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Evolutionary trajectories and diagnostic challenges of potentially zoonotic avian influenza viruses H5N1 and H9N2 co-circulating in Egypt

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ABSTRACT

In Egypt, since 2006, descendants of the highly pathogenic avian influenza virus (HP AIV) H5N1 of clade 2.2 continue to cause sharp losses in poultry production and seriously threaten public health. Potentially zoonotic H9N2 viruses established an endemic status in poultry in Egypt as well and co-circulate with HP AIV H5N1 rising concerns of reassortments between H9N2 and H5N1 viruses along with an increase of mixed infections of poultry.

Nucleotide sequences of whole genomes of 15 different isolates (H5N1: 7; H9N2: 8), and of the hemagglutinin (HA) and neuraminidase (NA) encoding segments of nine further clinical samples (H5N1: 2; H9N2: 7) from 2013 and 2014 were generated and analysed. The HA of H5N1 viruses clustered with clade 2.2.1 while the H9 HA formed three distinguishable subgroups within cluster B viruses. BEAST analysis revealed that H9N2 viruses are likely present in Egypt since 2009. Several previously undescribed substituting mutations putatively associated with host tropism and virulence modulation were detected in different proteins of the analysed H9N2 and H5N1 viruses.

Reassortment between HP AIV H5N1 and H9N2 is anticipated in Egypt, and timely detection of such events is of public health concern. As a rapid tool for detection of such reassortants discriminative SYBR-Green reverse transcription real-time PCR assays (SG-RT-qPCR), targeting the internal genes of the Egyptian H5N1 and H9N2 viruses were developed for the rapid screening of viral RNAs from both virus isolates and clinical samples. However, in accordance to Sanger sequencing, no reassortants were found by SG-RT-qPCR.

Abbreviations: BEAST, Bayesian Evolutionary Analysis Sampling Trees; BLAST, basic local alignment sequence tool; BSU, Beni-Suef University; DOPE, discrete optimised protein energy; GISAID, Global Initiative on Sharing all Influenza Data; HB, hemadsorbing sites; HPAIV, highly pathogenic avian influenza virus; HPD, highest posterior density; LPAIV, low pathogenic avian influenza virus; MAFFT, multiple alignment using fast fourier transform; MCC, maximum clade credibility; MCMC, Markov Chain Monte Carlo; MDCK, Madin-Darby canine kidney; MUSTANG, multiple structural alignment algorithm; NCBI, National Center of Biotechnology Information; NLQP, National Laboratory for Veterinary Quality Control on Poultry Production; PCR, polymerase chain reaction; PDB, protein data bank; PDZ, postsynaptic density protein 95, Drosophila disc large tumour suppressor, and zonula occludens 1 protein; PGS, potential glycosylation sites; SG-RT-PCRs, SYBR-Green reverse transcription real-time PCR assays; SYBR, N′,N′-dimethyl-N-[4-[(E)-(3-methyl-1,3-benzothiazol-2-ylidene) methyl]-1-phenylquinolin-1-ium-2-yl]-npropyl propane-1,3-diamine; Tm, melting temperature; TMRCA, time to most recent common ancestors; YASARA, yet another scientific artificial reality application.

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Nevertheless, the complex epidemiology of avian influenza in poultry in Egypt will require sustained close observation. Further development and continuing adaptation of rapid and cost-effective screening assays such as the SG-RT-qPCR protocol developed here are at the basis of efforts for improvement the currently critical situation.

1. Introduction

Avian influenza viruses (AIV), are members of the family *Orthomyxoviridae* and possess eight negative-sense single-stranded RNA segments encoding at least 10 viral proteins. AIV covers a broad range of avian host species and may on rare occasions also infect mammalian hosts (Alexander, 2007). Genetic and antigenic properties of the hemagglutinin (HA) and neuraminidase (NA) envelope glycoproteins of AIV allow the distinction of 16 and 9, respectively, subtypes. AIV infection in poultry usually runs a benign course inducing mild respiratory signs and/or a drop in egg production (hence, low pathogenic [LP] AIV). However, infections complicated by other opportunistic co-pathogens might enhance mortality (Pan et al., 2012). Amongst viruses of subtypes H5 and H7 highly pathogenic (HP) biotypes exist that may cause up to 100% mortality especially amongst galliform poultry (Alexander, 2007). In mammals including humans, occasional AIV infections caused by accidental spill-over transmission from avian hosts, may induce fatal disease but didn't result in sustained transmission chains yet (Cardona et al., 2009; Webster et al., 1992). Pandemic influenza viruses that have caused rapid global spread and, during some pandemics, significant death tolls amongst people, harboured gene segments from avian sources. This indicates that AIV, through genetic reassortment, may contribute to the pandemic potential of certain human influenza viruses (Hilleman, 2002; Shope, 1958; Taubenberger and Morens, 2006).

Since 2003, an epidemic of vast proportions of HPAIV H5N1 of Asian origin adversely affected the poultry industry in large areas of Asia, Europe and Africa. Descendants of this virus still remain endemic in several countries resulting in continuing huge losses of birds raised for human consumption (Swayne et al., 2011). Until to date, this virus evolved into 10 phylogenetic clades (0–9) and several subclades. Viruses of clade 2.2 escaped in 2005 and 2006 from South Eastern to Western Asia, Europe and Africa (WHO, 2014b). Along with disastrous outbreaks of HPAI in poultry public health problems arose due to the zoonotic potential of HPAIV H5N1: Since 2003, the virus caused 676 recorded human infections with 398 fatalities in 15 countries (WHO, 2014a). While currently there is no evidence for sustained human-to-human transmission of this virus, serosurveillance indicated that an under-reporting of human infections is possible (Dung et al., 2014). In Egypt, since 2006, HPAIV H5N1 of clade 2.2 caused serious losses in poultry production (Abdelwhab and Hafez, 2011). In addition, 185 laboratory confirmed human cases with 68 deaths were recorded as of December 2014 (WHO, 2014a). Despite serious nationwide attempts of eradication including blanket vaccination, HPAIV H5N1 gained endemic status in poultry in Egypt and continues to threaten poultry production and public health (Abdelwhab and Hafez, 2011). In 2011, in vaccinated commercial poultry, antigenic drift variants arose that formed distinct phylogenetic clusters termed 2.2.1.1 and 2.2.1.1a. Viruses that circulated amongst household and backyard poultry but also, though more rarely, in commercial farms belonged to subclade 2.2.1/C (WHO, 2014b). The latter clade prevails in poultry in Egypt since 2011 and continues to cause human cases while the variant viruses (2.2.1.1) apparently became extinct (Arafa et al., 2012).

Avian influenza viruses of subtype H9N2 are, along with the HPAIV H5N1, extremely widespread amongst poultry in all parts of Asia. Apart from its role as an important avian pathogen H9N2 virus also bears zoonotic potential (Kalthoff et al., 2010; WHO, 2013). Eurasian lineage H9N2 viruses became endemic in poultry in some Asian and Middle Eastern countries in the mid-1990s (Alexander, 2007). Several sublineages of H9N2 viruses evolved of which the most prevalent lineage "G1", represented by A/quail/Hong Kong/G1/97, further diversified into four co-circulating genetic groups A, B, C and D (Fusaro et al., 2011; Shahsavandi et al., 2012). Continued co-circulation of HPAIV H5N1 and H9N2 viruses in poultry populations over wide areas of Asia fostered genetic interactions of these viruses. Frequent reassortment events between these and further AI viruses contributed to a plethora of different genotypes of HPAIV H5N1 (Cameron et al., 2000; Guo et al., 2000; Iqbal et al., 2009; Lin et al., 2000; Munir et al., 2013). In Asia, recent reassortants that carried internal gene segments of H9N2, such as LP H7N9 (Lam et al., 2013) and H10N8 (Chen et al., 2014) revealed enhanced zoonotic potential and caused lethal human infections. Some of the Asian H9N2 lineages (e.g., G1 and Y280) carry markers of advanced adaptation to mammalian hosts at position 183, 190, 225 and 226 of the receptor-binding site of the hemagglutinin (HA) protein (H3 numbering) (Matrosovich et al., 2001).

In 2010, an incursion with such H9N2 viruses carrying human-like HA 226L was detected in poultry in Egypt (El-Zoghby et al., 2012; Monne et al., 2013a), and, similar to HPAIV H5N1, also H9N2 established an endemic status. In addition, sero-surveillance data indicated occupational exposure of humans to both H5N1 and H9N2 in Egypt (Gomaa et al., 2015). In January 2015, the first human H9N2 case was reported from Egypt (EMPRES, 2015). There is also evidence from the field (Arafa et al., 2012) and from experimental studies (Khalenkov et al., 2009) that past H9N2 infection in commercial poultry may induce some cross immunity against H5N1 whereby clinically masking consecutive infection with HPAIV H5N1 in galliform poultry. Reassortment between H9N2 and H5N1 viruses in Egypt is anticipated due to the increasingly parallel occurrence of these viruses in poultry holdings (Arafa et al., 2012; Kayali et al., 2014). Phenotypic properties of such reassortants cannot be predicted, however, given the impact of internal gene segments of H9N2 in the generation of highly zoonotic AIV in China the timely detection of such reassortants is certainly a matter of concern for public health (Fuller et al., 2013).

Therefore, molecular methods that allow the identification of such reassortants directly from clinical poultry samples have to be developed and validated. To this end, discriminative SYBR-Green reverse transcription real-time PCR assays (SG-RT-qPCR), targeting the internal genes of H5N1 and H9N2 for rapid screening of clinical samples were developed. In addition, aspects of the current evolution of H9N2 and H5N1 viruses in Egypt were analysed.

2. Materials and methods

2.1. Viruses and virus isolation

A total of 15 field isolates and 9 clinical samples from chickens, ducks, turkeys or quails in Egypt that tested positive for H5N1 or H9N2 viruses at the National Laboratory for Veterinary Quality Control on Poultry Production (NLQP, Ministry of Agriculture) and at the Faculty of Veterinary Medicine, Beni-Suef University (BSU), Egypt, were selected for sequence and evolutionary analysis (Table S1). Samples were collected between January 2013 and June 2014 from backyard poultry holdings and commercial farms in 10 governorates representing Upper and Lower Egypt (Table S1). Virus passaging from clinical samples was performed in 10-day old specific pathogen free (SPF) chicken eggs and/or Madin-Darby canine kidney (MDCK) cells, respectively, according to standard protocols (OIE, 2014). Allantoic fluids or cell culture supernatants were assayed for hemagglutinating activity (OIE, 2014) and isolates were stored at −70 °C until further use.

2.2. RNA extraction and virus subtyping

Viral RNA was extracted using the QIAamp Viral RNA Mini Kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions. RNA was eluted in 70 μL nuclease-free water, aliquoted at 10 μL each and stored at −20 °C until use. By RT-qPCR, presence of the influenza A virus matrix (M) gene was confirmed and positive samples were further subtyped using RT-qPCRs specific for H5 and H9 (Fereidouni et al., 2012; Monne et al., 2008). The RT-qPCR reactions were performed in 25 μL volumes using SuperScript® III One-Step RT-PCR kit with Platinum® Taq DNA Polymerase (Invitrogen) on a CFX96 thermocycler machine (Bio-Rad).

2.3. Sequencing of viral genomes

For amplification of the whole genome of H9N2 viruses forward and reverse primers for each gene fragment were designed (Table S2) based on the genomic sequence of A/quail/Egypt/113413v/2011 (H9N2); for H5N1 viruses primers published by Hoper et al. (2009) were used. Whole genomes of eight H9N2 and seven H5N1 viruses in addition to the HA and NA genes of another seven H9N2 and two H5N1 viruses were amplified using a one-step RT-PCR, size-separated in agarose electrophoresis excised and purified from gel using the QIAquick Gel Extraction Kit (Qiagen, Hilden, Germany). Purified PCR products were used directly for cycle sequencing reactions (BigDye Terminator v3.1 Cycle Sequencing Kit, Applied Biosystems). The reaction products were purified using NucleoSEQ columns (Macherey–Nagel GmbH & Co.) and sequenced on an ABI PRISM® 3100 Genetic Analyzer (Life Technologies).

