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Genetic characterisation of novel, highly pathogenic avian influenza (HPAI) H5N6 viruses isolated in birds, South Korea, November 2016

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A novel genotype of H5N6 influenza viruses was isolated from migratory birds in South Korea during November 2016. Domestic outbreaks of this virus were associated with die-offs of wild birds near reported poultry cases in Chungbuk province, central South Korea. Genetic analysis and animal studies demonstrated that the Korean H5N6 viruses are highly pathogenic avian influenza (HPAI) viruses and that these viruses are novel reassortants of at least three different subtypes (H5N6, H4N2 and H1N1).

In late October 2016, isolation was reported of highly pathogenic avian influenza (HPAI) H5N6 virus from wild migratory birds in South Korea for the first time [1] which subsequently has caused continuous outbreaks in domestic poultry. In Southeast Asia, HPAI H5 viruses have been continuously isolated from wild birds and domestic poultry since the first detection of A/Gs/Guangdong/1/1996 (Gs/GD/1996, H5N1) in poultry in 1996 [2]. These viruses cause high mortality resulting in serious economic losses in the poultry industry and they spread widely. The HPAI H5N1 subtype was stably maintained for more than a decade before it started to evolve into the novel reassortant HPAI H5Nx virus in 2008 [3]. The HPAI H5N5, which was the first H5Nx subtype isolated, is a member of clade 2.3.4 while most H5Nx recently circulating worldwide, including H5N2, H5N6 and H5N8, cluster into a sublineage of clade 2.3.4 designated as 2.3.4.4 [4]. The clade 2.3.4.4 H5N8 influenza virus was first reported in South Korea in 2014 and subsequently spread to East Asia, Europe, and further to North America and created novel H5Nx subtypes [5-7].

In addition to the H5N8 viruses, the clade 2.3.4.4 H5N6 virus that first emerged in China in 2013, spread to Laos and Vietnam in 2014/15 with evidence of sustained transmission and further geographical spread within both countries. The H5N6 virus caused fatalities in poultry and now appears to be endemic in mainland China, Laos, and Vietnam [8]. Although the H5N8 virus is contained in clade 2.3.4.4 haemagglutinin (HA) gene pools along with HPAI H5N6 viruses, it is relatively low pathogenic in mammalian hosts [5,9], and no human cases have been reported thus far. However, the avian influenza virus subtype H5N6 caused 16 human infections including six fatalities in China as of November 2016 [10].

