA genes of these viruses were used to generate isogenic 7+1 clade-specific PR8-H5 viruses, which only differed in the amino acid sequence of their HA proteins, and recombinant soluble trimeric sHA3 proteins that were expressed in HEK293T cells. In addition, we included a clade 2.2 HA consensus sequence (referred to as MSD05) in our analysis that except for 2 amino acid residues, of which 1 is located in HA1 (S196), is identical to A/duck/Wels/2025/2006 (Supplementary Fig 2). We were unable to generate the intended clade 2.1.3.2 recombinant virus containing the A/Indonesia/5/05 HA gene, and this HA protein could hence not be included in our analysis. Also a PR8 virus carrying the clade 2.3.4 Anhui05 LP HA gene could not be generated, hence we generated a recombinant virus in the TT05 background with the HP version of this HA gene.

By using formalin-inactivated preparations of the recombinant PR8-H5 viruses for the immunization of chickens we obtained a set of antisera that differed only in the composition of HA-specific antibodies. In addition, another set of antisera was generated by immunizing chickens with the different sHA3 proteins. Using these two sets of antisera and the PR8-H5 viruses, we performed HI and VN tests and calculated antigenic distances between the different H5 proteins as described previously (Sitaras et al., 2014) and as detailed in the Materials and Methods section for each of the 4 datasets (Supplementary Table 2A-D). The TT05 virus with the Anhui05 HA protein was only used in the VN assay, as this recombinant virus displayed very low hemagglutinating activity. The Pearson correlation coefficient was calculated to determine the correlation between the different antigenic distance data sets (Supplementary Table 2E). Highly significant, positive correlations were observed between all 4 data sets, with r values ranging from 0.46 to 0.76. Fig. 1A shows that the mean antigenic distance values determined by VN for both sera sets correlated well with those determined by HI ($r = 0.83$; $p < 0.0001$). The mean antigenic distance values determined for each sera set (mean of VN

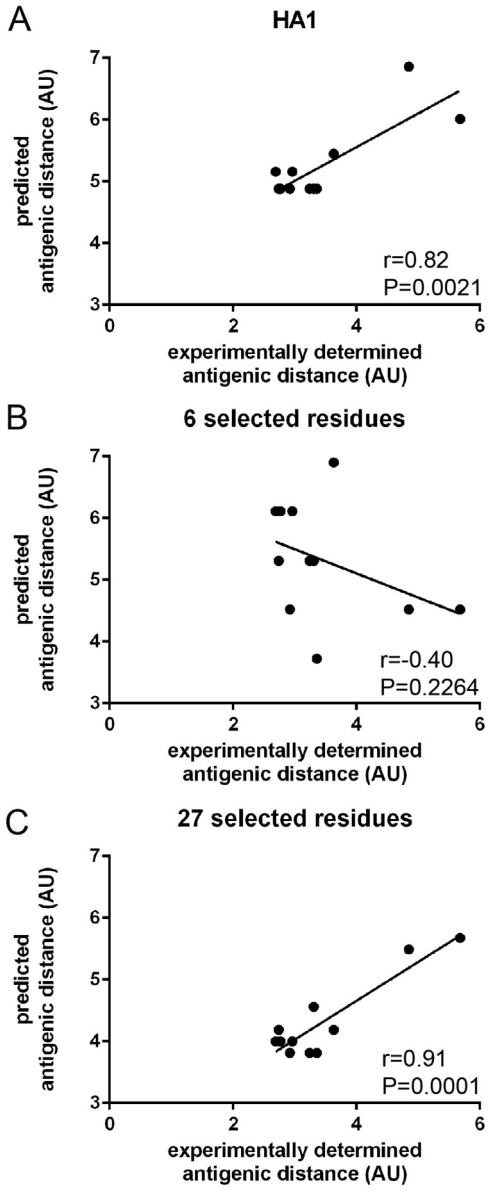

Fig. 5. Predicted antigenic distances for clade 2.3.4.4 H5N8 virus. Based on the correlations shown in Fig. 3, the antigenic distances between the H5 proteins of the novel clade 2.3.4.4 H5N8 virus and the other viruses used in this study were predicted by interpolation using the Graphpad Prism software. The predicted antigenic distances based on differences in the entire HA1 (A), 6 selected residues according to (Koel et al., 2014) (B) or 27 selected residues (C) were correlated to the experimentally determined antigenic distances (based on HI using the PR8-H5 sera; Table 2). The Pearson correlation coefficients r and the significance of the correlations are indicated.

and HI) also correlated well (r=0.81; $p < 0.0001$) (Fig. 1B).