2.4. Sequence and phylogenetic analyses

Sequences were assembled and edited using the Geneious software, version 7.1.7 (Kearse et al., 2012). Alignment and identity matrix analyses were performed using MAFFT (Katoh and Standley, 2013) and BioEdit (Hall, 1999). Sequences generated in this study were deposited in the Global Initiative on Sharing All Influenza Data (GISAID) database, and assigned accession numbers are shown in Table S1. Sequences of other H9N2 or H5N1 viruses required for further analyses were extracted from public databases and from the GISAID database, (see Acknowledgements section). Phylogenetic analyses were based on manually edited alignments of the full-length open reading frames. For maximum likelihood analysis of phylogenetic relationship a best fit model was chosen first on which further calculations and an ultrafast bootstrap equivalent analysis was based. The IQ-tree software version 1.1.3 was used for all operations (Minh et al., 2013; Nguyen et al., 2014). Finally, trees were viewed and edited using FigTree v1.4.2 software (http://tree.bio.ed.ac.uk/software/figtree/) and Inkscape 0.48.

Prediction of N-linked potentially glycosylated amino acids (N-X-S/T-X) in the HA and NA proteins was by the NetNGlyc 1.0 server (Gupta and Brunak, 2002).

2.5. Structural prediction of HA1 epitope sites

Representative structural models for the HA protein from H9N2 viruses from Egypt were derived using an existing crystal structure of the A/swine/Hong Kong/9/98 HA (PDB ID: 1JSH) (Ha et al., 2001) and full-length HA sequences from the Egyptian H9N2 isolates. Representative models for H5N1 HA protein were derived from A/Egypt/N03072/2010 (PDB ID: 4KW1) (Shore et al., 2013) and corresponding H5N1 sequences. Homology modelling was performed using a custom script based on the MODELLER engine (Eswar et al., 2006) with five rudimentary models generated and loop refinement performed on each model. The best model for each target sequence was selected on the basis of DOPE quality scores (Shen and Sali, 2006). Resulting models were structurally aligned in YASARA Structure using the MUSTANG algorithm (Konagurthu et al., 2006) and structural and antigenic site comparison performed. Residue numbering was changed to H3 numbering for H9N2, and H5 numbering for H5N1.

2.6. Analysis of reassortment using a SYBR® Green-based RT-qPCR assay

All sequenced isolates and 6 additional field samples (positive by RT-qPCR for H9N2 [$n = 4$] or H5N1 [$n = 2$]) were examined for reassortment events by a SYBR-Green RT-qPCR assay that was specifically designed and established in the frame of this study for the rapid detection of reassortment of the PB2, PB1, PA, NP, M and NS genes of H9N2 and H5N1 viruses in Egypt. Sets of forward and reverse primers highly specific for fragments of the six internal gene segments of either H9N2 or H5N1 viruses from Egypt were designed (Table S3) and evaluated *in silico* for specificity using the BLAST search tool on the National Center of Biotechnology Information (NCBI) website. Further primer properties were analysed using Oligo Calculator, online version 3.26 (Kibbe, 2007). One-step SYBR Green-based RT-qPCR assay was performed using the qScript™One-Step SYBR® Green kit (Quanta BioSciences, USA). To evaluate specificity of the chosen primer pairs RNA was extracted from the H9N2 isolate A/chicken/Egypt/NLQP139V-A R754/2013 (H9N2) and the H5N1 isolate A/chicken/Egypt/NLQP7 FL-AR747/2013 (H5N1), and RNA extracts were diluted so that, by M gene specific RT-qPCR, similar Cq values of 26–28 were obtained for both viruses. Then each primer set was tested against the homologous and the heterologous gene segment. The detection limit of the SYBR Green assays was determined by testing tenfold serial dilutions of viral RNA extracted from the Egyptian H9N2 and H5N1 viruses mentioned above. Cq values (mean of three separate runs) obtained with a standard RT-qPCR for the M gene (Fereidouni et al., 2012) were compared with melting point analyses of the SYBR Green assay for the six internal segments also for three separate runs. Finally, the assay was adjusted to a 10 μL reaction mixture containing 5 μL of SYBR Green reaction mix, 0.4 μL of each forward and reverse primer (20 pmol/reaction), 0.2 μL of qScript one step RT-enzyme, 2 μL of nuclease-free water and 2 μL of template RNA. The optimised thermal cycling conditions were as follows: reverse transcription at 50 °C for 10 min, initial

denaturation at 95 °C for 5 min, 40 cycles of PCR amplification at 95 °C for 10 s, 55 °C for 20 s, and 72 °C for 30 s, followed by a melting curve analysis from 65 °C to 95 °C with an increment of 0.5 °C. All SYBR-Green real time RT-qPCR reactions were conducted in CFX96 thermocycler machines (Bio-Rad).

2.7. Evolutionary analysis

All Egyptian H5N1 and H9N2 gene sequences available at the EpiFLu GISAID and the GenBank databases were retrieved. A selection of full or near full length sequences of a total of 122 and 77 H5N1 and H9N2 viruses, respectively, including sequences generated in this study, were used for analysis.

Selection pressure modes for each viral protein of H5N1 and H9N2 were determined using the software suite Datamonkey (Poon et al., 2009). A gene was admitted under neutral, positive or negative selection pressure when mutation rates of non-synonymous to synonymous substitutions ($dN/dS(\omega)$) was $\omega \approx 1$, $\omega > 1.0$ or $\omega < 1.0$, respectively. Evidence of positive selection of a site was detected using the single-likelihood ancestor counting (SLAC) method using the best fit model and setting the significance level to p-value <0.05.

Evolutionary rates and divergence times of the Egyptian H9N2 viruses were estimated using the Bayesian Markov Chain Monte Carlo (MCMC) method implemented in the Bayesian Evolutionary Analysis Sampling Trees (BEAST) program version 1.8 (Drummond et al., 2012). Rates of nucleotide substitutions per site per year (subs/site/year) and the 95% highest posterior density (HPD) for each parameter were estimated for each segment of all H9N2 viruses circulating in Egypt between 2011 and 2014, by using two molecular clock models, a strict constant and a relaxed uncorrelated lognormal one with gamma-distributed rate heterogeneity after identifying the best-fitting substitution model using JModelTest 2.1.7 (Darriba et al., 2012). The MCMC computation was done under a Bayesian skyline coalescent tree prior using simulations for 5×10^7–10^8 generations with sampling every 5000 steps for each of the Egyptian H9N2 segments.

To estimate the introduction time of group B H9N2 viruses into Egypt the approximate or exact dates of sampling (day/-month/year) were used. Uncorrelated lognormal relaxed clock models with gamma-distributed rate heterogeneity were run independently after identifying the best-fitting substitution model using jModelTest 2.1.7 (Darriba et al., 2012) for 10^8 generations with sampling every 1000 steps for group B HA segments. Convergence and effective sampling size were analysed using the Tracer v1.4 program (Drummond et al., 2012) Lower and upper bounds of the 95% highest posterior density interval (HPD) were used to assess the robustness of the data. Maximum clade credibility (MCC) trees for the group B HA segment were summarised after a 10% burn-in removal by using TreeAnnotator v1.8.0 (http://beast.bio.ed.ac.uk/TreeAnnotator) and the MCC trees were visualised with FigTree v1.4.0 (http://beast.bio.ed.ac.uk/figtree).

3. Results

In this study, a total of 138 gene segments from 8 H9N2 and 7 H5N1 virus isolates and 7 H9N2 and 2 H5N1 positive field samples (from which no virus isolate could be obtained) were sequenced to update genetic and phylogenetic developments of these co-circulating viruses from poultry in Egypt from 2013 to 2014. Samples originated from infected chickens ($n = 21$), ducks ($n = 1$), quails ($n = 1$) and turkeys ($n = 1$) in backyards and commercial farm sectors in ten governorates (Table S1).

3.1. Phylogenetic analysis

All Egyptian H9N2 viruses (2010–2014) including those analysed here are members of the G1-like lineage. Based on a clustering scheme proposed by Fusaro et al. (2011) for H9N2 viruses circulating in Middle Eastern and Central-Asian countries from 1998 to 2010, the PB2, PB1, PA and NP genes clustered in group A together with isolates from Israel, Saudi Arabia, and Jordan while the HA, NA, M and NS genes clustered in group B with recent Israeli viruses (Fig. 1.1 and 1.2 and S1). Within group B of the HA sequences the Egyptian viruses segregate into at least three further subgroups (B.Egy. 1–3; Fig. 1.1). Segregation is not correlated with time of occurrence, region or host species and seems to reflect a star-like diversification of these sequences. The NA N2 sequences of these viruses similarly reveal a shallow subclustering within group B (Fig. 1.2). However, here groups of older isolates (2010–2012) are separated from those obtained in 2013 and 2014.

Phylogenetic analysis of the HA and NA gene sequences of the sequenced H5N1 viruses revealed that they are continuing to cluster in clade 2.2.1 (Fig. 1.3 and 1.4). This is also true for the clustering of the internal gene segments of the H5N1 viruses (Fig. S2).

Thus, from the analysed isolates and datasets, no evidence for reassortment between the H9N2 and H5N1 viruses or an intrusion from other AI viruses could be obtained using both sequencing and phylogenetic analyses.

3.2. Sequencing-independent analysis of reassortment of internal gene segments by SYBR-Green RT-qPCRs (SG-RT-qPCR)

As an alternative to nucleotide sequence analysis for detection of reassortant viruses SG-RT-qPCRs specific for the internal gene segments of the Egyptian H9N2 and H5N1 lineage were developed. Since SYBR Green dye intercalates non-specifically into the DNA strands (including primer dimers), a melting curve analysis is required to confirm the specificity of amplificates. Specificity evaluation in a proof-of-concept manner using diluted viral RNA of both an Egyptian H9N2 and H5N1 virus revealed distinct melting points for each internal gene segment using the homologous but lack of specific amplification with the heterologous primer sets (Fig. 2 and Table S3). This confirms high lineage specificity of the primer pairs. In addition, all amplificates showed the expected size in agarose gel electrophoresis (not shown). Detection of specific fragments was also possible with higher diluted template RNA: Results obtained with tenfold serial dilutions of viral RNA indicated that the relative detection limit of the SYBR Green assays are about one log step lower than RT-qPCR for an M gene fragment (Table 1a and b). Nevertheless, samples with Cq values up to 32 (H5N1) or up to 35 (H9N2) in the M-specific RT-qPCR yielded specific amplificates in each of the SYBR Green assays. Based on these validation results the 15 isolates and an additional 6 field samples were examined (Table 2). The results obtained with SG-RT-qPCRs matched exactly those generated from nucleotide sequencing and phylogenetic analysis. Thus, no reassortants between H9N2 and H5N1 were detected.

