Progressive glycosylation of the haemagglutinin of avian influenza H5N1 modulates virus replication, virulence and chicken-to-chicken transmission without significant impact on antigenic drift

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Highly pathogenic H5N1 avian influenza virus (A/H5N1) devastated the poultry industry and continues to pose a pandemic threat. Studying the progressive genetic changes in A/H5N1 after long-term circulation in poultry may help us to better understand A/H5N1 biology in birds. A/H5N1 clade 2.2.1.1 antigenic drift viruses have been isolated from vaccinated commercial poultry in Egypt. They exhibit a peculiar stepwise accumulation of glycosylation sites (GS) in the haemagglutinin (HA) with viruses carrying, beyond the conserved 5 GS, additional GS at amino acid residues 72, 154, 236 and 273 resulting in 6, 7, 8 or 9 GS in the HA. Available information about the impact of glycosylation on virus fitness and pathobiology is mostly derived from mammalian models. Here, we generated recombinant viruses imitating the progressive acquisition of GS in HA and investigated their biological relevance in vitro and in vivo. Our in vitro results indicated that the accumulation of GS correlated with increased glycosylation, increased virus replication, neuraminidase activity, cell-to-cell spread and thermostability, however, strikingly, without significant impact on virus escape from neutralizing antibodies. In vivo, glycosylation modulated virus virulence, tissue tropism, replication and chicken-to-chicken transmission. Predominance in the field was towards viruses with hyperglycosylated HA. Together, progressive glycosylation of the HA may foster persistence of A/H5N1 by increasing replication, stability and bird-to-bird transmission without significant impact on antigenic drift.

INTRODUCTION

Influenza A viruses, members of the RNA virus family Orthomyxoviridae, infect a wide spectrum of hosts including mammals and birds with inter- and intra-species transmission. Influenza virus infection in humans is commonly controlled by vaccination and antivirals (e.g. oseltamivir and amantadine) (Webster & Govorkova, 2014). In poultry, depopulation of infected birds is recommended for the effective and rapid containment of avian influenza virus (AIV) outbreaks. However, the costs of culling are a major burden on the poultry industry particularly in developing countries. Therefore, extensive vaccination has been applied in several Asian and African countries to mitigate the socioeconomic impact of AIV in poultry. Although vaccination was generally successful to limit virus spread, the virus became enzootic in poultry in China, India, Bangladesh, Egypt, Indonesia and Vietnam (Swayne et al., 2011). Most of our knowledge about continuous evolution of influenza viruses in the face of vaccination is driven by studying the gradual genetic changes of human seasonal influenza viruses in vitro and by infection studies in mammal animal models (e.g. ferrets, mice). Little is known about the biological relevance of genetic changes fostering enzootic circulation of AIV in chickens where vaccination is extensively used.

Sequences generated in this study were assigned accession numbers in the GISAID: EPI824801–EPI824808.

One supplementary table is available with the online Supplementary Material.
In early 2006, highly pathogenic AIV (HPAIV) H5N1 (A/H5N1) of clade 2.2.1 was first reported in poultry and humans in Egypt. Severe socio-economic losses pushed the poultry industry towards the application of a blanket-vaccination strategy using several non-local H5 virus vaccines (Abdelwhab et al., 2016). During the last 10 years, viruses isolated from vaccinated birds, designated as clade 2.2.1.1, underwent significant antigenic drift due to immune pressure (WHO/OIE/FAO H5N1 Evolution Working Group, 2014). Few mutations in or adjacent to the immunogenic epitopes in the haemagglutinin (HA) protein were sufficient for the escape of these antigenic drift variants from humoral immune response (Cattoli et al., 2011) which may explain the increased prevalence of infections in vaccinated poultry (Abdelwhab et al., 2011; Arafa et al., 2012). In addition to the extensive alterations in the immunogenic epitopes, N-glycosylation patterns (N-linked glycans attached at a nitrogen of asparagine side chain and predicted as N-X-S/T motif, where X can be any amino acid except proline) were observed (Abdelwhab et al., 2012). The significance of this unique glycosylation pattern of A/H5N1 viruses in clade 2.2.1.1 has not been yet studied. It has been previously shown that alterations in the number and location of glycosylation of the HA of (human) influenza viruses are a viral strategy to evade the host immune response, modulate virulence and/or enhance receptor binding avidity/specificity (Kim & Park, 2012; Klenk et al., 2002; Matrosovich et al., 1999; Owen et al., 2007). These studies have mostly been performed in mice and ferrets, and only limited knowledge is available for poultry.

Therefore, in this study, we investigated the biological relevance of the increased number of HA glycosylation sites (GS) of the Egyptian A/H5N1 viruses using recombinant viruses generated by reverse genetics.

**RESULTS**

In this study, we generated four recombinant AIVs with different HA glycosylation patterns resembling the 2.2.1.1 A/H5N1 viruses isolated from vaccinated chickens in 2007–2014 in Egypt by reverse genetics using A/chicken/Egypt/NLQP-0879/2008 (H5N1) (designated hereafter as wtG8). The HA gene of wtG8 contains GS at positions 72–74, 154–156 and 236–238 and was used to generate three different H5N1 HPAIV recombinants which contain less GS (G6 contains GS at position 72–74 and G7 contains GS at positions 72–74 and 154–156) or more GS (G9 contains GS at positions 72–74, 154–156, 236–238 and 273–275). Using the mutated HA genes, four corresponding mutants with monobasic CS were generated (designated herein as LpG6, LpG7, LpG8 and LpG9). The impact of glycosylation on virus replication, virulence, transmission and escape from the host immune response was investigated *in vitro* and/or *in vivo*.