As averaging of the antigenic distance data sets reduced scatter and increased correlations (Fig. 1 and Supplementary Table 2E), we generated a mean antigenic distance table that incorporated all antigenic distance data (Table 2). Subsequently, we used this table to generate an antigenic distance dendrogram by hierarchical clustering (Fig. 2). Comparison between the clustering of the different HA proteins based on the phylogenetic tree shown in Supplementary Fig 2 (Fig. 2A) and the antigenic distances (Fig. 2B) showed that the clustering of HA proteins was more or less conserved, with the notable exception of Eg08, Anhui05 and VN08.

In addition, we determined the correlation between the genetic distance (percentage amino acid differences in HA1) and the antigenic distance (Fig. 3A; for individual values see Supplementary Table 3). In general, the genetic and antigenic distances correlate well (r=0.72; $p < 0.0001$). However, in agreement with the results of hierarchical clustering, it is also clear that some H5 pairs deviated from their predicted positioning in the scatter plot. For example, although the Eg08-Eg10 pair differs at only 7.76% of their amino acid residues in HA1, their antigenic distance is approximately 6 AU. In contrast, the Eg08-VN0408 pair differs at more than 13% of their amino acid residues in HA1, but displays an antigenic distance of only approximately 4 AU.

Next, we analysed whether the correlation between genetic and antigenic distances could be improved by focussing on selected residues that were previously identified as being important for antigenic recognition. First, we focussed on a set of 6 residues in HA surrounding the receptor binding site, which were previously reported to be of crucial importance for the antigenic recognition of clade 2.1 H5 proteins (Koel et al., 2014) (Fig 4 and Supplementary Fig 3). Variation at these 6 positions correlated however to a somewhat lower extent (r=0.63; $p < 0.0001$; Fig. 3B) than the variation in the complete HA1 (Fig. 3A). Extension of this small set of residues with 21 other residues in subtype H5 HA1 that were previously reported to be important for antigenic recognition (Cattoli et al., 2011b; Duvvuri et al., 2009; Kaverin et al., 2007, 2002; Lee et al., 2004) and that are not strictly conserved between the HP H5 proteins analysed (resulting in a total set of 27 residues; Supplementary Fig 3, Fig. 4 and Supplementary Table 4), increased the correlation with antigenic distance significantly (r=0.86; $p < 0.0001$; Fig. 3C). We conclude that although genetic and antigenic distances generally correlate well, the genetic distance per se is not necessarily a very reliable predictor for antigenic distance between individual virus pairs. Correlation between genetic and antigenic distances is increased, however, by focussing on a limited set of 27 residues in HA1 that have previously been reported to be important for antigenic recognition rather than on complete HA1.

Knowledge about the correlation between genetic and antigenic distances may be useful for predicting the antigenic distances between a novel virus and viruses used in this study. To determine the predictive value of the different correlations shown in Fig. 3, we determined the antigenic distances between the H5 proteins of the novel clade 2.3.4.4 H5N8 virus and the other viruses used in this study by interpolation using the standard curves shown in Fig. 3A-C. The predicted values were subsequently correlated with the antigenic distance values as determined experimentally for the H5N8 virus based on HI of a recombinant PR8 virus carrying the clade 2.3.4.4 H5 protein (Fig. 5). Although the predictions made on the basis of the genetic variation in the complete HA1 correlated well with the antigenic distances as determined by HI for this particular virus (r=0.82), this correlation was even higher when based on the 27 selected residues (r=0,91). In contrast, predictions based on the variation in only the 6 residues identified by Koel et al. (Koel et al., 2014) resulted in a negative correlation with the experimentally determined antigenic distances (r=−0,40).