3.3. Genetic characterisation of H9N2 and H5N1 viruses

3.3.1. Ribonucleoprotein complex (PB2, PB1, PA and NP)

Markers of mammalian adaption in the PB2 protein (K627 and N701) were not found in any of the H9N2 sequences. Instead, all isolates possessed valine at position 627 which is typical of viruses of the G1 lineage, group A (Table 3) (Wang et al., 2012). In addition, substitutions I504V and K526R, associated with enhanced polymerase activity in mice, were recognised in all isolates (Rolling et al., 2009; Zhou et al., 2013). Egyptian H9N2 isolates of this study encoded three variants of the PB1-F2 protein (verified by

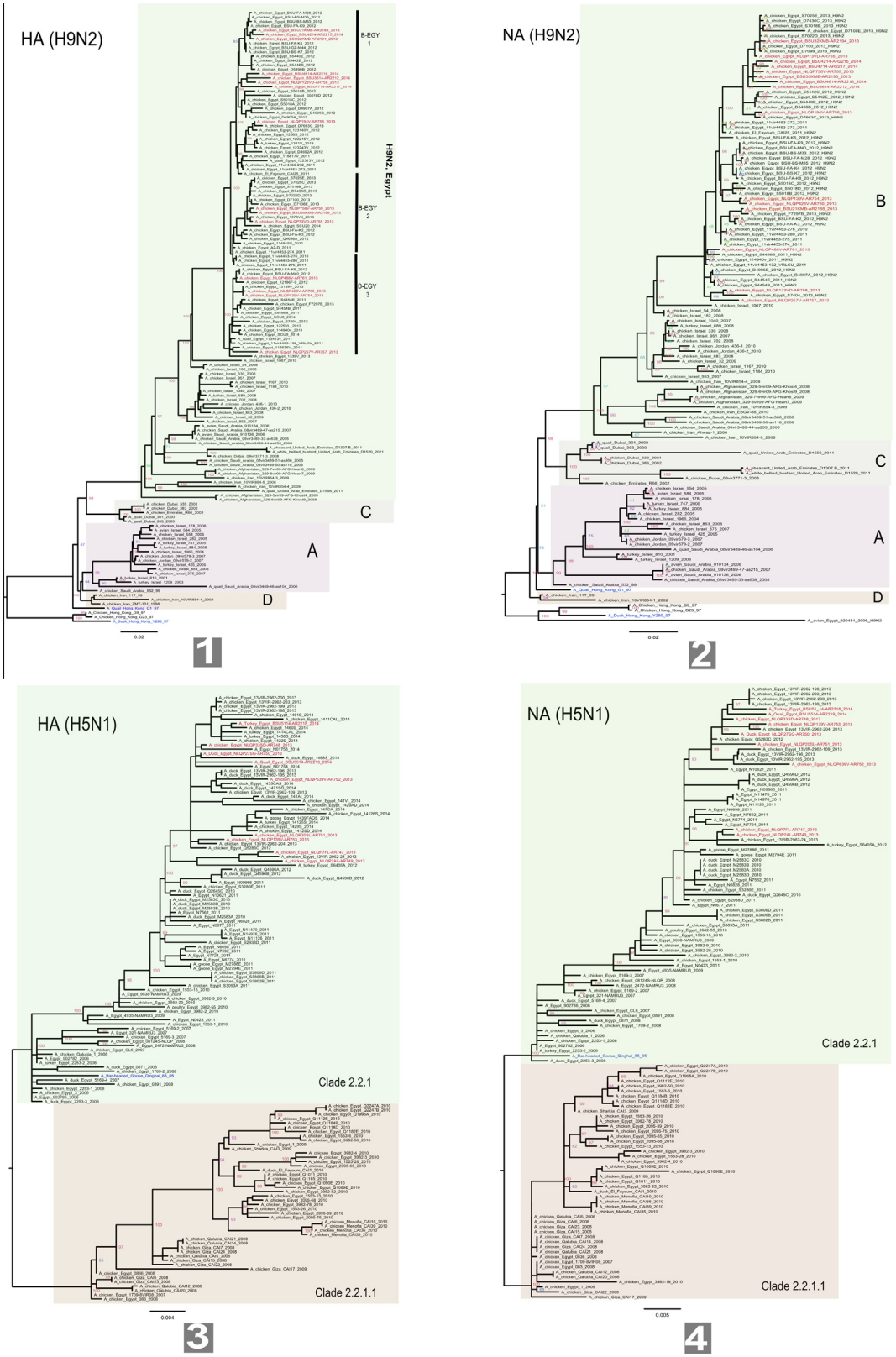

Fig. 1. Phylogenetic tree of the nucleotide sequences of the HA (1 and 3) and NA (2 and 4) gene segments of H9N2 and H5N1 viruses. Maximum likelihood calculations were done with the IQTree software using an ultrafast bootstrap approach. Best fit models were calculated in IQTRee as well. Cluster designation for H9N2 is based on Fusaro et al. (2011).

re-sequencing the encoding gene twice) that differed in length: (i) 52 amino acids (aa), isolates A/chicken/NLQP194V-AR756/Egypt/2013 and A/chicken/Egypt/NLQP758V-AR759/2013; (ii) 79 aa, isolate A/chicken/Egypt/NLQP257V-AR757/2013; and (iii) full length 90 aa ($n = 5$ isolates). All H9N2 isolates encoded a PB1 protein that showed mutation L13P which has been associated with

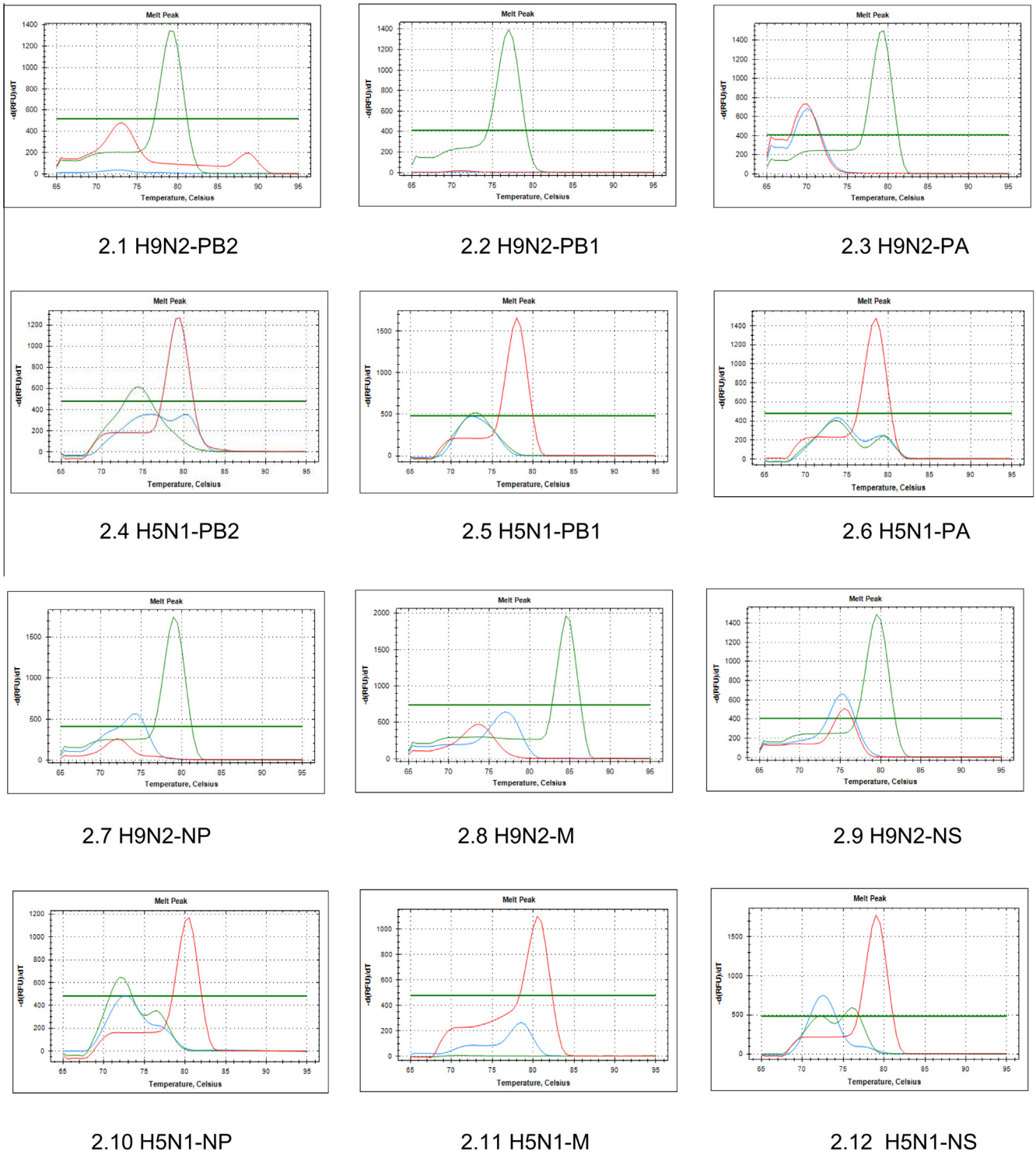

Fig. 2. Melting curve analysis of SYBR Green-based RT-qPCRs detecting and differentiating fragments of internal gene segments of H9N2 and H5N1 viruses from Egypt. Specificity of amplification is as follows: H9 – green, H5 – red, water control –blue.

enhanced replication in mammalian hosts (Gabriel et al., 2005). Moreover, the N66S substitution in the PB1-F2 protein associated with higher virulence in mice was present in all H9N2 isolates of this study (Schmolke et al., 2011). Sequence analysis of the PA protein revealed A404S, a marker for adaptation towards the human host, in only one isolate (A/chicken/Egypt/NLQP257V-A R757/2013 (H9N2)) (Finkelstein et al., 2007). Additional substitutions that have been described to affect virulence in avian and/or mammalian hosts were found in PA and NP encoding sequences (Chen et al., 2006; Rolling et al., 2009). Details are shown in Table 4. The viral protein PA-X was predicted to be of 252 amino acid length in all Egyptian H9N2 isolates.

In the PB2 protein of all sequenced H5N1 viruses, amino acid substitutions E627K and K318R were present while A661T was recorded in three isolates; these mutations have been associated with increased virus replication in mammalian cells (Gabriel

Table 1
Detection limit of SYBR Green RT-qPCRs targeting internal genome segments of H5N1 (a) and H9N2 (b) viruses from Egypt.

	Dilution	RT_qPCR M (Cq value)[a]	PB2-H5N1	PB1-H5N1	PA-H5N1	NP-H5N1	M-H5N1	NS-H5N1
(a)								
H5N1	−1	28.59 (SD 0.53)	+/+/+	+/+/+	+/+/+	+/+/+	+/+/+	+/+/+
	−2	32.08 (SD 0.10)	+/−/−	+/+/+	+/+/+	+/+/+	+/+/+	+/+/+
	−3	35.47 (SD 0.22)	+/−/−	+/−/−	+/−/−	+/−/−	+/−/−	+/−/−
	−4	37.91 (SD 1.06)	−	−	−	−	−	−
	−5	N/A	−	−	−	−	−	−
	Dilution	RT_qPCR M (Cq value)[a]	PB2-H9N2	PB1-H9N2	PA-H9N2	NP-H9N2	M-H9N2	NS-H9N2
(b)								
H9N2	−1	28.59 (SD 0.02)	+/+/+[b]	+/+/+	+/+/+	+/+/+	+/+/+	+/+/+
	−2	32.08 (SD 0.10)	+++	+/+/+	+/+/+	+/+/+	+/+/+	+/+/+
	−3	35.47 (SD 0.22)	+/+/+	+/+/+	+/+/−	+/+/+	+/+/+	+/+/−
	−4	37.91 (SD 1.06)	−	−	−	−	−	−
	−5	N/A	−	−	−	−	−	−

[a] Cq values represent the mean of three separate runs; SD – standard deviation; N/A – no specific amplificate detected.
[b] Detection of a specific amplificate showing the expected melting temperature is indicated by (+); (−) indicates failure of detection. Three independent runs have been performed (separated by slashes). The specific Tm for each segment is shown in Table 2.

et al., 2005; Gao et al., 2009; Shinya et al., 2004; Subbarao et al., 1993). All PB2 proteins coded for D701 which is a marker of avian adaptation while mutation L13P detected in the PB1 protein of all H5N1 isolates has been associated with enhanced replication in mammalian hosts (Gabriel et al., 2005). All analysed viruses encoded a full length PB1-F2 protein of 90 aa. Further mutations that were described to foster replication in mammals were present in the NP gene segment (I109V, K398Q and A184K; Table 3) (Chen et al., 2006; Shaw et al., 2002; Wasilenko et al., 2009).