**Generation of recombinant viruses**

Four viruses designated G6, G7, G8 and G9 and four corresponding viruses with monobasic cleavage sites (LpG6–LpG9) were rescued in 293T/MDCK (Madin–Darby canine kidney) cells. All Egyptian strains including those generated in this study carry the same six potential GS like G6, G7, G8 and G9 and carry one, two and three additional GS. G6–G9 differ correspondingly in their glycosylation pattern as indicated by the change in electrophoretic mobility of the HA protein on a polyacrylamide gel (Fig. 1a).

**Deglycosylation of the H5N1 HA protein decreased virus replication, neuraminidase (NA) activity, plaque size and thermostability *in vitro* without apparent impact on antigenic drift**

To investigate the impact of progressive glycosylation *in vitro*, we compared the replication kinetics, NA activity, plaque size and heat stability of the rescued recombinant viruses. The replication of G8 and G9 in CEK (chicken embryo kidney) as well as in DF1 at 37 °C was accelerated at each time point. The level of replication of G8 and G9 was approximately 10–100-fold higher than G6 and G7 particularly after 24 h (Fig. 1b, c). Moreover, G7 replicated to significantly higher titres (*P*<0.05) than G6 at 8 and/or 24 h post-infection (p.i.) in CEK or DF1, respectively. Likewise, G9 reached 10-fold higher titres than G8 at 8 h p.i. in CEK. At 39 °C, G8 and G9 produced 10–100-fold higher titres than G6 and G7 at 8 and 24 h p.i. but no statistically significant difference was observed at 48 and 72 h p.i. (Fig. 1d). The efficiency of G8 to enter the cells was higher than in the other recombinant viruses: 88% of G8 required only 5 min to enter the cells. Although G9 was less efficient than G8, it entered cells faster than G6 and G7, while the latter entered the cells faster than G6 (Fig. 1e). The quantity of virus released into the supernatant of CEK cells after single or multiple replication cycles was significantly higher (*P*<0.05) in G8 and G9 than in G6 and G7 (Fig. 1f). Similar results were obtained after titration of the virus from harvested cells, particularly at 8 h p.i. (data not shown). Estimating the NA activity of recombinant viruses showed that G8 and G9 had significantly higher activity than G6 and G7 (*P*<0.05) (Fig. 1g). Remarkably, G7 showed lower NA activity than G6. Plaque sizes correlated with the number of GS, and the average plaque sizes of G7, G8 and G9 were 1.9, 3.0 and 3.3 times larger than those produced by G6 (Fig. 1h). Moreover, incubation of viruses in 50 °C for up to 5 h indicated that G8 and G9 exhibited increased heat stability compared with G6 which lost infectivity dramatically (Fig. 1i). G7 was more stable than G6 but less than G8 and G9 particularly after 4 h. Likewise,
the haemagglutinating activity of G8 and G9 was more stable than G6 and G7 (data not shown).

To study the impact of progressive glycosylation on antigenic drift, LpG6–LpG9 were tested against anti-H5 serum samples using haemagglutination inhibition (HI) test. No remarkable differences in reactivity against homologous G6–G9 sera were observed. G6 serum showed 11, 11, 10 and 11 log₂ HI titre against G6, G7, G8 and G9, respectively, whereas HI test using anti-G7 and anti-G9 sera produced 10, 11, 11 and 11 and 10, 11, 11 and 11 log₂ HI titres against G6, G7, G8 and G9. All viruses reacted well with serum raised against wtG8 (Table 1). Strikingly, regardless of the glycosylation pattern, all viruses were poorly neutralized (as seen by low HI titres compared with virus controls) by sera directed against H5 viruses from clades 1, 2.2, 2.2.1.2 and 2.3.4.4, and not at all by antiserum against H5N9 of American lineage (Table 1). G8 shared the lowest identity with the HA of H5N9 with 79.5 and 85.5 % nucleotide and amino acid identity, respectively (Table 1).

Together, deglycosylation of the HA decreased virus replication, NA activity, heat stability and plaque size in vitro. Differences in virus replication are most likely due to impaired virus entry as well as virus release. All viruses reacted poorly with sera against different H5 viruses indicating antigenic drift regardless of the glycosylation pattern.

**Glycosylation of the H5N1 HA protein modulated virulence, transmission, virus excretion and tissue distribution in chickens**

It had been shown previously that the introduction or deletion of GS resulted in differences in virulence of H5N1 and pandemic H1N1 in mice (Chen et al., 2007; Zhang et al., 2013). Therefore, we determined the mean chicken
lethal dose (CLD$_{50}$) of G6, G7, G8 and G9 as $10^{3.0}$, $10^{2.8}$, $10^{2.3}$ and $10^{3.3}$ p.f.u. per bird, respectively. Subsequently, four birds were inoculated oculonasally with $10^3$/C2 CLD$_{50}$ of the respective viruses and six birds were added to each group at 1 day p.i. to assess chicken-to-chicken transmission. All oculonasally infected birds died by days p.i. 3 (G8), 4 (G9), 6 (G7) and 7 (G6) yielding pathogenicity indices of 2.5, 2.5, 2.1 and 2.1 and a mean death time of 3, 3.3, 5.3 and 5.5 days, respectively. Likewise, all contact birds in G8, G9 and G6 died by days p.i. 5, 5 and 7, respectively (Table 2). G7 killed five out of six contact chickens at 8 (two chickens) and 9 (three chickens) days p.i., while one bird survived and remained healthy, although anti-NP antibodies were detected by ELISA at the end of the experiment. Moreover, virus excretion from oropharyngeal swabs was detected earlier and at slightly higher levels in G8- and G9-infected birds than in G6-infected birds (Table 2). In the G7-infected group, none of the contact chickens excreted virus whereas all inoculated birds excreted virus at 4 days p.i. Results of immunohistochemistry showed differences in antigen distribution in lungs and pancreas. In the lungs, the level of NP antigen correlated with the number of GS where G6 and G7 exhibited less NP antigen than G8 and G9, particularly in endothelial cells (Fig. 2a, d, g and j). G6, G8 and G9 but not G7 were detected in the pancreas (Fig. 2f). All viruses had a strong tropism for the brain, although G8 was more readily detected in neurons and glia cells than the other viruses (Fig. 2b, e, h and k). The level of NP antigen in other organs was comparable (data not shown).

