Outbreaks of highly pathogenic avian influenza H5N1 clade 2.3.2.1c in hunting falcons and kept wild birds in Dubai implicate intercontinental virus spread

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Highly pathogenic avian influenza viruses (HPAIVs) of subtype H5N1 have continued to perpetuate with divergent genetic variants in poultry within Asia since 2003. Further dissemination of Asian-derived H5 HPAIVs to Europe, Africa and, most recently, to the North American continent has occurred. We report an outbreak of HPAIV H5N1 among falcons kept for hunting and other wild bird species bred as falcon prey in Dubai, United Arab Emirates, during the autumn of 2014. The causative agent was identified as avian influenza virus subtype H5N1, clade 2.3.2.1c, by genetic and phylogenetic analyses. High mortality in infected birds was in accordance with systemic pathomorphological and histological alterations in affected falcons. Genetic analysis showed the HPAIV H5N1 of clade 2.3.2.1c is a reassortant in which the PB2 segment was derived from an Asian-origin H9N2 virus lineage. The Dubai H5N1 viruses were closely related to contemporary H5N1 HPAIVs from Nigeria, Burkina-Faso, Romania and Bulgaria. Median-joining network analysis of 2.3.2.1c viruses revealed that the Dubai outbreak was an episode of a westward spread of these viruses on a larger scale from unidentified Asian sources. The incursion into Dubai, possibly via infected captive hunting falcons returning from hunting trips to central Asian countries, preceded outbreaks in Nigeria and other West African countries. The alarmingly enhanced geographical mobility of clade 2.3.2.1c and clade 2.3.4.4 viruses may represent another wave of transcontinental dissemination of Asian-origin HPAIV H5 viruses, such as the outbreak at Qinghai Lake caused by clade 2.2 (‘Qinghai’ lineage) in 2005.

INTRODUCTION

Highly pathogenic avian influenza viruses (HPAIVs) of subtype H5Nx have continued, since 2003, to circulate and diversify in poultry within South-east Asia. Further spread of different virus lineages of the Asian-derived H5Nx HPAIVs to Europe, Africa and, most recently, the North American continent has brought significant turbulence to poultry industries (Alders et al., 2014; Chmielewski & Swayne, 2011; University of Minnesota, 2015). Apart from causing severe economic losses, there exists remarkable zoonotic potential in several of these virus lineages. Considering the significant threat H5N1 viruses pose to the global economy and human health, public awareness of the potential risks of HPAI H5N1 viruses has increased considerably (Webster & Hulse, 2005). Continuous genetic and antigenic variation of the viral haemagglutinin (HA) and/or neuraminidase (NA) genes and proteins resulting in the emergence of new HPAIV H5Nx lineages has frequently been recognized in the past. During the last years of circulation of H5N1 viruses in poultry, 10 genetically distinct virus clades (numbered 0 to 9) with

Sequences established in this study for eight segments of six selected H5N1-positive samples have been submitted to the Global Initiative on Sharing All Influenza Data (GISAID) database and their assigned accession numbers are shown in Table 2.

One supplementary figure and two supplementary tables are available with the online Supplementary Material.
further diverged subclades down to (currently) a fifth order
as based on genetic diversification and phylogenetic analysis
of the HA gene have been recognized by the WHO/OIE/FAO
Evolution Working Group (Donis et al., 2015).