3.3.2. Envelope glycoproteins (HA, NA)

The HA of the H9N2 viruses studied here encoded amino acid residues H183 and L226 (H3 numbering) which were reported to correlate with a shift in affinity of the HA from the "avian" alpha 2–3 towards the "human-like" alpha 2–6 sialic acid linkage (Matrosovich et al., 2001). The exception was A/chicken/Egypt/NLQP73VD-AR755/2013 (H9N2) which showed Q at residue 226 signalling pronounced avian-type affinity. In addition, avian receptor-specific substitutions 158N, 190A and 227I (H3 numbering) were identified in the receptor binding site (RBS) differing from the lineage ancestor A/quail/Hong Kong/G1/97. All but one isolate showed the trypsin-sensitive R-x-x-R (R-S-S-R) motif at the proteolytic cleavage site (PCS). Isolate A/chicken/Egypt/BSU3614-AR2212/2014 (H9N2) encoded K-S-S-R. The H9 HA encoded seven potential glycosylation sites (PGS) in positions 21, 97, 133, 290, 297, 484 and 543 in the membrane distal part of the HA and lacked PGS at residues 198 and 210 (H3 numbering) compared to A/quail/Hong Kong/G1/97.

Based on a structural analysis of sequences of representative viruses from these subgroups most of the coding differences compared to A/swine/Hong Kong/9/98 (H9N2) are located in the stem region of the HA glycoprotein. Only one of the analysed viruses (A/chicken/Egypt/NLQP257V-AR757/2013) showed two (conserved) AA substitutions located in antibody epitopes in the HA1 region: M79L at the Cb antigenic site and A166V (H3 numbering) at the Sa binding site (Fig. 3A).

The NA N2 protein of A/quail/Hong Kong/G1/97 is reported to harbour three hemadsorbing sites (HB) ^{366}IKKDSRSG^{373}, ^{399}DSDIRS^{404} and ^{431}PQE^{433} (Matrosovich et al., 2001). In contrast to the neuraminidase of human influenza viruses, the NA of the avian viruses has separate sialic acid binding sites (hemadsorption site) in addition to its catalytic site (Uhlendorff et al., 2009). All Egyptian H9N2 viruses encoded IKKDSR**A**G while at the second location they showed variable substitutions as summarised in Table S5. Seven PGS were predicted at positions 44, 61, 69, 86, 146, 200 and 234 in all viruses but A/chicken/Egypt/NLQP123VD-AR758/2013 which lacked the PGS at position 69. Moreover, no deletions in the NA stalk domain were recognised relative to the sequence of the quail/G1 lineage ancestor virus which is known to lack aa 38 and 39 while viruses of the Y280 lineage of H9N2 viruses (A/Duck/Hong Kong/Y280/97) harbour a deletion at position 62–64 of the NA stalk region.

The HA of the H5N1 viruses harboured the known multibasic PCS motif PQGEKRRKKR/GLF in all viruses, except A/chicken/Egypt/NLQP2AL-AR749/2013 (H5N1) which encoded an additional arginine: P**R**GEKRRKKR/GLF. The analysed H5N1 viruses possessed six PGS at amino acid positions 11, 23, 165, 286, 484 and 543 (H5 numbering) while A/chicken/Egypt/NLQP20SL-AR751/2013 (H5N1) had an additional PGS at position 54. All H5N1 viruses showed markers of avian receptor specific binding: Q222 and G224 (H5 numbering) (Lin et al., 2012; Liu et al., 2009). A/chicken/Egypt/NLQP639V-AR752/2013 encoded R140I at antigenic site Ca (Shore et al., 2013) (Table S6). In addition, A184D was observed in antigenic site Sb in A/chicken/Egypt/NLQP7FL-AR747/2013 and in A/chicken/Egypt/NLQP2AL-AR749/2013 (Fig. 3B).

In the neuraminidase N1 stalk region of H5N1 viruses the previously described 20 aa-deletion (positions 49–68) was present. Downstream of that deletion one additional aa was deleted at position A56 (N1 numbering, not counting the previous deletion) in A/chicken/Egypt/NLQP7FL-AR747/2013 and A/chicken/Egypt/NLQP2AL-AR749/2013. This deletion had not been described before amongst the Egyptian H5N1 viruses. The same isolates also showed an A184D substitution in antigenic site Sb of the HA1 protein. No oseltamivir resistance markers were observed and all Egyptian H5N1 strains including those analysed herein encode PGS at positions 68, 126 and 215 (N1 numbering) in the NA protein (Abdelwhab et al., 2012).

3.3.3. Matrix and non-structural proteins

The deduced matrix protein sequences revealed several substitutions associated with enhanced mammalian host tropism (M1: I15; M2: G16, F55, V28) or increased virulence in mammalian hosts (M2: S64, and P69) (Tables 3 and 4) (Chen et al., 2007; Shaw et al., 2002). Furthermore, in three (A/chicken/Egypt/NLQP139V-AR754/2013, A/chicken/Egypt/NLQP 123VD-AR758/2013 and A/chicken/Egypt/NLQP426V-AR760/2013) of the eight H9N2 isolates an amantadine resistance marker (V27I) was found (Scholtissek et al., 1998). Such resistance marker has not been reported from Egyptian H9N2 viruses before. In contrast, no evidence for amantadine resistance was observed in the H5N1 M2 sequences (Ison, 2011).

Table 2
Melting curve analysis of SYBR Green RT-qPCR products obtained with primers specific for internal gene segments of either H9N2 or H5N1 viruses from Egypt. SYBR Green RT-qPCR was used as real time method to examine reassortment between H5N1 and H9N2 viruses.

	Virus[a]	Sample types	PB2		PB1		PA		NP		M		NS		Reassortant
			H9N2[b]	H5N1	H9N2	H5N1	H9N2	H5N1	H9N2	H5N1	H9N2	H5N1	H9N2	H5N1	
1	A/chicken/Egypt/NLQP7FL-AR747/2013(H5N1)	Isolate	<65[c]	79	<65	77.5	<65	78	<65	81	<65	80.5	<65	79	No
2	A/chicken/Egypt/NLQP33SD-AR748/2013(H5N1)	Isolate	<65	79.5	<65	79	<65	78.5	<65	81.5	<65	79.5	<65	79	No
3	A/chicken/Egypt/NLQP2AL-AR749/2013(H5N1)	Isolate	<65	79	<65	78	<65	78.5	<65	81.5	<65	80.5	<65	79	No
4	A/Duck/Egypt/NLQP27SG-AR750/2013(H5N1)	Isolate	<65	79.5	<65	79.5	<65	78.5	<65	81.5	<65	80.5	<65	79	No
5	A/chicken/Egypt/NLQP20SL-AR751/2013(H5N1)	Isolate	<65	79	<65	79	<65	78.5	<65	81.5	<65	80.5	<65	79	No
6	A/chicken/Egypt/NLQP639V-AR752/2013(H5N1)	Isolate	<65	79.5	<65	79	<65	78.5	<65	82	<65	80.5	<65	80	No
7	A/chicken/Egypt/NLQP139V-AR753/2013(H5N1)	Isolate	<65	79.5	<65	79	<65	78.5	<65	82	<65	81	<65	79.5	No
8	A/chicken/Egypt/NLQP139V-AR754/2013(H9N2)	Isolate	79	<65	77	<65	79	<65	79.5	<65	85	<65	80	<65	No
9	A/chicken/Egypt/NLQP73VD-AR755/2013(H9N2)	Isolate	78.5	<65	77	<65	79	<65	79.5	<65	85	<65	80	<65	No
10	A/chicken/Egypt/NLQP194V-AR756/2013(H9N2)	Isolate	79	<65	77	<65	79	<65	79	<65	84	<65	79.5	<65	No
11	A/chicken/Egypt/NLQP257V-AR757/2013(H9N2)	Isolate	78.5	<65	77	<65	78.5	<65	79.5	<65	85	<65	79.5	<65	No
12	A/chicken/Egypt/NLQP123VD-AR758/2013(H9N2)	Isolate	78.5	<65	76.5	<65	78.5	<65	79	<65	84	<65	80	<65	No
13	A/chicken/Egypt/NLQP758V-AR759/2013(H9N2)	Isolate	78.5	<65	77.5	<65	79	<65	79.5	<65	85	<65	80	<65	No
14	A/chicken/Egypt/NLQP426V-AR760/2013(H9N2)	Isolate	78.5	<65	77.5	<65	78.5	<65	79.5	<65	84.5	<65	80	<65	No
15	A/chicken/Egypt/NLQP488V-AR761/2013(H9N2)	Isolate	79	<65	77	<65	79	<65	79.5	<65	85	<65	80	<65	No
16	A/Turkey/Egypt/BSU5114-AR2218/2014(H5N1)	Swab	<65	78.5	<65	78.5	<65	77.5	<65	81	<65	80	<65	79	No
17	A/Quail/Egypt/BSU5514-AR2219/2014(H5N1)	Swab	<65	79.5	<65	78.5	<65	78.5	<65	81.5	<65	80.5	<65	79	No
18	A/chicken/Egypt/BSU21KMB-AR2188/2013(H9N2)	Swab	78	<65	76.5	<65	78	<65	78	<65	84	<65	79	<65	No
19	A/chicken/Egypt/BSU32KMB-AR2194/2013(H9N2)	Swab	77	<65	76.5	<65	78	<65	79	<65	83	<65	79.5	<65	No
20	A/chicken/Egypt/BSU35KMB-AR2196/2013(H9N2)	Swab	78.5	<65	77	<65	78.5	<65	79	<65	84	<65	79.5	<65	No
21	A/chicken/Egypt/BSU3614-AR2212/2014(H9N2)	Swab	78.5	<65	76.5	<65	79	<65	79	<65	84	<65	79.5	<65	No
	Average *T* °C melt		78.4	79.2	76.9	78.7	78.6	78.3	79.2	81.5	84.3	80.4	79.7	79.2	
	Standard deviation (SD)		0.54	0.36	0.35	0.61	0.39	0.35	0.44	0.35	0.64	0.41	0.33	0.35	

[a] Positions 1–15 represent virus isolates; position 16–21 are clinical field samples.
[b] Indicates which primer set has been used (see also Table S3 for primer sequences).
[c] Values indicate melting temperatures (°C) of PCR products. A temperature of <65 °C indicates that no specific amplificate has been obtained.

Table 3
Deduced amino acid markers associated with host tropism in internal and M2 proteins of H9N2 and H5N1 viruses isolated from poultry in Egypt.

Protein		Tropism[a]		Ancestral viruses[b]		Egyptian viruses[c]	
Name	Residue	Avian	Mammalian	H9N2 quail/G1	H5N1 BHG/65/05	H9N2	H5N1
PB2	64	M	T	M	I	M	I
	318	K	R	K	R	(R)^{5} (K)^{3}	R
	627	E	K	E	K	V	K
	661	A	T	T	A	A	(A)^{4} (T)^{3}
PB1	13	L	P	P	P	P	P
PB1-F2	73	K	R	K	K	(K)^{7} (E)^{1}	E
	82	L	S	L	L	S	L
PA	100	V	A	V	V	V	(V)^{4} (I)^{3}
	404	A	S	A	A	(A)^{7} (S)^{1}	A
	615	K	L	R	K	K	R
NP	34	D	N	G	S	G	(S)^{6} (R)^{1}
	61	I	L	I	I	(I)^{7} (L)^{1}	I
	109	I	V	I	I	(I)^{7} (V)^{1}	V
	214	R	K	R	R	K	R
	398	K	Q	Q	Q	Q	Q
M1	15	V	I	I	I	I	I
M2	16	E	G/D	G	E	G	G
	28	I	I/V	V	V	V	(I)^{6} (F)^{1}
	55	L	F	F	L	F	L
NS1	227	E	K/R	E	E	K	E

[a] Amino acids associated with increased tropism for avian or mammalian hosts is indicated.
[b] Amino acid constellation of viruses that bare ancestral to the current Egyptian H9N2 and H5N1 lineages: A/quail/Hong Kong/G1/97(H9N2) and A/Bar-headed-Goose/Qinghai-65/05(H5N1).
[c] Sequences refer to seven H5N1 isolates (No. 1–7 in Table S1) as well as eight H9N2 isolates (No. 10–17 in Table S1). If more than one substitution was detected in a site, the superscript indicates the number of virus showing the respective mutation.