**Table 1.** Impact of glycosylation on reactivity with different antisera

<table>
<thead>
<tr>
<th>Serum samples against</th>
<th>Clade</th>
<th>G6</th>
<th>G7</th>
<th>G8</th>
<th>G9</th>
<th>Homologous antigen</th>
<th>HA identity*</th>
</tr>
</thead>
<tbody>
<tr>
<td>A/Vietnam/1194/2004 (H5N1)</td>
<td>1</td>
<td>4</td>
<td>4</td>
<td>4</td>
<td>4</td>
<td>10</td>
<td>94.1/93.9</td>
</tr>
<tr>
<td>A/teal/Germany/WV632/2005 (H5N1)</td>
<td>2.2</td>
<td>5</td>
<td>4</td>
<td>5</td>
<td>11</td>
<td>88.3/88.6</td>
<td></td>
</tr>
<tr>
<td>A/chicken/Egypt/0879-NLQP/2008 (H5N1; wtG8)</td>
<td>2.2.1.1</td>
<td>14</td>
<td>13</td>
<td>13</td>
<td>13</td>
<td>11</td>
<td>100/100</td>
</tr>
<tr>
<td>A/chicken/Egypt/AR234-FAOF8NLQP/2014 (H5N1)</td>
<td>2.2.1.2</td>
<td>5</td>
<td>4</td>
<td>4</td>
<td>5</td>
<td>9</td>
<td>94.8/94.5</td>
</tr>
<tr>
<td>A/turkey/Germany-MV/R2472/2014 (H5N8)</td>
<td>2.3.4.4</td>
<td>3</td>
<td>3</td>
<td>3</td>
<td>4</td>
<td>9</td>
<td>89.9/90.4</td>
</tr>
<tr>
<td>A/duck/AB/329/2006 (H5N9)</td>
<td>American</td>
<td>&lt;1</td>
<td>&lt;1</td>
<td>&lt;1</td>
<td>&lt;1</td>
<td>9</td>
<td>79.5/85.5</td>
</tr>
<tr>
<td>Negative SPF serum</td>
<td>&lt;1</td>
<td>&lt;1</td>
<td>&lt;1</td>
<td>&lt;1</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

*Identity to G8 HA nucleotides/amino acids to given viruses. The HA gene of wtG8 was used to generate three different H5N1 HPAIV recombinants G6, G7 and G9.

MDT, mean death time; NA, not applicable because all birds died; PI, pathogenicity index.

**Table 2.** Mortality and virus excretion of G6–G9 inoculated and in contact birds

<table>
<thead>
<tr>
<th>Virus</th>
<th>CLD$_{50}$</th>
<th>PI</th>
<th>Birds</th>
<th>Mortality/infected animals</th>
<th>Mortality time range</th>
<th>MDT</th>
<th>Shedding* no. positive/no. examined (quantity p.f.u. ml$^{-1}$)</th>
</tr>
</thead>
<tbody>
<tr>
<td>G6</td>
<td>$10^{3.0}$</td>
<td>2.5</td>
<td>Infected</td>
<td>4/4</td>
<td>3–7</td>
<td>5.5</td>
<td>1/4 (2.7) 1/3 (2.5) 2/2 (2.4)</td>
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<tr>
<td></td>
<td></td>
<td></td>
<td>Contact</td>
<td>6/6</td>
<td>6–7</td>
<td>6.7</td>
<td>0/6 1/6 (2.0) 4/4 (2.8)</td>
</tr>
<tr>
<td>G7</td>
<td>$10^{2.8}$</td>
<td>2.5</td>
<td>Infected</td>
<td>4/4</td>
<td>5–6</td>
<td>5.3</td>
<td>0/4 4/4 (2.6) NA</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Contact</td>
<td>5/6</td>
<td>8–9</td>
<td>8.6</td>
<td>0/6 0/6 0/6</td>
</tr>
<tr>
<td>G8</td>
<td>$10^{2.3}$</td>
<td>2.1</td>
<td>Infected</td>
<td>4/4</td>
<td>3</td>
<td>3</td>
<td>1/4 (2.7) NA NA</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Contact</td>
<td>6/6</td>
<td>4–5</td>
<td>5.7</td>
<td>0/6 6/6 (3.1) NA</td>
</tr>
<tr>
<td>G9</td>
<td>$10^{3.3}$</td>
<td>2.1</td>
<td>Infected</td>
<td>4/4</td>
<td>3–4</td>
<td>3.3</td>
<td>3/4 (2.5) NA NA</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Contact</td>
<td>6/6</td>
<td>5</td>
<td>5</td>
<td>0/6 6/6 (2.3) NA</td>
</tr>
</tbody>
</table>

MDT, mean death time; NA, not applicable because all birds died; PI, pathogenicity index.