In 2004, H5N1 HPAIVs of clade 2.3.2.1 were detected and
isolated for the first time from a dead Chinese pond heron
in Hong Kong (WHO/OIE/FAO H5N1 Evolution Working
Group, 2012). Thereafter, viruses of this fourth-order clade
have spread geographically and further evolved in Asia
(WHO/OIE/FAO H5N1 Evolution Working Group,
2012). Clade 2.3.2.1 viruses had diversified into subclades
term 2.3.2.1a and 2.3.2.1b by 2011/2012. The most
recent addition to this list is represented by clade
2.3.2.1c, which emerged in Vietnam in 2012, following
reassortment between 2.3.2.1 viruses of genotypes a and b
(II) viruses, and became predominant in 2013 (Lee et al.,
2015). In addition, H5N1 viruses of clade 2.3.2.1c sporadically
occurred in Europe, i.e. in Bulgaria and Romania, in
poultry and wild birds in 2010, but vanished thereafter
(Reid et al., 2011). Most recently, 2.3.2.1c viruses were
found to cause outbreaks in Nigeria and other West African
countries (Monne et al., 2015). Also, these viruses
reappeared, in 2015, in Central Asia (Azerbaijan) and
south-eastern Europe (Romania, Bulgaria and possibly Turkey)
(OIE, 2015). The possible transmission routes from
Asia to Europe and Africa were not fully understood,
but an involvement of migratory wild birds and/or land-
based poultry was suspected (Chen et al., 2005; WHO/
Trans-species transmission of the 2.3.2.1c lineage to mammals
has occurred on at least two occasions: a Canadian nurse
who returned home from China and fell sick was finally
diagnosed with a clade 2.3.2.1c (A/Alberta/01/2014) infection
(Pabbaraju et al., 2014); earlier, a tiger in a zoo in China
was found positive for that clade (A/tiger/Jiangsu/
01/2013) (He et al., 2015).

In this study, an outbreak of HPAIV among hunting falcons and kept wild birds in Dubai during the autumn of 2014 was investigated. Viruses of subtype H5N1, clade 2.3.2.1c, were identified and analysed by genetic and phylo-
genetic means. Median-joining network analysis of 2.3.2.1c viruses suggested that the Dubai outbreak is related to an intercontinental westward spread of these viruses from unknown sources in Asia, and that the introduction of 2.3.2.1c viruses to Dubai preceded outbreaks in Nigeria and other West African countries.

RESULTS

Epidemiology

Signs of a systemic disease were noticed in falcons kept for
hunting in the United Arab Emirates (UAE) in November
and December 2014. These falcons, mainly gyr falcons
(Falco rusticolus) or hybrids of gyr and peregrine falcons
(Falco peregrinus), most probably had returned from a
hunting trip to central Asian countries together with free-
ranging houbara bustards (Chlamydotis undulata macqueenii), which are routinely used as prey. The peak season for hunting with falcons as a sport is in December, and many falconers traditionally hunt with their falcons in Asian countries. It is therefore considered not unlikely that on returning to UAE the falcons or the accompanying live prey birds had acquired the infection in their Asian hunting grounds and spread the disease to other avian species like quail, ducks, seagulls or stone curlews that were kept in the same prey-breeding premises in UAE. From such index holding, the infection likely spread to other prey-
breeding facilities across the entire country. However, it is not clear if there was only one or several foci of incursion and spread of HPAIV H5N1 at the same time. The outbreaks were contained in March 2015 by strict quarantine measures and by culling all susceptible birds in affected holdings. Poultry holdings were not affected since there are no contacts between the falcon-keeping and prey-
breeding facilities and poultry production units.

Clinical symptoms, pathology and histology

The most striking finding at necropsy of falcons and hou-
bara bustards was multifocal haemorrhagic necrosis in the
pancreas (Fig. 1a), while this was not seen in the other bird
species. Marked lung congestion with focal haemorrhages
was seen in all birds. Histological lesions corresponded
well with the gross findings in falcons and houbara bust-
tards: marked acute pancreatitis with atrophy of exocrine
tissue, with numerous eosinophilic necroses, but without
cellular infiltration (Fig. 1b). Marked haemosiderosis of
hepatic Kupffer cells was also observed in the falcons.
Besides lung congestion with focal haemorrhages, mild ca-
tarrhal tracheitis was seen in all bird species.