The C-terminus of the non-structural protein 1 of influenza viruses encodes a PDZ domain which enhances the virulence of the virus by targeting cellular PDZ proteins (Fan et al., 2013). All H9N2 isolates sequenced here encoded ^{227}KSEV^{230} like that of the 1918 H1N1 virus NS1 C-terminus. ^{222}ESEV^{225} was present in all H5N1 viruses except A/chicken/Egypt/NLQP639V-AR752/2013 (H5N1) which encoded E**P**EV. The latter is observed here and in three further strains represented in the database (EPI538761, EPI538777 and EPI538769) for the first time amongst Egyptian H5N1 viruses. EPEV has occurred in other H5N1 virus lineages in Asia and was associated with an increase of virulence (Jackson et al., 2008). The mutated motif was associated with an increase of virulence of H5N1 virus in mammals (Fanning and Anderson, 1999). The NS1 of the H5N1 viruses revealed a deletion at positions 80–84 which is known to be associated with high virulence in chickens and mice (Long et al., 2008; Seo et al., 2002). No such deletions were found in the NS1 of H9N2 viruses.

3.4. Selection pressure and evolutionary rates

Few proteins of H9N2 and H5N1 viruses circulating in Egypt are under positive selective pressure (Table 5A). Amongst the H9N2 and H5N1 viruses circulating to date in Egypt positive selection seems to focus on the PB1-F2 encoding sequence only. Increased rates of evolution were seen in the Pb1-F2, M2 and the HA1 proteins of the H5N1 2.2.1.1 clade (Table 5A); viruses within this clade represented vaccine escape mutants but this lineage is extinct by now. In the HA protein of H9N2 viruses only position 234/226 (H9/H3 numbering) was found to be under positive selection whereas in the HA of H5N1 position 129 (H5 numbering) was under positive selection. The estimated evolutionary rates using the strict and the uncorrelated lognormal relaxed clock model for the Egyptian H9N2 segments are indicated in Table 5B. The obtained results showed mean substitution rates for all segments that were within the range previously observed for avian influenza viruses (Fusaro et al., 2011). The highest rate of evolution was observed for the envelope glycoproteins HA (5.30) and NA (5.44) regardless of the clock model used. BEAST analyses to clarify tim e-to-most-recent-common-ancestors (TMRCA) revealed that the H9N2 strains circulating in Egypt between 2011 and 2014 likely originated from a common ancestor in July–August 2009 with roots in neighbouring countries in the Middle East region (Fig. 4).

4. Discussion

Despite efforts to limit the spread and eliminate H5N1 and H9N2 viruses in poultry in Egypt, these viruses remain deeply entrenched until today. Due to their widespread co-circulation (since 2010) and their zoonotic potential, Egypt is considered a hotspot for the generation of new sub- and genotypes of H5N1/H9N2 AIVs by reassortment and a potential epicentre for a future next influenza pandemic (Abdelwhab and Abdel-Moneim,

Table 4
Amino acid residues associated with virulence in the PB2, PB1-F2, PB1, PA, M2, NS1, and NS2 proteins in H9N2 and H5N1 viruses isolated from poultry in Egypt.

Protein		Virulence[a]		Ancestral viruses[b]		Egyptian viruses[c]	
Name	Residue	Higher	Lower	H9N2 quail/G1	H5N1 BHG/65/05	H9N2	H5N1
PB2	147	L	M	M	T	I	T
	250	G	V	V	V	V	V
	504	V	I	V	V	V	V
	627	K	E	E	K	V	K
	701	N	D	D	D	D	D
PB1	317	I	M/V	I	M	(I)^{5} (M)^{3}	M
PB1-F2	66	S	N	N	N	S	N
PA	127	V	I	V	V	V	V
	550	L	I	L	L	L	L
	672	L	F	L	L	L	L
M2	64	S/A/F	P	S	S	S	S
	69	P	L	P	P	P	P
NS1	42	S	A/P	S	S	S	S
	103	L	F	L	F	F	F
	106	I	M	I	M	M	M
	189	N	D/G	D	D	D	D
NS2	31	I	M	M	M	M	M
	56	Y	H/L	H	H	H	H

[a] Amino acids associated with variations in virulence for avian and/or mammalian hosts is indicated.
[b] Amino acid constellation of viruses that bare ancestral to the current Egyptian H9N2 and H5N1 lineages: A/quail/Hong Kong/G1/97(H9N2) and A/Bar-headed-Goose/Qinghai-65/05(H5N1).
[c] Seven H5N1 isolates (No. 1–7 in Table S1) as well as eight H9N2 isolates (No. 10–17 in Table S1) are summarised. If more than one substitution was detected in a site, the superscript indicates the number of virus showing the respective mutation.

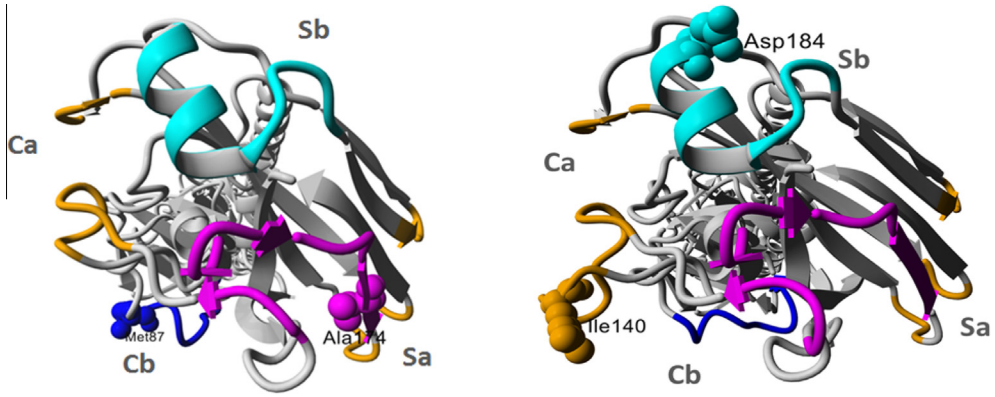

Fig. 3. Epitope configuration at the HA1 protein of H9N2 (A) and H5N1 (B) isolates from Egypt. (A) H9N2 isolate A/chicken/Egypt/NLQP257V-AR757/2013 – an M87L substitution at the Cb antigenic site (blue) and an A174V substitution at the Sa binding site (pink) distinguish this virus from other H9N2 viruses circulating in Egypt. (B) Substituting mutation R140I at the Ca antigenic site (orange) was observed in A/chicken/Egypt/NLQP639V-AR752/2013 while A184D at the Sb antigenic site (cyan) was observed in both A/chicken/Egypt/NLQP7FL-AR747/2013 and A/chicken/Egypt/NLQP2AL-AR749/2013.

Table 5A
SLAC analysis examining selective pressure acting on deduced proteins sequences of H9N2 viruses (2011–2014) and H5N1 viruses (2006–2014).

Viral gene	H9N2	H5N1 (all)	H5N1 (clade 2.2.1)	H5N1 (clade 2.2.1.1)
PB2	0.1304[a]	0.10136	0.08774	0.12266
PB1	0.32770	0.10714	0.11125	0.093646
PB1-F2	3.9533	2.5350	3.01222	1.98144
PA	0.09156	0.13941	0.15333	0.11894
HA	0.31924	0.30964	0.24308	0.4477
HA1	0.30179	0.49429	0.38654	0.67066
NP	0.14504	0.09508	0.09938	0.08378
NA	0.36189	0.25243	0.24789	0.24467
M1	0.10146	0.39087	0.49744	0.47724
M2	0.69202	0.83018	0.96156	1.16878
NS1	0.26047	0.57767	0.57656	0.59324
NS2	0.37105	0.19250	0.34540	0.11507

[a] Values indicate the $dN/dS(\omega)$ substitution rate obtained by SLAC analysis where $\omega = 1$ indicates neutral evolution, $\omega > 1$ indicates positive selection and $\omega < 1$ indicates an overall selective pressure on conservation.

Table 5B
Estimated evolutionary rates of nucleotide substitutions per site per year (subs/site/year) for H9N2 viruses circulating in Egypt between 2011 and 2014. Substitution rates were estimated under a strict clock (SC) and under an uncorrelated lognormal relaxed clock (UL) model. The 95% HPD intervals are given.

Genome segment	SC[a]-skyline[b]		UL[c]-skyline	
	Substitution rates (subs/site/year)		Substitution rates (subs/site/year)	
	Mean ($\times 10^{-3}$)	95% HPD[d] ($\times 10^{-3}$)	Mean ($\times 10^{-3}$)	95% HPD ($\times 10^{-3}$)
PB2	1.81	1.12–2.56	3.72	1.89–6.01
PB1	1.46	0.88–2.14	3.63	2.18–5.02
PA	2.468	1.79–3.12	2.99	1.89–4.31
HA	4.293	3.55–5.05	5.30	4.26–6.37
NP	2.477	1.79–3.19	3.79	2.22–5.63
NA	4.16	3.13–5.30	5.44	3.79–7.01
M	1.882	1.05–2.81	2.16	1.05–3.35
NS	2.433	1.51–3.41	2.83	1.57–4.23

[a] SC = strict molecular clock.
[b] Skyline = Bayesian skyline coalescent prior.
[c] UL = uncorrelated lognormal molecular clock.
[d] HPD = highest posterior density.

2015). Therefore, powerful diagnostic tools would be helpful to detect such events as early as possible. In this study, we developed a rapid sensitive assay for detection of H5N1/H9N2 reassortants and analysed and updated knowledge on the co-evolutionary trajectories of H5N1 and H9N2 in Egypt.

The inspection of sequence data in data bases and the whole genome nucleotide sequencing each of eight H9N2 and seven Egyptian H5N1 isolates from 2013 to 2014 did not give evidence for the existence of such reassortants. The sequencing results were confirmed by a newly developed SYBR Green RT-qPCR based assay which detected fragments of internal gene segments in a subtype discriminative way. These assays also allowed a sequencing-independent genotyping of viruses in six field samples from which no isolates could be grown. Detection of reassortants is usually accomplished by nucleotide sequence analysis of partial or full length gene segments. Such tasks require, in general, the availability of a virus isolate and an appropriately equipped and highly specialised laboratory for either Sanger-based or next generation sequencing. Detection of reassortants, therefore, is often delayed and coincidental. Rapid molecular diagnostic tool for detection of reassortant viruses would be advantageous. The SG-RT-qPCR tool was shown to produce results that fully correlate with sequence analysis when using RNA of viral isolates. In addition, it is also suitable for rapid screening of clinical (swab) samples provided they harbour viral loads that correlate with Ct values of up to 32 (H5N1) or up to 35 (H9N2) based on generic M gene specific RT-qPCR. SG-RT-qPCR is rapid, cost-effective (compared to sequencing), and, most importantly, circumvents the need for virus isolation.