*Number of birds which excreted virus in oropharyngeal swabs using plaque test at 2, 4 and 6 days p.i. Between parentheses, the average of excreted viruses from positive birds expressed as p.f.u. ml$^{-1}$ was given. The HA gene of wtG8 was used to generate three different H5N1 HPAIV recombinants G6, G7 and G9.
Sequence analysis of the HA of A/H5N1 viruses indicated the uniqueness of the time-dependent glycosylation pattern in the HA1 head domain of Egyptian viruses

By comparison of sequences obtained from countries enzootic with A/H5N1 available at GenBank, a similar pattern of progressive glycosylation was not observed in 1538 sequences from Bangladesh (n=133), China (n=443), India (n=84), Indonesia (n=365) and Vietnam (n=513) (Table S1, available in the online Supplementary Material). Only 2 G7-like sequences and 17 G8-like sequences were observed in Indonesia and Vietnam, respectively. To study the prevalence of GS in the Egyptian viruses, 938 sequences were collected from the Global Initiative on Sharing All Influenza Data (GISAID) and GenBank databases and analysed. G6 appeared in 2007 as the first GS variant, while all viruses analysed in this study co-circulated in 2008–2009 when the prevalence was 53, 54 and 61 for G6, G8 and G9, respectively, out of 181 sequences in the 2.2.1.1 clade from 2007 to 2014. Only seven viruses resembling G7 were detected. This 2.2.1.1 clade was not detected after 2014 (Table 3). Phylogenetic analysis of the HA protein revealed clustering and co-circulation of G8 and G9 together in a group distinct from the ancestral G6 and G7 (Fig. 3). On the tertiary structure, it was predicted that the variable GS are located in the HA1 head domain and the conserved GS reside in the stalk domain of HA1 and HA2 (Fig. 4). GS72 is located within the immunogenic epitope E, and GS154 resides within the immunogenic epitope D at the tip of the head domain, very close to the receptor binding domain and antigenic sites, whereas GS236 is located on a side of the head domain closely related to the receptor binding domain. GS273 is located on the connection of head and stalk domain close to immunogenic epitope C (Duvvuri
Table 3. Prevalence of different glycosylation pattern of A/H5N1 2.2.1.1 from Egypt from 2006 to 2014

<table>
<thead>
<tr>
<th>Year</th>
<th>Total sequences</th>
<th>G6</th>
<th>G7</th>
<th>G8</th>
<th>G9</th>
</tr>
</thead>
<tbody>
<tr>
<td>2007</td>
<td>9</td>
<td>9</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>2008</td>
<td>65</td>
<td>36†</td>
<td>3‡</td>
<td>25</td>
<td>1</td>
</tr>
<tr>
<td>2009</td>
<td>43</td>
<td>10</td>
<td>5</td>
<td>6</td>
<td>22</td>
</tr>
<tr>
<td>2010</td>
<td>60</td>
<td>1</td>
<td></td>
<td>23§</td>
<td>36</td>
</tr>
<tr>
<td>2011</td>
<td>3</td>
<td>2</td>
<td>1</td>
<td></td>
<td></td>
</tr>
<tr>
<td>2014</td>
<td>1</td>
<td></td>
<td></td>
<td></td>
<td>1</td>
</tr>
<tr>
<td>Total</td>
<td>181</td>
<td>55</td>
<td>9</td>
<td>56</td>
<td>61</td>
</tr>
</tbody>
</table>

*Full HA protein length of different A/H5N1 viruses was collected from Influenza Virus Resource.
†Two sequences contain GS as G6 and at position 236.
‡Two sequences contain GS as G7 and at position 273.
§Two sequences contain GS as G8 and at position 165 or 193.

Moreover, the G6–G9 viruses have 16 amino acid differences compared with the HI antigens used in this study, while all mutations but one are in the head domain of the HA1 (Fig. 4).

DISCUSSION

Most of our knowledge about influenza virus adaptation in the face of vaccination is based on studies on seasonal influenza viruses and in vivo experiments using mammals. The enzootic A/H5N1 in poultry in Egypt where vaccination is extensively used to control the disease is a good opportunity to track progressive adaptive genetic changes in the viral genome after long-term residence in chickens. Here, we investigated the progressive accumulation of GS in the HA of A/H5N1 clade 2.2.1.1 viruses which caused severe losses in vaccinated commercial poultry in Egypt.

Variable glycosylation, in both location and number, of (human) influenza viruses particularly in the HA head domain is a strategy to mask antigenic sites for immune evasion and modulate receptor binding as well as virulence (Ohuchi et al., 1997b). In this study, the unique glycosylation pattern of the HA in the clade 2.2.1.1 A/H5N1 in poultry in Egypt was studied in vitro and in vivo. In vitro, the loss of two and one GS in G6 and G7, respectively, reduced the levels of virus replication and plaque sizes in cell culture compared to viruses with eight or nine GS. This may be due to decreased virus entry and/or decreased virus release, most likely due to the interaction of HA and NA with cell receptors. It is known that influenza virus entry is mediated by binding of the trimeric HA to sialic acid receptors on the cell surface, while NA is responsible for progeny virus release. A subtle balance between HA and NA activities is important for successful replication (Hulse et al., 2004; Mishin et al., 2005; Mitnaul et al., 2000; Thompson et al., 2004; Wagner et al., 2000). Therefore, the large oligosaccharide side chains in the HA may interfere with receptor binding by steric hindrance in the same monomer or inter- or intratrimeric structures (Ohuchi et al., 1997a; Tate et al., 2014). Similar to the results in the current study, loss of N-glycans adjacent to the HA receptor binding site (i.e. residues 123 and/or 149 corresponding to residues 129 and 154 in this study, respectively) of a historic H7N1 decreased virus release, reduced virus titres from infected cells and produced smaller plaques (Wagner et al., 2000) which were attributed to a highly impaired release of progeny virions due to an increased affinity of the HA for the cell receptor (Wagner et al., 2000). This effect was abrogated by an increased NA activity using more active NA (Wagner et al., 2000). Also, hyperglycosylation in the H1N1 HA head domain (residues 131 and 144 corresponding to residues 129 and 140 in this study, respectively) decreased the receptor binding avidity of the virus and required compensatory mutations in the HA and/or NA (Das et al., 2011). Thus, we assume that high titres of G8 and G9 were due to decreased affinity to cell receptor and increased NA activity. However, the impact of glycosylation on other steps in the replication cycle (e.g. pH activation, trafficking and assembly) (Hebert et al., 1997; Schuy et al., 1986) cannot be excluded. Loss of glycosylation of the HA also influenced thermostability which may be important for proper protein folding at higher temperature or establishing infection in inner organs of infected birds. It has been shown previously that glycosylation (mostly in the stem region) of the HA increased thermostability of AIV (Wagner et al., 2002; Zhang et al., 2015b).