3.2. Correlation between antigenic distance and in vivo cross-protection

To examine the extent to which the H5 genetic and antigenic distances correlate with in vivo cross-protection, we performed an animal experiment, in which chickens were vaccinated twice with sHA3 and challenged 3 weeks after the last vaccination with highly pathogenic virus from different H5N1 clades. To this end, we selected a number of sHA3 vaccines and highly pathogenic challenge strains to study the effect of a low, intermediate or high antigenic distance on in vivo cross-protection. The vaccines consisted of baculovirus-expressed sHA3 from strains MSD05, VN11 and Eg08, whereas the set of challenge strains consisted of TT05 (generated by reverse genetics) and three isogenic 7+1 TT05-H5 recombinants carrying the HA gene from VN11, Eg08, and VN0408, respectively. Antigenic distances for heterologous vaccine-virus pairs ranged from 1.59 (MSD05-TT05) to

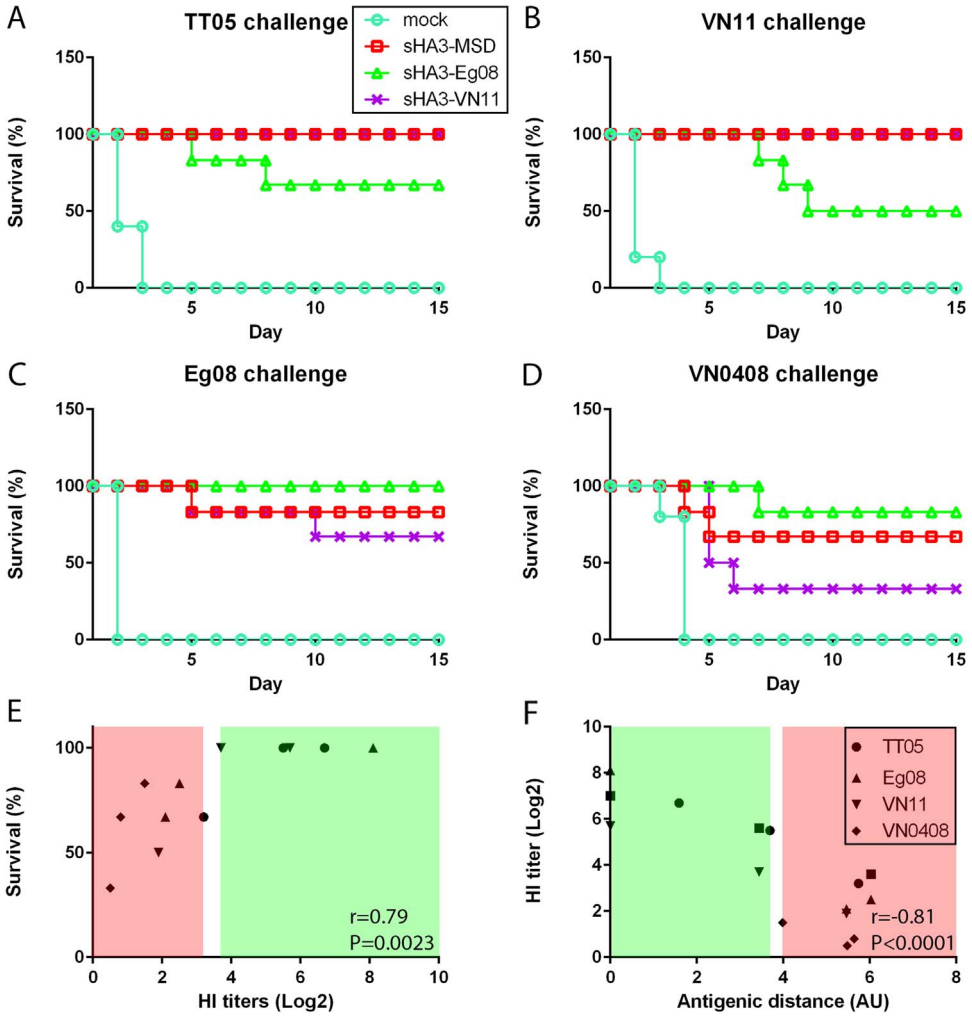

Fig. 6. Survival of chickens receiving different vaccine-challenge combinations. Chickens were vaccinated twice with the indicated sHA3 proteins and subsequently challenged with HP TT05-H5 recombinant viruses. Survival curves are shown for chickens challenged with TT05-H5 recombinants containing the TT05 (A), VN11 (B), Eg08 (C) or the VN0408 (D) H5 protein. Mean HI titres (homologous and heterologous) of the sera of these groups of animals against the indicated PR8-H5 viruses were correlated with survival (E) and with antigenic distance (F). The Pearson correlation coefficients r and the significance of the correlations are indicated. Parts of the graphs that contain groups that were 100% protected or less than 100% protected against the lethal challenge were coloured green and red, respectively. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

6.03 (MSD05-Eg08) AU (Table 2). Fig. 6 shows the survival curves of vaccinated animals and non-vaccinated control animals after challenge with the different highly pathogenic TT05-based viruses. Whereas all non-vaccinated animals died within 2–4 days, survival of the vaccinated animals was dependent on the particular combination of vaccine and challenge strain. As expected, survival correlated with HI titres of sera of the vaccinated animals against the challenge viruses at the day of challenge (Fig. 6E). Chickens were protected against lethality when the mean HI titres of a group were 3.7 or higher. As expected, the HI titres of these vaccinated chickens were shown to inversely correlate with the antigenic distances (Fig. 6F).