There is a continuing apparent absence of H9N2/H5N1 reassortants in Egypt. However, this is based on analysis of a limited number of fully sequenced isolates. Future use of the SG-RT-qPCR to a much wider scale of samples my give an altered picture. More frequent reassortment events were reported from Bangladesh (Monne et al., 2013b), Pakistan (Iqbal et al., 2009) and China (Bi et al., 2011). Moreover, H9N2 viruses featured as donors in a series of reassortments that lead, e.g., to the generation of highly zoonotic H7N9 and H10N8 viruses in China since early 2013 (Qi et al., 2014; Wu et al., 2013). In addition, the newly emerging H5N8 HP viruses that were spread from Southeast Asia to Europe and North America have formed several new reassortant lineages involving the NA as well as internal gene segments within only a few months of circulation (Adlhoch et al., 2014). The occurrence of H5N1/H9N2 reassortants with unpredictable gene constellations and phenotypes poses a potential threat to both poultry and human populations in general, and to those in Egypt in particular. In Egypt extensive co-circulation of the two viruses is observed since 2011 and the high density of poultry and human populations along the Nile

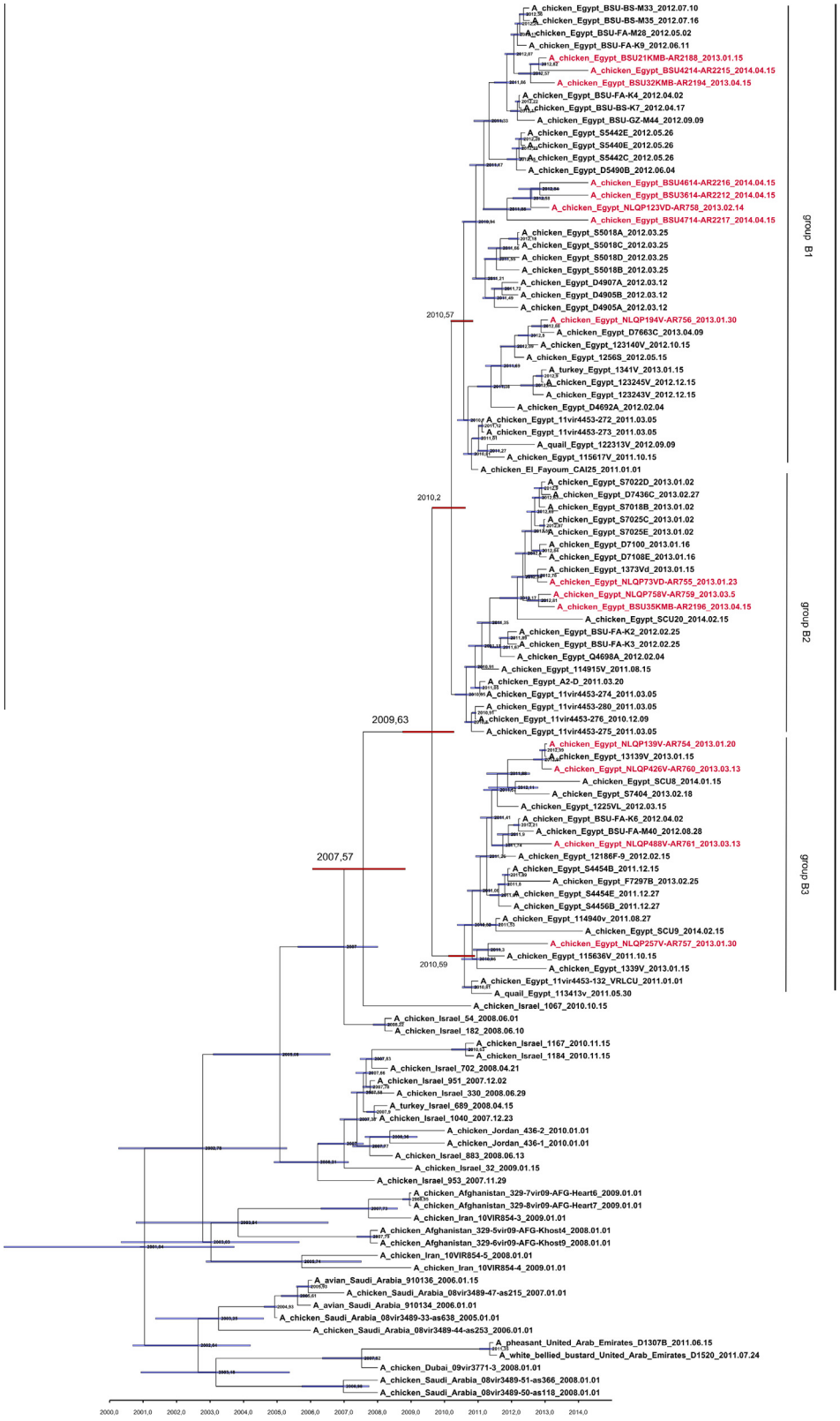

Fig. 4. Maximum clade credibility (MCC) tree based on nucleotide sequences of the HA gene segment of H9N2 viruses. BEAST analysis was carried out to determine time to the most recent common ancestor (TMRCA; indicated by horizontal blue bars overlaying nodes). TMRCA calculations were based on an uncorrelated lognormal relaxed clock model. The tree was scaled to time using the collection dates (day/month/year) of all samples.

valley enables frequent and close contacts between poultry, livestock and humans. Nevertheless there may be several factors that hamper extensive viral reassortments in Egypt: H5N1 in Egypt is highly pathogenic and infected non-vaccinated chickens, particularly in the backyard sector, die rapidly and are removed as hosts for reassortment while infected water fowl does not necessarily succumb to the infection and may excrete virus for prolonged times creating, in theory, a fertile ground for reassortment. However, H9N2 infections have so far been largely confined to the chicken sector of the Egyptian poultry industry and infections have not regularly been reported in domestic waterfowl to date (Abdelwhab and Abdel-Moneim, 2015). As such the main replication pools of H9N2 (chickens) and H5N1 (backyard poultry, ducks) are separated. Nevertheless, bivalent vaccination of chickens against both H5N1 and H9N2 as practiced in some sectors of the Egyptian poultry industry is highly recommended to prevent mixed infections and hence the risk of reassortment.

Currently (January 2015) an alarming increase of human cases of H5N1 infections in Egypt is observed (ProMED-mail, 2015) that cannot be easily explained with changing behaviours of poultry rearing and marketing. A close observation of the viruses in Egypt is essential to detect emerging variants as early as possible. Phylogenetic analysis of the Egyptian H9N2 and H5N1 viruses in this study did not yield evidence for new lineages developing in 2013 and 2014. However, virulence and host tropism are polygenic traits in influenza viruses that are still difficult to dissect by using single molecular markers. Markers known to be associated with increased affinity of avian influenza viruses to the human host have been extensively described in Egyptian H9N2 and H5N1 viruses (El-Shesheny et al., 2014; Kandeil et al., 2014) and were also detected in viruses from 2013 to 2014 sequenced in this study (Table 4).

In addition to the above mentioned substitutions several mutations previously not described in Egyptian H9N2 and H5N1 viruses were discovered in the frame of the current study: For the H9N2 viruses these comprise PA A404S and NP I61L. Moreover, the PB1-F2 protein of Egyptian H9N2 isolates revealed three variants of which two were truncated to 52 aa and 79 aa, respectively, apart from the intact 90 aa protein. The 52 aa form with an N-terminal truncation is rarely represented in avian viruses (including H5N1) in the data bases but has been frequently described in swine H1N1 viruses (Chakrabarti and Pasricha, 2013). The 79 aa protein, truncated at its C-terminus, was previously recorded in swine H3N2 viruses (Harder et al., 2013). The HA of H9N2 viruses sequenced in this study showed aa H183 and L226 (H3 numbering) which would correlate with a shift in affinity of the HA from the avian to the human sialic acid receptor type (Wan et al., 2008). Only one H9N2 virus, A/chicken/Egypt/NLQP73VD-AR755/2013, carried Q226 indicating a preference for the avian receptor. Another strain, A/chicken/Egypt/NLQP257V-AR757/2013, revealed two physicochemically conserved aa substitutions located in antibody epitopes of the HA1 region, M79L in the antigenic site Cb and A166V (H3 numbering) at the Sa epitope. These mutations have not been detected in any other H9N2 virus from Egypt so far, and due to the conservation of chemical properties, their effect on antigenicity is unclear. Also unique to Egyptian H9N2 viruses M2 V27I presumed to be associated with amantadine resistance was detected in three isolates; no further amantadine resistance markers were present (Belshe et al., 1988).

Previously undescribed in the HA of Egyptian H5N1 viruses was an additional, sixth, PGS at position 54 in isolate A/chicken/Egypt/NLQP20SL-AR751/2013. Furthermore, downstream of the 20 aa deletion present in the stalk domain of Egyptian N1 NA proteins, one additional deletion at position 56 (N1 numbering) was identified in isolates A/chicken/Egypt/NLQP7FL-AR747/2013 and A/chicken/Egypt/NLQP2AL-AR749/2013. The same mutation is recorded in another isolate from 2013 (EPI538758). Phenotypic consequences of this additional deletion are not clear but may be associated with on-going adaptation to chickens as a host, amongst other factors. It should be noted that in the same two isolates showing deletion A56 a substitution was present in antigenic site Sb of the HA1:A184D (Shore et al., 2013).

Several scenarios were proposed for the introduction of H9N2 into poultry in Egypt. (i) Smuggling of infected poultry from neighbouring countries (El-Zoghby et al., 2012), (ii) wild birds as a source (Abdel-Moneim et al., 2012), (iii) undetected long term circulation of the virus (Monne et al., 2013a). H9N2 seropositive poultry was detected for the first time in 2009 (Afifi et al., 2013). BEAST analysis carried out here narrowed the possible introduction date of H9N2 viruses into Egypt to July–August 2009 (Fig. 4). H9N2 viruses from Egypt and Israel share a common ancestor that is predicted to have existed in mid 2007 although there is a gap of sequence data for that region between 2007 and 2009. There is a single report of an H9N2 virus detected in a chicken in Egypt in 2006 but no HA sequences of this virus are available. As such it is likely to assume that H9N2 viruses have been introduced by unknown routes from neighbouring countries of Egypt in the Middle East region in 2009 and that these viruses could have been in (limited) circulation for almost 2 years until detected at a larger scale in commercial poultry in Egypt.

5. Conclusions

The endemic status of avian influenza viruses H9N2 and of HP H5N1 persisted in Egypt through 2014. While phylogenetically no significant changes versus previous years were observed, a number of new substituting mutations were detected in several viral proteins. BEAST analysis allowed narrowing the introduction date of H9N2 viruses into Egypt to summer 2009. Nucleotide sequence analysis of whole genomes of 15 isolates did not yield evidence for genome reassortment of these viruses. The successful development of SG-RT-qPCRs for the specific detection and differentiation of the internal gene segments of H9N2 and H5N1 aided in analysing further 9 clinical samples from which no isolate was available. Again, no reassortants were found. The SG-RT-qPCR will aid in screening on a wider scope as these PCRs are directly applicable also to field samples whereby circumventing needs for virus isolation. The epidemiology of AIV in poultry in Egypt will require enduring close observation. Further development and continued adaptation of rapid and cost-effective screening assays are at the basis of any efforts in improving the currently critical situation.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.meegid.2015.06.004.

References

Abdel-Moneim, A.S., Afifi, M.A., El-Kady, M.F., 2012. Isolation and mutation trend analysis of influenza A virus subtype H9N2 in Egypt. Virol. J. 9, 173.

Abdelwhab, E.M., Abdel-Moneim, A.S., 2015. Epidemiology, ecology and gene pool of influenza A virus in Egypt: will Egypt be the epicentre of the next influenza pandemic? Virulence 6, 6–18.

Abdelwhab, E.M., Arafa, A.S., Stech, J., Grund, C., Stech, O., Graeber-Gerberding, M., Beer, M., Hassan, M.K., Aly, M.M., Harder, T.C., Hafez, H.M., 2012. Diversifying evolution of highly pathogenic H5N1 avian influenza virus in Egypt from 2006 to 2011. Virus Genes 45, 14–23.

Abdelwhab, E.M., Hafez, H.M., 2011. An overview of the epidemic of highly pathogenic H5N1 avian influenza virus in Egypt: epidemiology and control challenges. Epidemiol. Infect. 139, 647–657.

Adlhoch, C., Gossner, C., Koch, G., Brown, I., Bouwstra, R., Verdonck, F., Penttinen, P., Harder, T., 2014. Comparing introduction to Europe of highly pathogenic avian influenza viruses A(H5N8) in 2014 and A(H5N1) in 2005. Euro. Surveill., 19

Afifi, M.A., El-Kady, M.F., Zoelfakar, S.A., Abdel-Moneim, A.S., 2013. Serological surveillance reveals widespread influenza A H7 and H9 subtypes among chicken flocks in Egypt. Trop. Anim. Health Prod. 45, 687–690.

Alexander, D.J., 2007. An overview of the epidemiology of avian influenza. Vaccine 25, 5637–5644.