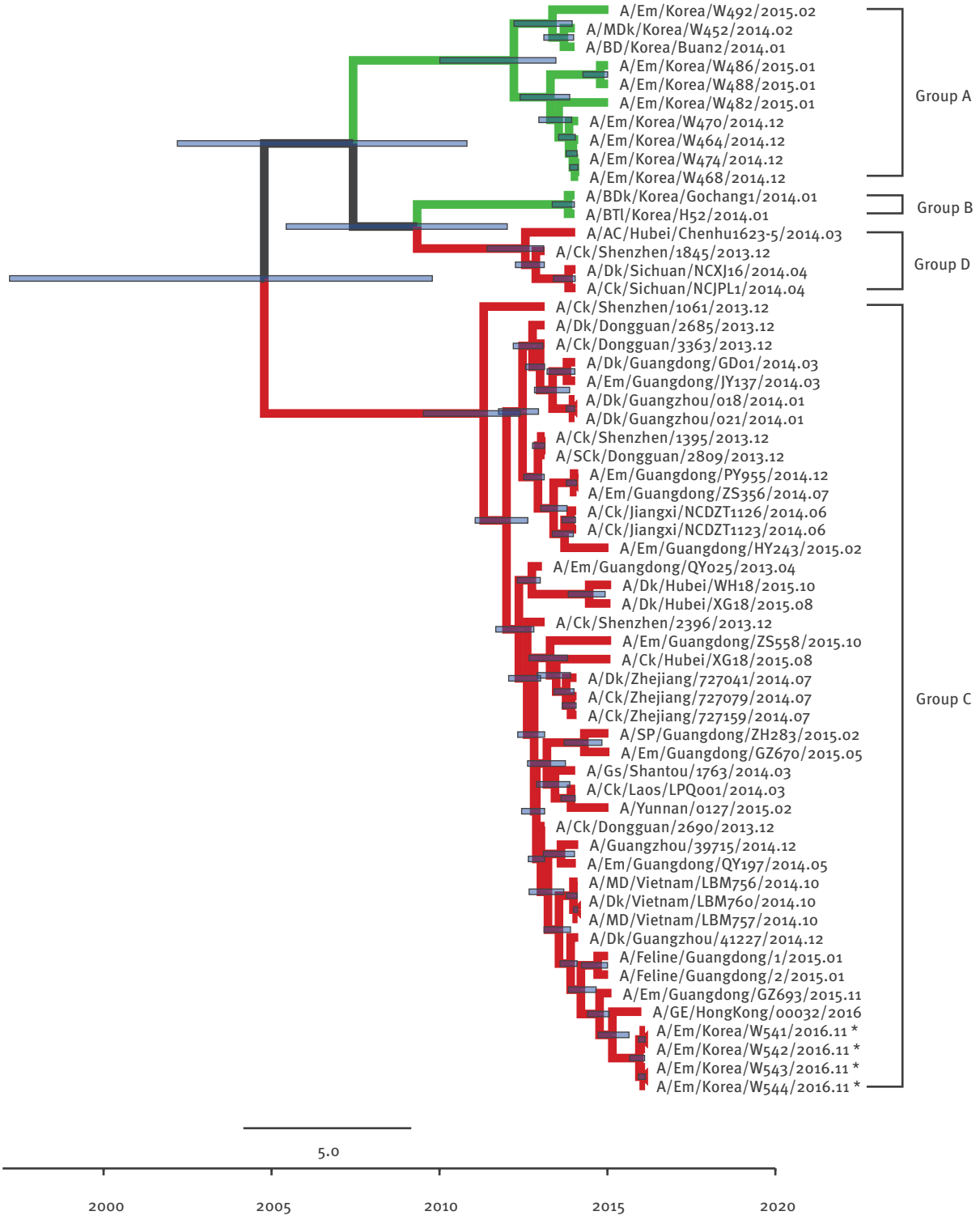
During late October 2016, the clade 2.3.4.4 H5N6 influenza virus was first detected in faecal specimens of migratory wild birds in South Korea and has subsequently caused poultry outbreaks in South Korea from mid-November 2016 [11]. The first reported poultry cases in Chungbuk province in central South Korea were associated with nearby die-offs of wild birds leading to speculation that migratory waterfowl were the source of infection. We report here the genetic characterisation of the H5N6 viruses isolated from faecal specimens of migratory wild birds during these first outbreaks and the investigation of their pathogenic potential in chickens.

Genetic characterisation of novel avian influenza A(H5N6) viruses

Four H5N6 viruses were isolated from faecal samples obtained from migratory bird habitats in Chungbuk Province during a surveillance study conducted on 18 November 2016. Full-length genomic sequence analysis revealed that the viruses showed 99.9% to 100% nucleotide (nt) homology to one another (data not shown). One representative virus, A/Environment/

Figure 1

Phylogenetic tree of H5 segment of novel H5N6 viruses, South Korea, November 2016