All vaccines provided 100% homologous protection, but the level of heterologous protection was dependent on the specific vaccine-challenge virus pair (Fig. 6). The data indicate, at least under the conditions of this vaccination-challenge experiment, that 100% protection against mortality was achieved when the antigenic distance between vaccine and challenge strain (Table 2) was less than 4 AU (Fig. 7A). Survival also correlated with the genetic distances between vaccine and challenge virus based on complete HA1 (Fig. 7B), and the 27 (Fig. 7C) and 6 (Fig. 7D) selected residues. Survival correlated best with genetic distances based on the 27 selected residues that were previously shown to be important for antigenic recognition. When survival was correlated with the genetic distance based on complete HA1 or on the 6 residues, the absolute r value was somewhat lower and the window between groups of animals that were fully protected and those with lower than 100% protection was smaller than when genetic distances were based on the 27 selected residues.

3.3. Correlation between antigenic distance and virus shedding

Shedding of challenge virus was analysed by determining the amount of viral RNA in choana and cloaca swabs by means of RT-PCR. The data showed that non-vaccinated animals shed high amounts of virus before they died at 2–4 days post challenge (Fig. 8A and B). Virus titres in cloaca swabs were lower than in choana swabs, but overall they showed a good correlation (r=0.67; p=0.0048) (Supplementary Fig 4A). Vaccinated animals showed a clear reduction in virus shedding and, as expected, the lowest virus titres were seen for the homologous combination. For the cloaca swabs (Fig. 8D), but not for the choana swabs (Fig. 8C), a significant correlation between the antigenic distance and the amount of virus that was shed could be observed, with the exception of sHA3-VN11 vaccinated animals that were challenged with TT05-VN0408 (r=0.47; P=0.1175 or r=0.72; P=0.0133 with or without inclusion of the TT05-VN0408 vaccine-challenge virus pair, respectively). In this case virus shedding was significantly higher than in sHA3-MSD-vaccinated animals (Figs. 8A