Arafa, A.S., Hagag, N.M., Yehia, N., Zanaty, A.M., Naguib, M.M., Nasef, S.A., 2012. Effect of cocirculation of highly pathogenic avian influenza H5N1 subtype with low pathogenic H9N2 subtype on the spread of infections. Avian Dis. 56, 849–857.

Belshe, R.B., Smith, M.H., Hall, C.B., Betts, R., Hay, A.J., 1988. Genetic basis of resistance to rimantadine emerging during treatment of influenza virus infection. J. Virol. 62, 1508–1512.

Bi, Y., Lu, L., Li, J., Yin, Y., Zhang, Y., Gao, H., Qin, Z., Zeshan, B., Liu, J., Sun, L., Liu, W., 2011. Novel genetic reassortants in H9N2 influenza A viruses and their diverse pathogenicity to mice. Virol. J. 8, 505.

Cameron, K.R., Gregory, V., Banks, J., Brown, I.H., Alexander, D.J., Hay, A.J., Lin, Y.P., 2000. H9N2 subtype influenza A viruses in poultry in Pakistan are closely related to the H9N2 viruses responsible for human infection in Hong Kong. Virology 278, 36–41.

Cardona, C.J., Xing, Z., Sandrock, C.E., Davis, C.E., 2009. Avian influenza in birds and mammals. Comp. Immunol. Microbiol. Infect. Dis. 32, 255–273.

Chakrabarti, A.K., Pasricha, G., 2013. An insight into the PB1F2 protein and its multifunctional role in enhancing the pathogenicity of the influenza A viruses. Virology 440, 97–104.

Chen, G.W., Chang, S.C., Mok, C.K., Lo, Y.L., Kung, Y.N., Huang, J.H., Shih, Y.H., Wang, J.Y., Chiang, C., Chen, C.J., Shih, S.R., 2006. Genomic signatures of human versus avian influenza A viruses. Emerg. Infect. Dis. 12, 1353–1360.

Chen, H., Bright, R.A., Subbarao, K., Smith, C., Cox, N.J., Katz, J.M., Matsuoka, Y., 2007. Polygenic virulence factors involved in pathogenesis of 1997 Hong Kong H5N1 influenza viruses in mice. Virus Res. 128, 159–163.

Chen, H., Yuan, H., Gao, R., Zhang, J., Wang, D., Xiong, Y., Fan, G., Yang, F., Li, X., Zhou, J., Zou, S., Yang, L., Chen, T., Dong, L., Bo, H., Zhao, X., Zhang, Y., Lan, Y., Bai, T., Dong, J., Li, Q., Wang, S., Zhang, Y., Li, H., Gong, T., Shi, Y., Ni, X., Li, J., Zhou, J., Fan, J., Wu, J., Zhou, X., Hu, M., Wan, J., Yang, W., Li, D., Wu, G., Feng, Z., Gao, G.F., Wang, Y., Jin, Q., Liu, M., Shu, Y., 2014. Clinical and epidemiological characteristics of a fatal case of avian influenza A H10N8 virus infection: a descriptive study. Lancet 383, 714–721.

Darriba, D., Taboada, G.L., Doallo, R., Posada, D., 2012. JModelTest 2: more models, new heuristics and parallel computing. Nat. Methods 9, 772.

Drummond, A.J., Suchard, M.A., Xie, D., Rambaut, A., 2012. Bayesian phylogenetics with BEAUti and the BEAST 1.7. Mol. Biol. Evol. 29, 1969–1973.

Dung, T.C., Dinh, P.N., Nam, V.S., Tan, L.M., Hang Nle, K., Thanh le, T., Mai le, Q., 2014. Seroprevalence survey of avian influenza A(H5N1) among live poultry market workers in northern Viet Nam, 2011. Western Pac. Surveill. Response J. 5, 21–26.

El-Shesheny, R., Kandeil, A., Bagato, O., Maatouq, A.M., Moatasim, Y., Rubrum, A., Song, M.S., Webby, R.J., Ali, M.A., Kayali, G., 2014. Molecular characterization of avian influenza H5N1 virus in Egypt and the emergence of a novel endemic subclade. J. Gen. Virol. 95, 1444–1463.

El-Zoghby, E.F., Arafa, A.S., Hassan, M.K., Aly, M.M., Selim, A., Kilany, W.H., Selim, U., Nasef, S., Aggor, M.G., Abdelwhab, E.M., Hafez, H.M., 2012. Isolation of H9N2 avian influenza virus from bobwhite quail (*Colinus virginianus*) in Egypt. Arch. Virol. 157, 1167–1172.

EMPRES, 2015. Animal Influenza Update. Availabe online at: <http://empres-i.fao.org/empres-i/home> (accessed 13.02.15).

Eswar, N., Webb, B., Marti-Renom, M.A., Madhusudhan, M.S., Eramian, D., Shen, M.Y., Pieper, U., Sali, A., 2006. Comparative protein structure modeling using modeller. Curr. Protoc. Bioinf. (Chapter 5, Unit 5 6)

Fan, S., Macken, C.A., Li, C., Ozawa, M., Goto, H., Iswahyudi, N.F., Nidom, C.A., Chen, H., Neumann, G., Kawaoka, Y., 2013. Synergistic effect of the PDZ and p85beta-binding domains of the NS1 protein on virulence of an avian H5N1 influenza A virus. J. Virol. 87, 4861–4871.

Fanning, A.S., Anderson, J.M., 1999. PDZ domains: fundamental building blocks in the organization of protein complexes at the plasma membrane. J. Clin. Invest. 103, 767–772.

Fereidouni, S.R., Harder, T.C., Gaidet, N., Ziller, M., Hoffmann, B., Hammoumi, S., Globig, A., Starick, E., 2012. Saving resources: avian influenza surveillance using pooled swab samples and reduced reaction volumes in real-time RT-PCR. J. Virol. Methods 186, 119–125.

Finkelstein, D.B., Mukatira, S., Mehta, P.K., Obenauer, J.C., Su, X., Webster, R.G., Naeve, C.W., 2007. Persistent host markers in pandemic and H5N1 influenza viruses. J. Virol. 81, 10292–10299.

Fuller, T.L., Gilbert, M., Martin, V., Cappelle, J., Hosseini, P., Njabo, K.Y., Abdel Aziz, S., Xiao, X., Daszak, P., Smith, T.B., 2013. Predicting hotspots for influenza virus reassortment. Emerg. Infect. Dis. 19, 581–588.

Fusaro, A., Monne, I., Salviato, A., Valastro, V., Schivo, A., Amarin, N.M., Gonzalez, C., Ismail, M.M., Al-Ankari, A.R., Al-Blowi, M.H., Khan, O.A., Maken Ali, A.S., Hedayati, A., Garcia Garcia, J., Ziay, G.M., Shoushtari, A., Al Qahtani, K.N., Capua, I., Holmes, E.C., Cattoli, G., 2011. Phylogeography and evolutionary history of reassortant H9N2 viruses with potential human health implications. J. Virol. 85, 8413–8421.

Gabriel, G., Dauber, B., Wolff, T., Planz, O., Klenk, H.D., Stech, J., 2005. The viral polymerase mediates adaptation of an avian influenza virus to a mammalian host. Proc. Natl. Acad. Sci. USA 102, 18590–18595.

Gao, Y., Zhang, Y., Shinya, K., Deng, G., Jiang, Y., Li, Z., Guan, Y., Tian, G., Li, Y., Shi, J., Liu, L., Zeng, X., Bu, Z., Xia, X., Kawaoka, Y., Chen, H., 2009. Identification of amino acids in HA and PB2 critical for the transmission of H5N1 avian influenza viruses in a mammalian host. PLoS Pathog. 5, e1000709.

Gomaa, M.R., Kayed, A.S., Elabd, M.A., Zeid, D.A., Zaki, S.A., El Rifay, A.S., Sherif, L.S., McKenzie, P.P., Webster, R.G., Webby, R.J., Ali, M.A., Kayali, G., 2015. Avian influenza A(H5N1) and A(H9N2) seroprevalence and risk factors for infection among Egyptians: a prospective, controlled seroepidemiological study. J. Infect. Dis. 211, 1399–1407.

Guo, Y.J., Krauss, S., Senne, D.A., Mo, I.P., Lo, K.S., Xiong, X.P., Norwood, M., Shortridge, K.F., Webster, R.G., Guan, Y., 2000. Characterization of the pathogenicity of members of the newly established H9N2 influenza virus lineages in Asia. Virology 267, 279–288.

Gupta, R., Brunak, S., 2002. Prediction of glycosylation across the human proteome and the correlation to protein function. Pac. Symp. Biocomput., 310–322

Ha, Y., Stevens, D.J., Skehel, J.J., Wiley, D.C., 2001. X-ray structures of H5 avian and H9 swine influenza virus hemagglutinins bound to avian and human receptor analogs. Proc. Natl. Acad. Sci. USA 98, 11181–11186.

Hall, T., 1999. BioEdit: a user-friendly biological sequence alignment editor and analysis program for Windows 95/98/NT. Nucleic Acids Symp. Ser. 41, 95–98.

Harder, T.C., Grosse Beilage, E., Lange, E., Meiners, C., Dohring, S., Pesch, S., Noe, T., Grund, C., Beer, M., Starick, E., 2013. Expanded cocirculation of stable subtypes, emerging lineages, and new sporadic reassortants of porcine influenza viruses in swine populations in Northwest Germany. J. Virol. 87, 10460–10476.

Hilleman, M.R., 2002. Realities and enigmas of human viral influenza: pathogenesis, epidemiology and control. Vaccine 20, 3068–3087.

Hoper, D., Hoffmann, B., Beer, M., 2009. Simple, sensitive, and swift sequencing of complete H5N1 avian influenza virus genomes. J. Clin. Microbiol. 47, 674–679.

Iqbal, M., Yaqub, T., Reddy, K., McCauley, J.W., 2009. Novel genotypes of H9N2 influenza A viruses isolated from poultry in Pakistan containing NS genes similar to highly pathogenic H7N3 and H5N1 viruses. PLoS One 4, e5788.

Ison, M.G., 2011. Antivirals and resistance. influenza virus. Curr. Opin. Virol. 1, 563–573.

Jackson, D., Hossain, M.J., Hickman, D., Perez, D.R., Lamb, R.A., 2008. A new influenza virus virulence determinant: the NS1 protein four C-terminal residues modulate pathogenicity. Proc. Natl. Acad. Sci. USA 105, 4381–4386.

Kalthoff, D., Globig, A., Beer, M., 2010. (Highly pathogenic) avian influenza as a zoonotic agent. Vet. Microbiol. 140, 237–245.

Kandeil, A., El-Shesheny, R., Maatouq, A.M., Moatasim, Y., Shehata, M.M., Bagato, O., Rubrum, A., Shanmuganatham, K., Webby, R.J., Ali, M.A., Kayali, G., 2014. Genetic and antigenic evolution of H9N2 avian influenza viruses circulating in Egypt between 2011 and 2013. Arch. Virol. 159, 2861–2876.

Katoh, K., Standley, D.M., 2013. MAFFT multiple sequence alignment software version 7: improvements in performance and usability. Mol. Biol. Evol. 30, 772–780.

Kayali, G., Kandeil, A., El-Shesheny, R., Kayed, A.S., Gomaa, M.M., Maatouq, A.M., Shehata, M.M., Moatasim, Y., Bagato, O., Cai, Z., Rubrum, A., Kutkat, M.A., McKenzie, P.P., Webster, R.G., Webby, R.J., Ali, M.A., 2014. Active surveillance for avian influenza virus, Egypt, 2010–2012. Emerg. Infect. Dis. 20, 542–551.

Kearse, M., Moir, R., Wilson, A., Stones-Havas, S., Cheung, M., Sturrock, S., Buxton, S., Cooper, A., Markowitz, S., Duran, C., Thierer, T., Ashton, B., Meintjes, P., Drummond, A., 2012. Geneious basic: an integrated and extendable desktop software platform for the organization and analysis of sequence data. Bioinformatics 28, 1647–1649.