Phylogenetic analyses

Maximum-likelihood-based phylogenetic analyses of the
HA gene confirmed that HPAIV H5N1 viruses from Dubai, 2014, fell into clade 2.3.2.1c (Fig. 2a). Closest
relationships were seen with contemporary viruses from
West African countries, e.g. A/chicken/Nigeria/15VIR39-
2/2015, and from Romania and Bulgaria, 2015. The
human case (A/Alberta/01/2014) and the second mamma-
lian-derived sequence, A/tiger/Jiangsu/01/2013, were more
distantly related. These viruses together with the ones from Dubai formed a phylogenetic subcluster distinct
from viruses of clade 2.3.2.1c (Fig. 2a), and the internal
gene segments, except PB2, showed the same phylogenetic
relationships as described for HA (Figs 3 and S1, available
in the online Supplementary Material). Among the internal
gene segments of the Dubai isolates, the PB2 sequences
showed a divergent phylogeny and did not cluster with
those of other, older, 2.3.2.1c viruses; instead, a BLASTN2
search revealed that a PB2 gene from an H9N2 subtype
lineage recently detected in Shandong province in China
[A/chicken/Shandong/qd0917/2013 (H9N2), accession number
KM609853] appeared to be the most closely related (98 % nucleotide identity; data not shown). In the phylogenetic analysis PB2 consequently fell into a cluster with recent H9N2 viruses from East Asia (Fig. 3). Therefore, the 2.3.2.1c viruses from Dubai represent reassortants between 2.3.2.1 viruses and an H9N2 virus, probably from South-east Asia. A similar finding had been reported for the closely related viruses from Nigeria, 2015 (Monne et al., 2015).

Genetic characterization

Deduced amino acid sequences of the whole genome (except PB2) of six Dubai isolates were compared with closely related viruses within the emerging subcluster in 2.3.2.1c (Table S1) and with further recent 2.3.2.1c viruses from Vietnam and Bangladesh. Viruses within the new subcluster are distinguished by 23 fixed amino acid mutations scattered along the genome among all Dubai viruses and the Nigerian virus as compared with other recent clade 2.3.2.1c viruses (Table 1; further details on markers associated with tropism and virulence are illustrated in Table S1). In addition, further sporadic amino acid substitutions were observed in one or more of the Dubai viruses (not shown).

The amino acid sequence of the HA cleavage site (PQRERRRKR/GLF) of the Dubai viruses revealed multiple basic amino acids, which is characteristic of HPAIVs (Suguitan et al., 2012). The receptor-binding pocket of the HA protein showed markers of avian receptor-specific binding – Q222 and G224 (H5 numbering) (Table S1) – although substitution mutations D94N, S133A and S155N observed in the HA protein have previously been reported to be associated with increased binding to mammalian α-2,6-sialic acid receptor types (Su et al., 2008; Wang et al., 2010; Yang et al., 2007). However, among three substitution mutations at positions 68, 189 and 397 of the HA that are unique to the Dubai, Nigerian and recent European 2.3.2.1c viruses, R189K is predicted to reduce α-2,6-sialic acid binding (Wang et al., 2010). Substitution D68G is predicted to play a role in antigenic drift, and has been identified in an escape mutant (Nakajima et al., 2007). The locations of these mutations are depicted in an HA homology model (Fig. 2b). There were no differences in the number (n = 7) and location of potential N-linked glycosylation sites in the HA protein compared with older viruses of clade 2.3.2.1c.

In the NA protein, no mutations conferring antiviral resistance have been observed. Three new substitution mutations (A59T, D264E and I398M) distinguish the NA protein of the Dubai viruses from recent Vietnamese H5 viruses of clade 2.3.2.1c (Table 1); the biological significance of these mutations remains to be elucidated.

The polymerase protein PB2 contained amino acid 627E, which is characteristic of a preference for replication in avian hosts (Kim et al., 2010), although substitution mutation L89V as previously shown could compensate at least in part for enhanced polymerase activity in mammalian hosts (Li et al., 2009). The PB1-F2 protein of the Dubai viruses is truncated at 57 aa in length which is similar to that of the A/human/Alberta/2013, but the Nigerian virus encoded a full-length 90 aa protein.

The predicted amino acid sequence of the M1 protein revealed substitutions N30D and T215A, which are associated with increased virulence in mice (Fan et al., 2009). The M2 protein mutation V27I of Dubai viruses has been previously reported to reduce susceptibility to the amantadine drugs (Liu et al., 2010). All Dubai viruses expressed serine at position 42 of the NS1 protein, and this mutation was shown to play a key role in viral escape from antiviral immune response of the mammalian host (Jiao et al., 2008). There is an ‘avian-like’ ESEV motif at the NS1 C terminus, which may increase the virulence of H5 viruses in...
Reassortant HPAIV H5N1 in Dubai