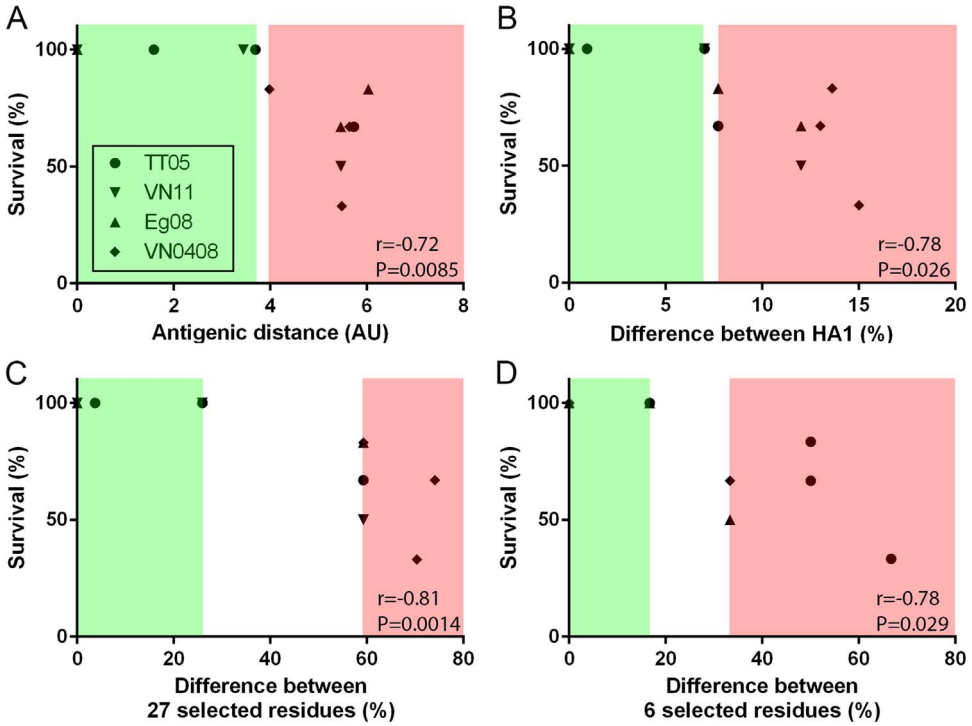

Fig. 7. Correlation of survival with antigenic distance and genetic differences. Chickens were vaccinated twice with the indicated sHA3 proteins and subsequently challenged with HP TT05-H5 recombinant viruses containing the TT05, VN11, Eg08 or the VN0408 H5 protein. Survival of the chickens (as shown in Fig. 6) was correlated with antigenic distance (as shown in Table 2) (A), and with differences (%) between the H5 proteins of the sHA3 vaccine and the challenge virus in HA1 (B), 27 selected residues (C), and 6 selected residues according to [30] (D). The Pearson correlation coefficients r and the significance of the correlations are indicated. The parts of the graphs that contain groups that were 100% protected or less than 100% protected against the lethal challenge were coloured green and red, respectively. Challenge viruses are indicated in the box. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

and B) despite the fact that the antigenic distances between the vaccines and the challenge strains were very similar (5.64 for MSD vs VN0408; 5.48 for VN11 vs VN0408; Table 2). Statistically significant correlations between virus shedding and HI titres could be observed for cloaca and choana swabs (Supplementary Fig. 4B and 4C), indicating that high (homologous or heterologous) HI titres protect against shedding. In agreement herewith, virus shedding also correlated with survival (Supplementary Fig. 4D and 4E).

4. Discussion

H5N1 HPAIVs have displayed a remarkable genetic diversification since their re-emergence in 2003, resulting in their classification into more than 10 first-order clades and numerous higher order clades. While those genetic classifications are very useful particularly for determining evolutionary relationships between these viruses, their use for making antigenic comparisons, in particular for selecting the appropriate strains for vaccine development, is not *a priori* obvious. In the present study we analysed the correlation between genetic and antigenic distances for more than 10 (contemporary) Eurasian-lineage H5N1 HPAIVs belonging to different higher-order clades and determined the correlation thereof with vaccine efficacy in chickens for selected vaccine-challenge strain combinations. Our results indicate that genetic and antigenic distances do not always correlate and that some viruses deviate considerably from the expected antigenic distance relative to other viruses. This deviation was less when genetic distances were not based on variation in the entire HA1, but limited to a selection of 27 residues out of the many residues that were previously reported to be important for antigenic recognition. Focussing on only the 6 residues surrounding the receptor binding site that were identified earlier (Koel et al., 2014) as being important for antigenic recognition did not result in improved correlation with antigenic distance. In agreement herewith, antigenic distances could better be predicted for the novel clade 2.3.4.4 H5N8 virus when based on the 27 selected residues than on the basis of the entire HA1 or of the 6 residues surrounding the receptor binding site. Recombinant soluble sHA3 vaccine preparations were shown to protect chickens against a lethal challenge with heterologous HPAIV H5N1 strains when the antigenic distance between vaccine and challenge strain did not exceed 4 AU. Protection also correlated better with genetic distances based on the 27 selected residues in HA1, than on the entire HA1 sequence. With one exception, antigenic distance correlated well with shedding of virus in the cloaca, not with that in the choana. These results provide novel insights in the correlation between genetic and antigenic distances on the one hand and vaccine efficacy on the other, and offer opportunities to better predict vaccine performance against novel HPAIV H5 viruses.

Scatterplots of genetic and antigenic distances between HA proteins (Fig. 3) and hierarchal clustering of the different HA proteins based on these genetic or antigenic distances (Fig. 2) revealed that genetic distance does not necessarily correlate well with antigenic distance. Notably, the HA proteins of Eg08 (clade 2.2.1.1) and Eg10 (2.2.1) were found to differ more in their antigenicity (6.17 AU) than was expected based on their HA1 genetic distance (Fig. 3A). This is in agreement with observations of others that viruses belonging to clade 2.2.1.1 are antigenically quite distinct from their immediate ancestors (Abdelwhab et al., 2012; Balish et al., 2010; Beato et al., 2013; Cattoli et al., 2011a; Watanabe et al., 2012). Interestingly, the HA protein of Eg08 was antigenically more closely related to the HA protein of the clade 7.1 strain VN0408 (3.99 AU), even though these latter two strains display more differences at the amino acid level in the HA1 domain than the Eg08-Eg10 pair.