One of the major biological consequences of HA glycosylation is to mask antigenic recognition sites and thus results in evasion of the virus from antibody-mediated immune response (Li et al., 2013; Wang et al., 2009). Surprisingly, and contrary to expectation, all our mutants, irrespective of their glycosylation pattern, reacted poorly with heterologous sera from different clades/lineages indicating significant antigenic drift. This drift was not due to the glycosylation at positions 72–74, since glycosylation at this position alone did not alter the antigenicity of the Egyptian viruses, and other mutations directly in the immunogenic epitopes (e.g. residues 140, 141, 144, 162 and A184E) were required for significant evasion from the humoral antibodies as proven by reverse genetic studies (Cattoli et al., 2011). Together, our data demonstrate that HA glycosylation was likely required for virus fitness and stability rather than immune evasion.

In this study, we did not investigate the impact of single GS on virus fitness and pathobiology but rather followed the trajectory of progressive glycosylation imitating the field situation. While increased virulence in chickens of HPAIV H5N2/Pennsylvania/1983 was due to deglycosylation in the vicinity of the polybasic HA cleavage site thereby enhancing HA cleavage (Webster et al., 1986), most of our information...
Fig. 3. Phylogenetic analysis of the HA gene of the Egyptian H5N1 viruses of different clades. Representative viruses were selected from 934 HA sequences available in the GenBank and GISAID to represent the Egyptian A/H5N1 clades. Viruses in clade 2.2.1 and 2.2.1.2 are shown in black. Viruses in clade 2.2.1.1 were selected to represent G6-like (yellow), G7-like (green), G8-like (red) and G9-like (blue) viruses. Virus used in this study is highlighted in grey. A maximum-likelihood tree was generated by IQ-TREE after selection of the best-fit model. The current tree was generated by MrBayes after selection of the best-fit model implemented in Topali v.2 and further edited using FigTree and Inkscape free software. Potential GS for each virus in clade 2.2.1.1 were predicted by NetNGlyc 1.0 Server.
about the impact of HA glycosylation on virulence derives from mammalian models (e.g., mice, ferrets) (Matsuoka et al., 2009; Zhang et al., 2015a). Results of animal experiments in this study indicated that glycosylation modulated virulence in chickens as estimated by CLD50. GS154 and GS236 increased virulence of the G7 and G8 viruses compared with G6, whereas GS273 reduced CLD50 of G9 by 10-fold compared with G8. Several studies have shown that the addition of GS to the HA head domain (residues 131, 154 and/or 169) resulted in attenuation of A/H5N1 viruses in mice but did not regulate high virulence in chickens (Matsuoka et al., 2009; Yen et al., 2009; Zhang et al., 2015a). It is not known how GS273 decreased virulence of G9, but seasonal human influenza viruses have more GS on the head of HA compared to the pandemic H1N1 viruses assuming a role in adaptation and lower virulence (Tate et al., 2014). A possible explanation is that hyperglycosylation enhances susceptibility of G9 to innate immune response (e.g., via lectins) and thus reduces virulence (Tate et al., 2014). Another explanation is that the evasion from humoral immunity as seen in outbreaks caused by G8-like viruses may be balanced by attenuation of infection caused by progeny G9-like viruses. As in human influenza viruses, optimal HA glycosylation patterns are probably the result of competing pressures associated with evasion from antibody immune response, maintenance of virus fitness and attenuation of virulence following recognition by the innate immune system (Tate et al., 2014). It is worth mentioning that naturally occurring G6 and G7 viruses carry compensatory mutations in, for example, HA and NA that would restore virulence/transmission.

Except in lungs and pancreas, all recombinant viruses in this study exhibit comparable organ distribution. However, antigen concentrations in lungs correlated with a higher number of GS in the HA, being lower in G6- and G7-infected birds than in G8- and G9-infected birds. Glycosylation affected the transmission of G6 and, in particular, G7 from infected birds to their cage mates as indicated by delayed death time and virus excretion at 4 days p.i. in contact birds (if any) compared to those infected by G8 or G9. Therefore, it is likely that glycosylation of the Egyptian H5N1 2.2.1.1 is important for efficient virus replication and transmission. Likewise, numerous GS in the HA protein increased replication and shedding of AIV in chickens (Matsuoka et al., 2009). H7N1 virus carrying GS123 (corresponding to residue 129 in this study) was selected over H7N1 virus possessing GS149 (corresponding to GS154 in this study) in experimentally infected chickens and turkeys indicating a replication advantage of certain GS patterns in vivo (Iqbal et al., 2012).