Fig. 2. (a) Unrooted phylogenetic tree of the nucleotide sequences of the HA gene segment. Maximum-likelihood calculations were done with the IQ-TREE software (Nguyen et al., 2014) under the best-fit model according to the Akaike criterion (GTR + I + G4). Numbers at nodes represent measures of robustness based on an ultrafast bootstrap approach implemented in IQ-TREE. Viruses from Dubai are shown in red; closely related viruses are coloured in blue. (b) 3D structural homology model for the HA protein of A/Falcon/Dubai/AR3430-2293/2014 as created using SWISS-MODEL (Biasini et al., 2014) with A/tiger/Jiangsu/01/2013 (H5N1) serving as a template. Amino acids distinguishing the Dubai sequence from the modelling template are shown in blue (RasTop software, http://www.geneinfinity.org/rastop/).
Fig. 3. Unrooted phylogenetic tree of the nucleotide sequences of NA, PB1 and PB2 gene segments based on maximum-likelihood calculations (IQ-TREE software) under the best-fit model according to the Akaike criterion (NA and PB1, GTR + K2 + F; PB2, GTR + G). Numbers at nodes represent measures of robustness based on an ultrafast bootstrap approach implemented in IQ-TREE. Viruses from Dubai are shown in red; closely related viruses are coloured in blue. Origin according to subtype of the PB2 sequences is indicated to the right of the tree.
mammals. In addition to a deletion of aa 80–84 in NS1, further substitution mutations D87E, L98F, I101M have also been described to increase virulence in mice (Seo et al., 2002; Spesock et al., 2011).

Network analysis

The analysis established very similar relationships to those produced by maximum-likelihood phylogenetic calculations, and the three subclades of 2.3.2.1 viruses were clearly discernible (Fig. 4). Median-joining (MJ) network analysis for the subclade had to be based on HA sequences alone since very few viruses from this cluster were represented in the databases with a whole genome sequence. This prevented a more refined resolution using concatenated genome segments as demonstrated for example for H7N7 viruses in The Netherlands (Jonges et al., 2011). In our study, both nucleotide and amino acid sequences were used for network analysis (Fig. 4a, b). The nucleotide-based network revealed patterns of the three subclades a, b and c in clade 2.3.2.1. In the amino acid-based network (Fig. 4b) a more refined picture of possible transmission and spreading events emerged, showing that the viruses from West African countries as well as from Bulgaria and Romania were obviously derived from ancestors detected in Dubai. These sequences are linked by very few median vectors, indicating a relatively stringent relationship that does not depend on hypothetical but unsampled or extinct ancestral sequences. Finally, a third analysis was based on concatenated full-genome nucleotide sequences of the Dubai viruses established in this study, including three closely related viruses with full-length genome sequences (Fig. 4c). This study provided a ‘higher magnification’ of the relationships between the Dubai viruses and revealed almost linear relationships. A falcon sequence is close to the root of the other sequences, which can be further divided into two groups comprising falcon AR3430/2014 and viruses from quail, seagull and a stone curlew on one hand, and a second falcon sequence (2506/2014) as well as houbara and duck viruses on the other.

DISCUSSION

Outbreaks of HPAIV H5N1 or other avian influenza viruses involving falcons reared for hunting and several species of kept wild birds bred as falcon prey are not without precedent in the Arabian Peninsula (Aamir et al., 2007; Khan et al., 2009; Marjuki et al., 2009; Wernery et al., 2013). A previous outbreak in Saudi Arabia and Kuwait was caused by H5N1 viruses of clade 2.2 (Khan et al., 2009; Marjuki et al., 2009). The current scenario affecting the UAE appears to be similar in the extent of affected species and the epidemiological patterns, but the HPAIVs causing the outbreak are grouped into clade 2.3.2.1c.