While several studies have identified residues in HA1 of H5 that are important for antibody binding (Cattoli et al., 2011b; Duvvuri et al., 2009; Kaverin et al., 2007, 2002; Lee et al., 2004), it is not clear to what extent changes at these positions correlate with antigenic distances. We now show that the correlation between genetic and antigenic distances

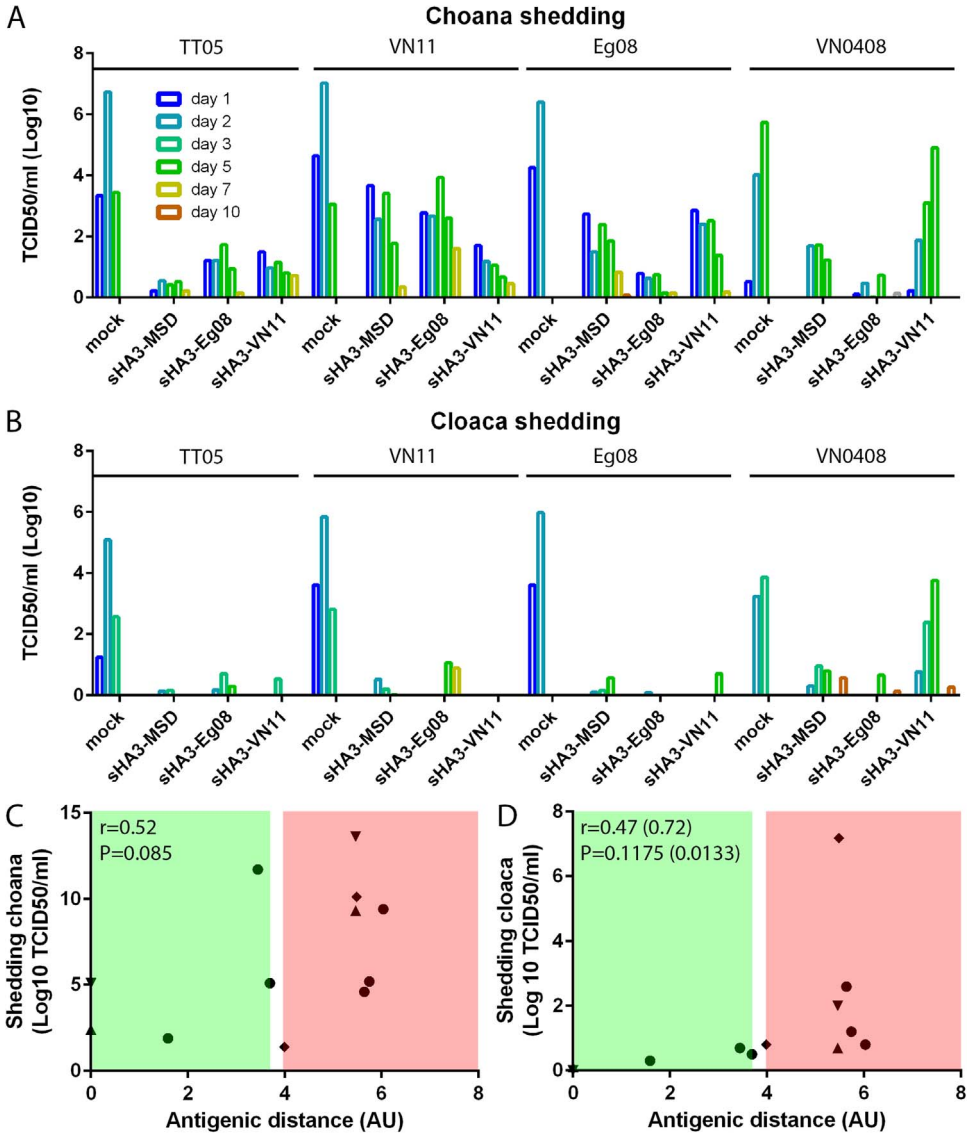

Fig. 8. Shedding of virus by vaccinated animals. Chickens were vaccinated twice with the indicated sHA3 proteins and subsequently challenged with HP TT05-H5 recombinant viruses containing the TT05, VN11, Eg08 or the VN0408 H5 protein. Virus shedding in choana (A) or cloaca (B) is graphed. Cumulative shedding in choana (C) or cloaca (D) was correlated with antigenic distance (as shown in Table 2). The Pearson correlation coefficients r and the significance of the correlations are indicated. The values obtained when TT05-VN0408 vaccine-challenge virus pair was excluded from the analysis are indicated between brackets. The parts of the graphs that contain groups that were 100% protected or less than 100% protected against the lethal challenge were coloured green and red, respectively. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