Khalenkov, A., Perk, S., Panshin, A., Golender, N., Webster, R.G., 2009. Modulation of the severity of highly pathogenic H5N1 influenza in chickens previously inoculated with Israeli H9N2 influenza viruses. Virology 383, 32–38.

Kibbe, W.A., 2007. OligoCalc: an online oligonucleotide properties calculator. Nucleic Acids Res. 35, W43–W46.

Konagurthu, A.S., Whisstock, J.C., Stuckey, P.J., Lesk, A.M., 2006. MUSTANG: a multiple structural alignment algorithm. Proteins 64, 559–574.

Lam, T.T., Wang, J., Shen, Y., Zhou, B., Duan, L., Cheung, C.L., Ma, C., Lycett, S.J., Leung, C.Y., Chen, X., Li, L., Hong, W., Chai, Y., Zhou, L., Liang, H., Ou, Z., Liu, Y., Farooqui, A., Kelvin, D.J., Poon, L.L., Smith, D.K., Pybus, O.G., Leung, G.M., Shu, Y., Webster, R.G., Webby, R.J., Peiris, J.S., Rambaut, A., Zhu, H., Guan, Y., 2013. The genesis and source of the H7N9 influenza viruses causing human infections in China. Nature 502, 241–244.

Lin, Y.P., Shaw, M., Gregory, V., Cameron, K., Lim, W., Klimov, A., Subbarao, K., Guan, Y., Krauss, S., Shortridge, K., Webster, R., Cox, N., Hay, A., 2000. Avian-to-human

transmission of H9N2 subtype influenza A viruses: relationship between H9N2 and H5N1 human isolates. Proc. Natl. Acad. Sci. USA 97, 9654–9658.
Lin, Y.P., Xiong, X., Wharton, S.A., Martin, S.R., Coombs, P.J., Vachieri, S.G., Christodoulou, E., Walker, P.A., Liu, J., Skehel, J.J., Gamblin, S.J., Hay, A.J., Daniels, R.S., McCauley, J.W., 2012. Evolution of the receptor binding properties of the influenza A(H3N2) hemagglutinin. Proc. Natl. Acad. Sci. USA 109, 21474–21479.
Liu, J., Stevens, D.J., Haire, L.F., Walker, P.A., Coombs, P.J., Russell, R.J., Gamblin, S.J., Skehel, J.J., 2009. Structures of receptor complexes formed by hemagglutinins from the Asian influenza pandemic of 1957. Proc. Natl. Acad. Sci. USA 106, 17175–17180.
Long, J.X., Peng, D.X., Liu, Y.L., Wu, Y.T., Liu, X.F., 2008. Virulence of H5N1 avian influenza virus enhanced by a 15-nucleotide deletion in the viral nonstructural gene. Virus Genes 36, 471–478.
Matrosovich, M.N., Krauss, S., Webster, R.G., 2001. H9N2 influenza A viruses from poultry in Asia have human virus-like receptor specificity. Virology 281, 156–162.
Minh, B.Q., Nguyen, M.A., von Haeseler, A., 2013. Ultrafast approximation for phylogenetic bootstrap. Mol. Biol. Evol. 30, 1188–1195.
Monne, I., Ormelli, S., Salviato, A., De Battisti, C., Bettini, F., Salomoni, A., Drago, A., Zecchin, B., Capua, I., Cattoli, G., 2008. Development and validation of a one-step real-time PCR assay for simultaneous detection of subtype H5, H7, and H9 avian influenza viruses. J. Clin. Microbiol. 46, 1769–1773.
Monne, I., Hussein, H.A., Fusaro, A., Valastro, V., Hamoud, M.M., Khalefa, R.A., Dardir, S.N., Radwan, M.I., Capua, I., Cattoli, G., 2013a. H9N2 influenza A virus circulates in H5N1 endemically infected poultry population in Egypt. Influenza Other Respir. Viruses 7, 240–243.
Monne, I., Yamage, M., Dauphin, G., Claes, F., Ahmed, G., Giasuddin, M., Salviato, A., Ormelli, S., Bonfante, F., Schivo, A., Cattoli, G., 2013b. Reassortant avian influenza A(H5N1) viruses with H9N2-PB1 gene in poultry, Bangladesh. Emerg. Infect. Dis. 19, 1630–1634.
Munir, M., Zohari, S., Iqbal, M., Abbas, M., Perez, D.R., Berg, M., 2013. The non-structural (NS) gene segment of H9N2 influenza virus isolated from backyard poultry in Pakistan reveals strong genetic and functional similarities to the NS gene of highly pathogenic H5N1. Virulence 4, 612–623.
Nguyen, L.T., Schmidt, H.A., von Haeseler, A., Minh, B.Q., 2014. IQ-TREE: a fast and effective stochastic algorithm for estimating maximum likelihood phylogenies. Mol. Biol. Evol.
OIE, 2014. Chapter 2.3.4. Avian Influenza. Available online at: <http://www.oie.int/fileadmin/Home/eng/Health_standards/tahm/2.03.04_AI.pdf> (accessed 22.11.14).
Pan, Q., Liu, A., Zhang, F., Ling, Y., Ou, C., Hou, N., He, C., 2012. Co-infection of broilers with *Ornithobacterium rhinotracheale* and H9N2 avian influenza virus. BMC Vet. Res. 8, 104.
Poon, A.F., Frost, S.D., Pond, S.L., 2009. Detecting signatures of selection from DNA sequences using Datamonkey. Methods Mol. Biol. 537, 163–183.
ProMED-mail, 2015. Avian Influenza Human. Available online at: <http://www.promedmail.org> (accessed 25.01.15).
Qi, W., Zhou, X., Shi, W., Huang, L., Xia, W., Liu, D., Li, H., Chen, S., Lei, F., Cao, L., Wu, J., He, F., Song, W., Li, Q., Li, H., Liao, M., Liu, M., 2014. Genesis of the novel human-infecting influenza A(H10N8) virus and potential genetic diversity of the virus in poultry, China. Euro. Surveill. 19.
Rolling, T., Koerner, I., Zimmermann, P., Holz, K., Haller, O., Staeheli, P., Kochs, G., 2009. Adaptive mutations resulting in enhanced polymerase activity contribute to high virulence of influenza A virus in mice. J. Virol. 83, 6673–6680.
Schmolke, M., Manicassamy, B., Pena, L., Sutton, T., Hai, R., Varga, Z.T., Hale, B.G., Steel, J., Perez, D.R., Garcia-Sastre, A., 2011. Differential contribution of PB1-F2 to the virulence of highly pathogenic H5N1 influenza A virus in mammalian and avian species. PLoS Pathog. 7, e1002186.
Scholtissek, C., Quack, G., Klenk, H.D., Webster, R.G., 1998. How to overcome resistance of influenza A viruses against adamantane derivatives. Antiviral Res. 37, 83–95.
Seo, S.H., Hoffmann, E., Webster, R.G., 2002. Lethal H5N1 influenza viruses escape host anti-viral cytokine responses. Nat. Med. 8, 950–954.
Shahsavandi, S., Salmanian, A.H., Ghorashi, S.A., Masoudi, S., Ebrahimi, M.M., 2012. Evolutionary characterization of hemagglutinin gene of H9N2 influenza viruses isolated from Asia. Res. Vet. Sci. 93, 234–239.
Shaw, M., Cooper, L., Xu, X., Thompson, W., Krauss, S., Guan, Y., Zhou, N., Klimov, A., Cox, N., Webster, R., Lim, W., Shortridge, K., Subbarao, K., 2002. Molecular changes associated with the transmission of avian influenza a H5N1 and H9N2 viruses to humans. J. Med. Virol. 66, 107–114.
Shen, M.Y., Sali, A., 2006. Statistical potential for assessment and prediction of protein structures. Protein Sci. 15, 2507–2524.
Shinya, K., Hamm, S., Hatta, M., Ito, H., Ito, T., Kawaoka, Y., 2004. PB2 amino acid at position 627 affects replicative efficiency, but not cell tropism, of Hong Kong H5N1 influenza A viruses in mice. Virology 320, 258–266.
Shope, R.E., 1958. Influenza: history, epidemiology, and speculation. Public Health Rep. 73, 165–178.
Shore, D.A., Yang, H., Balish, A.L., Shepard, S.S., Carney, P.J., Chang, J.C., Davis, C.T., Donis, R.O., Villanueva, J.M., Klimov, A.I., Stevens, J., 2013. Structural and antigenic variation among diverse clade 2 H5N1 viruses. PLoS One 8, e75209.
Subbarao, E.K., London, W., Murphy, B.R., 1993. A single amino acid in the PB2 gene of influenza A virus is a determinant of host range. J. Virol. 67, 1761–1764.
Swayne, D.E., Pavade, G., Hamilton, K., Vallat, B., Miyagishima, K., 2011. Assessment of national strategies for control of high-pathogenicity avian influenza and low-pathogenicity notifiable avian influenza in poultry, with emphasis on vaccines and vaccination. Rev. Sci. Tech. 30, 839–870.
Taubenberger, J.K., Morens, D.M., 2006. 1918 Influenza: the mother of all pandemics. Emerg. Infect. Dis. 12, 15–22.
Uhlendorff, J., Matrosovich, T., Klenk, H.D., Matrosovich, M., 2009. Functional significance of the hemadsorption activity of influenza virus neuraminidase and its alteration in pandemic viruses. Arch. Virol. 154, 945–957.
Wan, H., Sorrell, E.M., Song, H., Hossain, M.J., Ramirez-Nieto, G., Monne, I., Stevens, J., Cattoli, G., Capua, I., Chen, L.M., Donis, R.O., Busch, J., Paulson, J.C., Brockwell, C., Webby, R., Blanco, J., Al-Natour, M.Q., Perez, D.R., 2008. Replication and transmission of H9N2 influenza viruses in ferrets: evaluation of pandemic potential. PLoS One 3, e2923.
Wang, J., Sun, Y., Xu, Q., Tan, Y., Pu, J., Yang, H., Brown, E.G., Liu, J., 2012. Mouse-adapted H9N2 influenza A virus PB2 protein M147L and E627K mutations are critical for high virulence. PLoS One 7, e40752.
Wasilenko, J.L., Sarmento, L., Pantin-Jackwood, M.J., 2009. A single substitution in amino acid 184 of the NP protein alters the replication and pathogenicity of H5N1 avian influenza viruses in chickens. Arch. Virol. 154, 969–979.
Webster, R.G., Bean, W.J., Gorman, O.T., Chambers, T.M., Kawaoka, Y., 1992. Evolution and ecology of influenza A viruses. Microbiol. Rev. 56, 152–179.
WHO, 2013. Cumulative Number of Confirmed Human Cases for Avian Influenza A(H5N1) Reported to WHO, 2003–2013. Available online at: <http://www.who.int/influenza/human_animal_interface/EN_GIP_20130604CumulativeNumberH5N1cases.pdf> (accessed 26.08.13).
WHO, 2014a. Cumulative Number of Confirmed Human Cases of Avian Influenza A(H5N1) Reported to WHO. Available online at: <http://www.who.int/influenza/human_animal_interface/H5N1_cumulative_table_archives/en/> (accessed December, 24).
WHO, 2014b. Revised and updated nomenclature for highly pathogenic avian influenza A (H5N1) viruses. Influenza Other Respir. Viruses 8, 384–388.
Wu, A., Su, C., Wang, D., Peng, Y., Liu, M., Hua, S., Li, T., Gao, G.F., Tang, H., Chen, J., Liu, X., Shu, Y., Peng, D., Jiang, T., 2013. Sequential reassortments underlie diverse influenza H7N9 genotypes in China. Cell Host Microbe 14, 446–452.
Zhou, B., Pearce, M.B., Li, Y., Wang, J., Mason, R.J., Tumpey, T.M., Wentworth, D.E., 2013. Asparagine substitution at PB2 residue 701 enhances the replication, pathogenicity, and transmission of the 2009 pandemic H1N1 influenza A virus. PLoS One 8, e67616.