Data from a previous experimental infection of falcons with H5N1 HPAIV revealed that pancreas, proventriculus, gizzard and brain were the most affected organs (Bertran et al., 2012). Depending on the route of experimental infection, different lesions were observed: multifocal in the pancreas and multifocal petechia on the proventriculus–ventriculus junction mucosa in falcons infected via the nasochoanal route; in falcons that ingested infected prey one bird that died 5 days post-infection did not show significant lesions, whereas multifocal petechia on the proventriculus–ventriculus junction mucosa and also multifocal haemorrhagic necrosis in the pancreas were found in birds that succumbed later. This is similar to the lesions seen in our natural infection falcon cases. However, no gross lesions were observed in our falcon cases on the proventriculus–ventriculus junction mucosa. Only one of the experimentally infected falcons developed multifocal haemorrhagic pancreatic necrosis (Bertran et al., 2012), whereas this was a pathognomonic finding in our falcon cases. Experimental infection with H5N1 HPAIV (Bertran et al., 2012) revealed histological lesions mainly in the brain, with multifocal areas of malacia in the cortex, associated with spongiosis of the neuropil, chromatolysis, gliosis and caryolysis. The cerebellum frequently showed multifocal areas of chromatolysis of Purkinje neurons, sometimes associated with necrosis of the Purkinje cell layer and non-suppurative inflammatory infiltrate. However, except for mild gliosis, no histological lesions were observed in the central nervous system of our falcon cases. These differences in the distribution of macroscopic and microscopic lesions might be due to different tissue tropism of the viral strains (Kwon et al., 2011). Vaccination using inactivated H5N1-specific vaccines has been shown to prevent clinical signs of disease following experimental HPAIV H5N1 infection (Lierz et al., 2007).

HPAIVs of clade 2.3.2.1 are widely distributed throughout South-east Asia; since 2010 representatives of this clade have also been found sporadically outside this region, affecting poultry and/or wild birds in the Tyva Republic of Russia as well as at sites on the northwestern coasts of the

<table>
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<th>Protein</th>
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<tr>
<td>PB1</td>
<td>N158S, M179I, M317V, M348L, R391K, Q569H</td>
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<tr>
<td>PB1-F2</td>
<td>R38Q, L43R, I45T, P48Q; truncated in all Dubai viruses at position 57</td>
</tr>
<tr>
<td>HA</td>
<td>D68G, R189K, R397K</td>
</tr>
<tr>
<td>NP</td>
<td>Y343I (not in A/Nigeria/2015)</td>
</tr>
<tr>
<td>NA</td>
<td>A59T, D264E, I389M</td>
</tr>
<tr>
<td>M2</td>
<td>F47L</td>
</tr>
<tr>
<td>NS1</td>
<td>M107L</td>
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<tr>
<td>NS2</td>
<td>G36E</td>
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the Black Sea in Bulgaria and Romania, suggesting a widespread occurrence of this lineage in central Asia also (Donis et al., 2015). In 2013, a novel 2.3.2.1c reassortant lineage carrying the PB2 gene segment of an Asian lineage H9N2 virus was described for the first time (Pabbaraju et al., 2014). The current study showed that descendants of this virus lineage had caused outbreaks of HPAIV in hunting falcons and several kept wild bird species in Dubai in November and December 2014. The viruses formed a distinct phylogenetic subcluster together with contemporary viruses from West Africa, Romania and Bulgaria. The group of the Dubai viruses is characterized by a total of 23 aa substitution mutations scattered across the genome as compared with other recent viruses of clade 2.3.2.1c circulating in Vietnam and Bangladesh (Table 1). These mutations are predicted to correlate with enhanced virulence in mice (matrix protein N30D and T215A; non-structural protein-1 P42S, deletion 80–84, D87E, L98F

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**Fig. 4.** MJ phylogenetic network of HPAIV H5 clade 2.3.2.1. The MJ network was constructed on the basis of nucleotide (a) and amino acid (b) sequences of the haemagglutinin and by concatenating whole genome sequences (c). Network (a) includes all the most parsimonious trees linking the sequences within clade 2.3.2.1. The length of branches is drawn in proportion to the number of mutations separating the entities. Virus entities in each subclade are shown in a distinct colour, clade 2.3.2.1a (green), clade 2.3.2.1b (cyan) and clade 2.3.2.1c (yellow). The node size corresponds to the frequency of the variants in the population after star reduction. Red circles symbolize median vectors that may or may not have existed but are not represented in the sequence databases. (b) A magnified part of the whole network focussing on the Dubai viruses; the corresponding network is based on amino acid sequences. Numbers in red depict the position of amino acid substitutions distinguishing the different entities. (c) A network analysis based on full-genome, concatenated nucleotide sequences spanning the longest ORF for each segment. All Dubai viruses sequenced in this study as well as three closely related viruses for which full genome sequences were available were used. The numbers of nucleotide mutations between the connected viruses are shown in blue; edges without numbering indicate one or two mutations.
and I101M). In addition, amino acid markers associated with increased virus binding to mammalian receptor type α-2,6-sialic acid (D94N, S133A and S155N) were present in the HA protein. In contrast, HA substitution mutation R189K, unique in clade 2.3.2.1 for the Dubai viruses, is predicted to reduce α-2,6-sialic acid binding (Fig. 2b, Table 1).