increased when genetic distances were based on a set of 27 selected residues in HA1 (Figs. 3 and 4 and Supplementary Fig 3) rather than on complete HA1. All but one of the selected residues (position 226) are surface-exposed at the HA1 globular head domain and most of them are closely located to the receptor binding site in the same or the adjacent HA monomer, while variation at/close to some of these residues will result in differential N-linked glycosylation. N-linked glycans may interfere with antibody recognition (Kim and Park, 2012; Tate et al., 2014), but may also affect folding of the HA trimer. Of note, of the 5 potential N-glycosylation sites belonging to the 27 selected residues, the Eg08-Eg10 pair (antigenic distance 6.17 AU) and the Eg08-VN0408 pair (antigenic distance 3.99 AU) differ at 4 and 1 of these, respectively (Supplementary Fig 3 and Supplementary Table 4). When limiting genetic distances only to the variation observed for the 6 residues that were previously identified to be important for antigenic variation in clade 2.1 H5N1 viruses, correlation with antigenic distances was not improved. Although the molecular basis for antigenic change of clade 2.1. viruses closely resembles that of seasonal human influenza viruses (Koel et al., 2013, 2014), our results suggest that other residues are also important. It will be of interest to analyse in more detail, which residues in H5 proteins contribute to antigenic variation and whether these residues are of equal importance for viruses belonging to other clades. Based on the correlation between antigenic and genetic distances we were able to accurately predict the antigenic distances between the novel clade 2.3.4.4 H5N8 viruses and viruses belonging to other clades. Again, these predictions correlated better with the experimentally determined antigenic distances when they were based on the 27 selected residues in HA1. Such predictions may be of importance when selecting vaccine strains in outbreak situations, when there is insufficient time to grow and antigenically characterize these viruses in detail.

Survival of chickens after challenge with a heterologous HPAIV H5N1strain correlated with several parameters. As expected, survival correlated well with HI titres that were detected in the vaccinated animals against the challenge viruses at the day of challenge. Groups of animals with HI titres higher than 3.7 were fully protected, in agreement with the generally accepted notion that HI titres higher than 4 correlate with protection (OIE, 2012). Recently, it was shown

that HI titres higher than 3 provide protection to transmission because of a reduction in infectivity (Sitaras et al., 2016). Antigenic distance between vaccine and challenge strain was shown to inversely correlate with HI titres and with survival. Full protection against heterologous challenge was observed when the antigenic distance measured with this set of sera was lower than 4 AU. Although genetic distance generally correlated well with protection, correlations improved (with a higher r, lower P, and larger window between fully and partially protected groups) when the antigenic distances were based on the antigenically important residues, which were also used to accurately predict the antigenic distance of H5N8 towards other HPAIV H5N1. Also, shedding correlated with survival, with least shedding being observed when homologous vaccine-challenge strain combinations were used. The correlation between antigenic distance and shedding was less obvious. No significant correlation was observed between antigenic distance and shedding in choana. Antigenic distance appeared to correlate well with shedding in cloaca, with one exception (sHA3-VN11 vaccine and VN0408 challenge). Why relatively high levels of VN0408 shedding were detected after vaccination with sHA3 of VN11, but not MSD (despite similar antigenic distances between vaccine and challenge strain) is unclear.

Based on the antigenic distance table generated in this study (Table 2) we can predict for individual sHA3-virus combinations whether chickens will be protected. Although it remains to be established to what extent the antigenic distances as generated in this study are predictive for the protective capacities of other vaccines, a similar antigenic profiling approach as performed in this study may be used to predict the protective capacity of other (commercially available) vaccines. Furthermore, although antigenic and genetic distances do not necessarily correlate, our results indicate that such correlations may be improved by selectively including those residues in HA1 that are known to be important for antigenic recognition. Analyses as performed in this study may thus allow to accurately predict, based on sequence analysis, the protective capacity of heterologous vaccine-virus strain combinations.

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Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.virol.2017.01.012.

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