Interestingly, decreased fitness of G7 may explain the lower prevalence of G7-like viruses in poultry in Egypt compared to other viruses which may indicate negative selective pressure and domination by G8- and G9-like viruses. Also, few 2.2.1.1 viruses lacking GS154 or with a different GS pattern from that investigated in the current study as shown in Table 3 (i.e. two G6-like sequences possessed GS236, two G7-like sequences possessed GS273 and two G8-like sequences possessed GS165 or GS193) were detected. Nevertheless, the vast majority of G8- and G9-like viruses followed the same trajectory by possessing GS at position 154 or additional GS in the HA, being lower in G6- and G7-infected birds than in G8- and G9-infected birds. Glycosylation affected the transmission of G6 and, in particular, G7 from infected birds to their cage mates as indicated by delayed death time and virus excretion at 4 days p.i. in contact birds (if any) compared to those infected by G8 or G9. Therefore, it is likely that glycosylation of the Egyptian H5N1 2.2.1.1 is important for efficient virus replication and transmission. Likewise, numerous GS in the HA protein increased replication and shedding of AIV in chickens (Matsuoka et al., 2009). H7N1 virus carrying GS123 (corresponding to residue 129 in this study) was selected over H7N1 virus possessing GS149 (corresponding to GS154 in this study) in experimentally infected chickens and turkeys indicating a replication advantage of certain GS patterns in vivo (Iqbal et al., 2012).

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![Predicted location of different GS on the HA tertiary structure of the Egyptian A/H5N1.](image)

**Fig. 4.** Predicted location of different GS on the HA tertiary structure of the Egyptian A/H5N1. The HA trimers are shown in cyan, green and magenta. The conserved GS in all Egyptian strains are shown in yellow and the progressively accumulated GS are in blue. To impose mutations, HA of wtG8 was used for generation of the PDB file by SWISS MODEL (http://swissmodel.expasy.org/) and then viewed and edited manually in Geneious.
In conclusion, the glycosylation of the HA of H5N1 clade 2.2.1.1 viruses was essential for efficient virus replication by increasing both virus entry and release and heat stability but with no apparent impact on virus escape from neutralizing antibodies. *In vivo*, glycosylation further modulated virulence, tropism and transmission in/to chickens with hyperglycosylated virus exhibiting lower virulence but more efficient chicken-to-chicken transmission. Overall, those adaptive changes likely enabled the long-term circulation of the enzootic A/H5N1 in poultry.

**METHODS**

**Viruses and cells.** A/chicken/Egypt/NLQP-0879/2008 (H5N1) (wtG8) was obtained from the repository of the Friedrich-Loeffler-Institut (FLI), Germany, and the National Laboratory for Veterinary Quality Control on Poultry Production (NLQP), Egypt. wtG8 was isolated during the national surveillance from H5-vaccinated chickens. It was extensively studied as an immune escape variant of the Egyptian A/H5N1 in chickens vaccinated with homologous or heterologous vaccines (Abdelwahab et al., 2011; Grund et al., 2011). MDCKII, chicken fibroblast (DF1) and human embryonic kidney 293 cells (HEK239T) were obtained from the Collection of Cell Lines in Veterinary Medicine of the FLI. CEK cells were prepared from kidneys of 18-day-old specific-pathogen-free (SPF) embryonated chicken eggs (ECE) (Lohmann Animal Health, Germany) according to the standard procedures.

**Virus isolation and propagation.** Virus isolation and/or propagation was conducted via inoculation of the allantoic membrane of SPF ECE and incubated at 37°C. Eggs were candled daily for 3–5 days, eggs with dead embryos were kept at 4°C and allantoic fluids (AF) were collected the next day. Haemagglutination test using 1% chicken erythrocytes was done as recommended (OIE, 2015). AF with an HA titre of over 16 used. The HA gene of wtG8 was used to generate HPAIV recombinants (G6, G7 and G9) and four corresponding mutants with monobasic CS (LpG6, LpG7, LpG8 and LpG9). In conclusion, the glycosylation of the HA of H5N1 clade 2.2.1.1 viruses was essential for efficient virus replication by increasing both virus entry and release and heat stability but with no apparent impact on virus escape from neutralizing antibodies. *In vivo*, glycosylation further modulated virulence, tropism and transmission in/to chickens with hyperglycosylated virus exhibiting lower virulence but more efficient chicken-to-chicken transmission. Overall, those adaptive changes likely enabled the long-term circulation of the enzootic A/H5N1 in poultry.

**Generation of mutants.** The wtG8 virus was subjected to two successive plaque purification rounds in MDCKII followed by a passage in ECE via the allantoic route for 3–4 days at 37°C. The viral RNA of wtG8 was extracted from the AF using QIAamp Viral RNA kit according to the manufacturer’s guidelines (Qiagen). Each gene segment was amplified and cloned in pHWScoB plasmid as described previously (Stech et al., 2008). Plasmids containing wtG8 gene segments were transformed into *Escherichia coli* TOP10 (Invitrogen), XL1-Blue or SURE2 (Stratagene). All plasmids were then used to infect ECE Plasmid Mini or Midi kit. DNA concentration was adjusted to about 1 μg μl⁻¹. The HA gene of wtG8 was used to generate HPAIV recombinants (G6, G7 and G9) and four corresponding mutants with monobasic CS (LpG6, LpG7, LpG8 and LpG9). All mutations were conducted using QuikChange II XL Site-Directed Mutagenesis kit (Agilent Technologies) according to the consensus sequence of the wild-type isolates, and 1424NT185 was changed to 1398DNA156 2364N728 was changed to 2364DA2388 and 27NC275 was changed to 27NC257. Primers used for generation of each mutant were available upon request. All recombinant viruses were rescued in mixed 293T and MDCKII cell culture using Lipofectamine 2000 and Opti-MEM (Stech et al., 2008). The recombinant viruses were propagated in ECE, aliquoted and stored at −80°C. The titres of these working stocks were determined by plaque assay.