On a wider perspective the Dubai incursion seems to be part of a larger westward sweep of this virus lineage reaching out again to the northwestern shores of the Black Sea and, for the first time, also to West African countries (Monne et al., 2015). In the latter region the virus continued to spread in 2015 and currently affects at least five countries (Nigeria, Ivory Coast, Niger, Burkina Faso and Ghana) (OIE, 2015).

MJ network analysis was used to define more precisely the origin and spread of the Dubai 2.3.2.1c viruses. The contemporary 2.3.2.1c viruses detected in Vietnam in 2014 are not in the direct ancestry of the Dubai viruses. Instead, a pool of precursor viruses seems to have existed from which the outbreaks in Dubai, south-eastern Europe and West Africa have originated. The full power of MJ network analysis could be employed only partially as only HA sequences were available from a reasonable number of viruses of subclade 2.3.2.1. Analysis of HA amino acid sequences at least yielded evidence that the Dubai viruses are in the direct ancestry of viruses from West Africa and south-eastern Europe. Obviously, the viruses gained a temporary foothold on the Arab Peninsula before moving further on in a westward direction to reach Africa. Network analysis after concatenation of further gene segments was possible only for the Dubai viruses and three further closely related sequences (Fig. 4c). Evidence was obtained that at least two waves of infection have occurred and that a falcon virus was closest to the root of the analysis.

These results allow some educated speculation about possible incursion pathways into Dubai. Falconry is a major business not only in the UAE but across much of the Arabian Peninsula. There are manifold connections as regards the international trade of hunting falcons and traditional hunting trips with falcons, including contacts with countries in Inner Asia where precursors of the Dubai 2.3.2.1c viruses have occurred. No HPAIV outbreaks in poultry holdings or live bird markets have been reported in the UAE. Therefore local poultry is highly unlikely as a source of the infection. In contrast, the succession of events suggests that either falcons returning from hunting trips to central Asian countries and/or wild birds used as live prey for the hunting falcons during hunting trips are a more likely source of incursion. This would be similar to events in the USA, where kept gyr falcons became infected with HPAIV H5N2 by feeding on an American wigeon (Anas americana) hunted by one of the falcons (Ip et al., 2015). Migrating wild birds often come into focus as a potential culprit when spreading routes of HPAI H5N1 viruses are discussed. The Arabian Peninsula lies along the Central Asian flyway, a major migration route for birds from Central Asian breeding grounds to African wintering sites. However, the epidemiological hallmarks of the Dubai outbreak point toward kept hunting falcons or their bred prey as a source of an infection that had likely been acquired during their recent hunting trip to Central Asian countries. The 2.3.2.1c viruses occurred in Dubai before the outbreaks in Nigeria, but it remains unclear whether the Dubai outbreak served as a stepping stone for the virus on its way to Africa or whether a direct introduction of the viruses from Asia into Nigeria has occurred, as suggested by Monne et al. (2015).

It is remarkable that, so far, these viruses have not been detected in Egypt despite a comparatively intense surveillance for avian influenza virus (AIV) in this country. Egypt suffers, since 2006, from intense endemic infections of HPAIV H5N1 in poultry populations, but these viruses belong to clade 2.2.1 and are clearly distinguished from 2.3.2.1c viruses. Although an upsurge of HPAIV cases in poultry and correspondingly also in humans in Egypt has been reported since November 2014, this was not caused by 2.3.2.1c but by a newly emerging cluster of endemic Egyptian viruses, now designated 2.2.1.2 (Arafa et al., 2015). Nevertheless, in light of the alarming spread of 2.3.2.1c viruses in West Africa, introduction of these viruses into Egypt is an eminent threat: presence and co-circulation of two, antigenically divergent HPAIV H5N1 lineages in Egypt would further complicate efforts to eradicate these viruses.