**Western blot analysis.** MDCKII cells were infected at an m.o.i. of 1 and incubated at 37°C and 5% CO₂ for 24 h. Cells were harvested followed by centrifugation at 6000 r.p.m. (Biofuge Pico Rotor 75003328) for 10 min. The pellet was suspended in PBS containing 2-mercaptoethanol and was incubated at 95°C for 10 min followed by centrifugation at 14000 r.p.m. (Biofuge Pico Rotor 75003328) for 5 min. Proteins were separated by SDS and 8% polyacrylamide gels and electrotransferred to nitrocellulose membranes using a TransBlot cell (Bio-Rad). Virus proteins were detected using polyclonal chicken antiserum against AIVH5 at a concentration 1:500 and peroxidase-conjugated IgY-specific goat IgG (Dianova) at a concentration 1:20,000. Immunodetection was achieved by chemiluminescence using Supersignal West Pico Chemiluminescent Substrate kit (Pierce, ThermoScientific) and images were captured using a Bio-Rad VersaDoc Imaging System and Quantity One software.

**Plaque assay.** Titration of viruses was performed in MDCKII cells by 10-fold serial dilutions. The cells were infected for approximately 1 h, then washed twice with PBS and overlaid with minimal essential medium (MEM) containing 1.2% BSA (Sigma) and 1.8% Bacto-agar (1:1) (Becton Dickinson). Plates were incubated at 37°C and 5% CO₂ for 3 days and then fixed with crystal violet. For measuring the plaque sizes, Nikon Instruments’ NIS Elements Basic Research software was used.

**Replication kinetics.** Infection of CEK and DF1 cells was conducted at an m.o.i. of 0.001 in two to four independent assays. After 1 h, cells were washed with citrate-buffered saline (CBS), pH 3.0, to inactivate extracellular virions. Then, the cells were washed twice with PBS 1×, and MEM with 0.2% BSA was added and incubated for 1, 8, 24, 48 and 72 h at 37°C and 5% CO₂. Harvested cells and supernatants were stored at −80°C. Progeny viruses were determined by plaque assay.

**Virus entry assay.** MDCKII cells in six-well plates and indicated viruses were prechilled for 30 min at 4°C. Cells were then infected with 300–500 p.f.u. on ice and incubated for 60 min at 4°C. Supernatants were removed, prewarmed medium was added and incubation was continued at 37°C for 0, 5, 10, 20, 30 and 60 min. At each time point, supernatant was removed, cells were washed with CBS, pH 3.0, for 2 min followed by 2× washing with PBS, pH 7.2, and fresh medium was added. After 2 h, medium was removed, the cells were washed with PBS and overlaid by agarose medium for 3 days.

**Virus release.** To determine the quantity of virus released from infected cells after single or multiple replication cycles, CEK cells were infected with recombinant G6–G9 viruses at an m.o.i. of 0.001 for 1 h at 37°C. Cells were then washed with CBS and twice with PBS, and MEM with 0.2% BSA was added and incubated for 1, 8 and 24 h at 37°C and 5% CO₂. At indicated time points, the supernatants were collected. Cells were harvested separately and dispersed in MEM. All samples were stored at −80°C until use. The assay was run in duplicates and repeated twice.

**NA activity assay.** The activity of the NA of LpG6, LpG7, LpG8 and LpG9 was determined by using NA-Fluor Influenza Neuraminidase Assay kit (Applied Biosystems). Virus suspensions were adjusted to 10⁶ p.f.u. ml⁻¹ and the test was conducted according to the manufacturer’s recommendations. Briefly, 50 μl of each virus was twofold serially diluted in 50 μl 1× assay buffer in Thermo Scientific NUNC 96-well black flat bottom plates. Then, 50 μl of 200 μM NA-Fluor substrate working solution was added. The plates were incubated at 37°C for 75 min, protected from light. The reaction was terminated by adding 100 μl of NA-Fluor stop solution. Reading was performed in Infinite M200 PRO (Tecan) with an excitation wavelength range from 350 to 365 nm and an emission wavelength range from 440 to 460 nm. Each virus dilution was plotted versus the average of the relative fluorescence unit values after subtraction of the background value. The assay was run in duplicate and repeated twice. Shown in Fig. 1 are the average and standard deviations of relative fluorescence unit values of two independent replicates.
Thermostability. Aliquots of selected viruses were incubated at 50°C for 0, 1, 2, 3, 4 and 5 h and then kept at −80°C until use. The decrease in HA titre and infectivity was investigated by HA test and plaque test, respectively. The HA test was conducted in duplicate. The experiment was repeated twice and the results show the average and standard deviation of all experiments for each virus.

HI test. To study the impact of the glycosylation pattern on immune escape, LpG6–LpG9 were tested against polyclonal antisera raised in chickens against different heterologous H5 viruses as well as homologous G6–G9 inactivated antigens. Sera against G6–G9 were generated by single vaccination of SPF chickens with indicated viruses. Finally, sera were collected 3–4 weeks post-immunization, inactivated at 56°C for 1 h and stored at −20°C until use. HI test was conducted in 96-microwell plastic plates using 8 haemagglutinating units of each recombinant virus as well as positive control viruses and 1% chickens’ erythrocytes according to the standard protocol (OIE, 2015). The HI titre is the reciprocal of the highest dilution of sera which completely inhibit HA activity of the virus. The HI titres were determined in duplicate and the results were given as the mean value of each serum against the given antigen.

Ethics statement. Animal experiments were carried out in the BSL3+ animal facilities of the FLI following the German Regulations for Animal Welfare after approval by the authorized ethics committee of the State Office of Agriculture, Food Safety and Fishery in Mecklenburg – Western Pomerania (LALLF M-V). All experiments were approved by the Commissioner for Animal Welfare at the FLI representing the Institutional Animal Care and Use Committee.

Determination of virulence in chickens. The impact of the glycosylation pattern on virulence of the recombinant viruses was investigated by determination of the CLD<sub>50</sub>. Therefore, four 6-week-old SPF White Leghorn chickens were inoculated via the ocularonasal route with 0.2 ml inoculum of 10-fold serial dilutions (about 10<sup>3</sup> to 10<sup>7</sup> p.f.u. per bird) of each G6–G9 virus. Birds were observed daily for a 10 day observation period. Clinical scoring was done as follows: 0 for healthy birds, 1 for birds with one clinical sign (depression, respiratory disorders, diarrhoea, cyanosis of the comb or wattles, facial oedema or central nervous signs), 2 for birds that showed more than one clinical sign and 3 for dead birds. The CLD<sub>50</sub> was calculated using the standard method by Reed & Muench.

Chicken-to-chicken transmission. The transmissibility of G6–G9 was studied in chickens. Four 6-week-old SPF chickens were infected with 10× CLD<sub>50</sub> of the respective viruses. One day after infection, six chickens were added to the infected birds and were always handled and sampled first. All birds were observed for 10 days. Oropharyngeal and cloacal swabs were collected from surviving chickens at 2, 4, 6, 8 and 10 days p.i. Viral titres were determined by plaque assay.

Histopathology and immunohistochemistry. Three to four 6-week-old SPF chickens were infected with 200 µl of 10× CLD<sub>50</sub> of G6, G7, G8 or G9 via the ocularonasal route. At 3 days p.i., all birds were euthanized by isoflurane (CP Pharma) inhalation and blood withdrawal. Samples from trachea, lung, heart, spleen, liver, pancreas, duodenum, jejunum, cecal tonsils, bursa of Fabricius, thymus and brains were formalin fixed, processed for paraffin wax embedding and subjected to histopathologic examination and immunohistochemistry. Primary anti-influenza NP antibodies and secondary biotinylated goat anti-rabbit IgG1 (Vector) antibody (1:200) were used to detect H5N1 antigens in tissues (Breithaupt et al., 2011). The signals of nucleoprotein antigen were semiquantitatively by scoring on a 0–4 severity scale for tissues: 0, negative; 1, single cells; 2, scattered foci; 3, numerous foci; 4, coalescing foci or diffuse and on a scale of 0–3 for endothelium: 0, negative; 1, single blood vessel; 2, multiple blood vessels; 3, diffuse, as described previously (Breithaupt et al., 2011).

ELISA. At the end of the experiment, sera from all surviving chickens were tested for influenza A NP antibodies using a commercial ELISA kit (ID Screen Influenza A Antibody Competition Multi-species, IDvet) according to the manufacturer’s guidelines.

Sequence and molecular analysis. Amplification of all gene segments of the wtG8 and recombinant viruses was conducted using a one-step reverse transcription PCR kit (Hoffmann et al., 2001). Amplicons were purified from 1% (w/v) agarose gels using the QIAquick Gel Extraction kit (Qiagen). Sequences were generated using an ABI BigDye Terminator v.1.1 Cycle Sequencing kit (Applied Biosystems). Sequences were analysed to exclude any unwanted mutation. Newly sequenced gene segments of wtG8 were assigned numbers from EP1824801 to EP1824808 in the GISAID. All available HA of Egyptian A/H5N1 in the GenBank and GISAID until 16 July 2016 were retrieved. All sequences were aligned using MAST (Katho & Standa, 2014), visualized and edited by BioEdit 7.1.7 (Hall, 1999). The pNGS in all Egyptian as well as Asian countries enzootic with A/H5N1 viruses was predicted by NetNGlyc 1.0 Server (Nguyen et al., 2015). Selected viruses representing different clades were chosen to generate a short tree. The tree was further visualized and edited using FigTree (http://tree.bio.ed.ac.uk/software/figtree/) and Inkscape 0.91 (www.inkscape.org). Mutations mentioned in this study in the HA were imposed on the tertiary structure of the HA protein of wtG8 virus using SWISS MODEL (http://swissmodel.expasy.org/) and then viewed and edited manually in Geneious v.8.1.3.

Statistical analysis. Data were analysed using R version 2.14.0 from the R Foundation for Statistical Computing available at the R project website (http://www.r-project.org) and differences were considered significant at a P value of <0.05. Inter- and intra-group variations of positive birds after examination of viral shedding in oral swabs were compared using pairwise Fisher’s exact tests with Bonferroni correction. Meanwhile, variations in replication kinetics, NA activity and heat stability were assessed using an ANOVA with post hoc Tukey’s tests. Statistical differences for amount of virus excretion, plaque sizes and pathogenicity indices were evaluated using Kruskal–Wallis test and Wilcoxon tests with Bonferroni correction. Significant differences in clinical scoring between infected groups were assessed by comparing the mean clinical score per bird during a 10 day observation period.

ACKNOWLEDGEMENTS

This work was conducted at the Institute of Molecular Virology and Cell Biology, FLI, Federal Research Institute for Animal Health, Germany, in the frame of the postdoctoral fellowships granted to El-Sayed M. Abdelwhab by Alexander von Humboldt foundation and the Deutsche Forschungsgemeinschaft (DFG). The authors are grateful to Dajana Helke for laboratory technical assistance; to Doreen Fiedler, Frank Klipp, Christian Loth, Harald Mantei and Ralph Henkel for their help during the animal experiment; to Günter Strebelow for his assistance in sequencing of viruses in this study and to Barbara Klupp for her valuable suggestions (all from the Friedrich-Loeffler-Institute, Insel Riems, Germany). M. Matrosovich, Marburg, is thanked for his comments and fruitful scientific discussion during the German FluResearchNet meeting.

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