The observed high geographical mobility of certain HPAIV H5 lineages of Asian origin should be both an incentive and a warning to continue and to arrange for new internationally concerted and intensified AIV surveillance programmes in poultry and wild bird populations. Hunting falcons and bred prey populations should essentially be included in such programmes (Kohls et al., 2011). Molecular characterization and whole genome sequencing of AIVs detected provide essential data required to better understand viral transmission networks and identify potential incursion pathways. However, many tesserae are still missing for a better view on the mosaic of spread of H5Nx HPAIVs.

**METHODS**

**Origin of clinical samples.** In November–December 2014, the Central Veterinary Research Laboratory, Dubai (CVRL) received cloacal and oropharyngeal swabs from sick kept birds (hunting falcons, quail, ducks, houbara, seagulls and stone curlews) for bacteriological and virological investigations. Subsamples were passed on to the Friedrich Loeffler Institute in Germany and to Hong Kong University for further molecular analysis.

**Virus isolation and molecular subtyping.** Tissue samples taken from each bird were homogenized and then pooled for virological assay. One millilitre of each clarified tissue suspension, diluted 1 : 10 in minimal essential medium without FBS was inoculated onto separate monolayer cultures of primary chicken embryo fibroblasts (CEF) and 0.2 ml into the allantoic cavity of 9–11-days-old embryonated chicken eggs.
CEF cells showing 100% cytopathic effect after 24 h of incubation and embryonated eggs that died within 36 h of incubation were tested for haemagglutination titres. Samples were tested by PCR (see below) and by a commercial influenza antigen detection assay. Viral RNA was extracted from clinical samples using the QIAamp Viral RNA Mini kit (Qiagen) as recommended by the manufacturer. One-step reverse transcription PCR (RT-PCR) and real-time RT-PCR (RT-qPCR) for identification of type A influenza virus (Fereidouni et al., 2012) HA and NA subtypes (Fereidouni et al., 2012; Gall et al., 2009) was carried out. All manipulations involving potentially infectious materials were performed under the appropriate Biosafety level 3 laboratory conditions at CVRL or the other institutions involved.

Pathomorphological and histological investigations. Dead birds were submitted for post-mortem to CVRL. During necropsy, samples were taken from liver, lung, kidney and intestine for bacterial culture, from brain, liver and lung for virus isolation and from faeces for parasitological examination using routine methods. Samples from all organs including brain were fixed in formalin and processed for routine (haemotoxylin and eosin) histopathology.

Genetic and phylogenetic analyses. Whole genome sequencing of all eight segments of six selected H5N1-positive samples was carried out using primers designed by Höper et al. (2009). The PCR products were purified from agarose gels using the QIAquick gel extraction kit (Qiagen) and sequenced using specific PCR primers (sequences available on request) with the BigDye Terminator v. 3.1 Cycle Sequencing kit (Applied Biosystems) on a 3130-Gene Analyzer (Applied Biosystems). Sequences were assembled and edited using the Geneious software, version 7.1.7. Alignments were performed using BioEdit (Hall, 1999) and MAFFT (Katoh & Standley, 2013). Sequences established in this study have been submitted to the Global Initiative on Sharing All Influenza Data (GISAID) database and assigned accession numbers are shown in Table 2. Sequences of other H5N1 viruses sharing All Influenza Data (GISAID) database and assigned accession numbers are found in Table S2.

MJ network analysis. HA nucleotide and amino acid data of the HA of representative database entries of viruses of clades 2.3.2.1 a–c were aligned with MAFFT and evaluated by Fluxus-Net DNA Alignment software for informative sites used for MJ network construction. Analysis was carried out using the MJ method implemented in Network v. 4.613 software (available at www.fluxus-engineering.com/sharenet.htm; Bandelt et al., 1999), including two rounds of star re-distribution and raising the epsilon parameter to 10.

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REFERENCES


### Table 2: Identity of H5N1 HPAIVs sequenced in this study

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M. M. Naguib and others