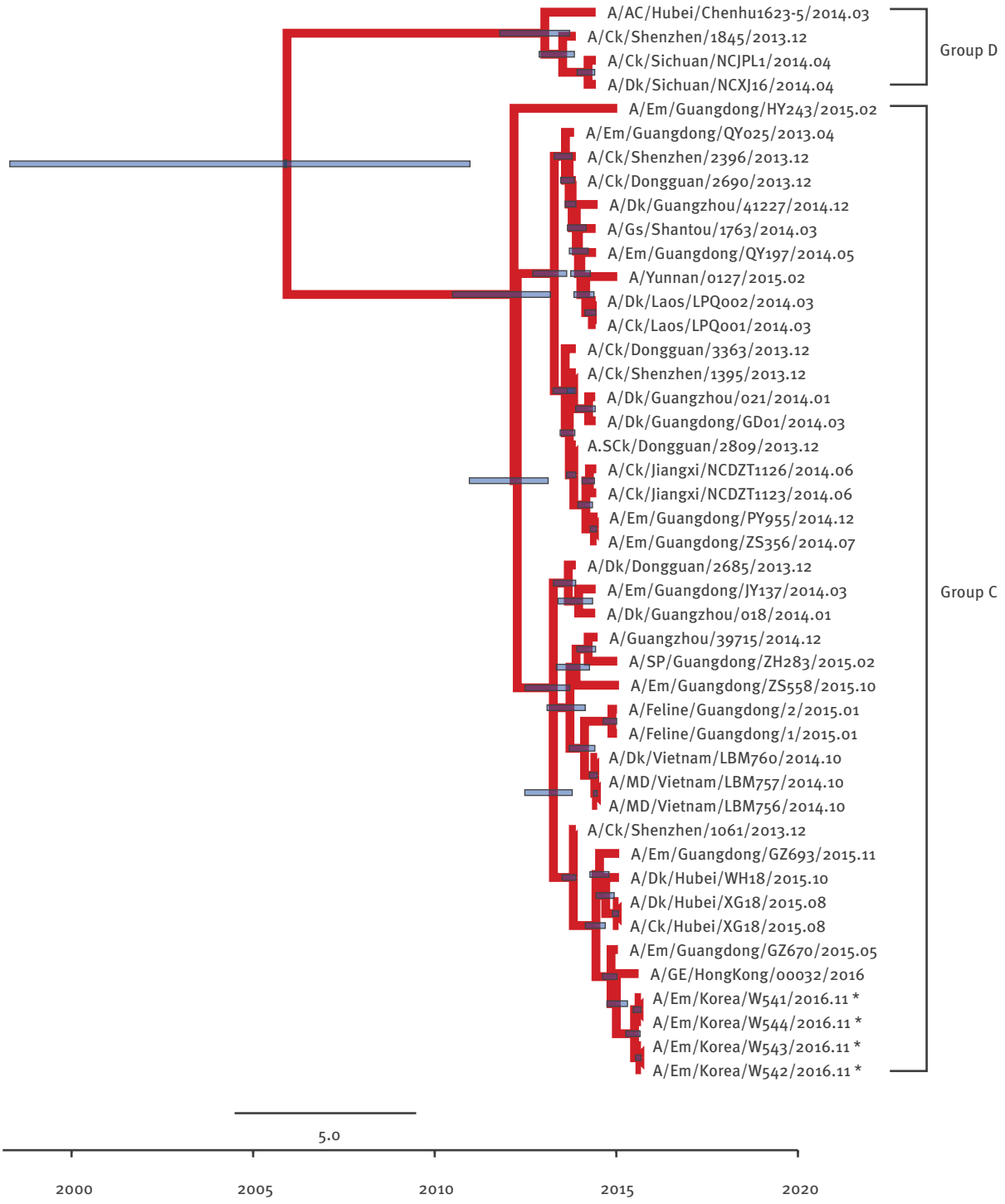


AC: anas crecca; BDk: breeder duck; BD: broiler duck; BTl: baikal teal; Ck: chicken; Dk: duck; Em: environment; GE, great egret; Gs, goose; HA: haemagglutinin; MD, mallard; nt: nucleotide; SCk: silkie chicken; SP: syrrhaptes paradoxus.

To investigate the origins of novel H5N6 viruses (A/Em/Korea/W541/2016, A/Em/Korea/W542/2016, A/Em/Korea/W543/2016, and A/Em/Korea/W544/2016: marked with asterisks), full-length nt sequences of each segment were compared with available H5Nx and high blast scoring virus sequences from the GenBank. The deposited GenBank accession numbers of HA genes are KY272997-KY273000. Time-scaled phylogenies (dates shown on the horizontal axis) were inferred using strict-clock Bayesian Markov chain Monte Carlo analysis. Times of most recent common ancestors with 95% highest posterior density intervals are shown by the horizontal bars at each node (violet line). The month of isolation is indicated at the end of the viral nomenclature. The green line indicates the H5N8 subtype while the red line indicates the H5N6 subtype.

Figure 2

Phylogenetic tree of N6 segment of novel H5N6 viruses, South Korea, November 2016

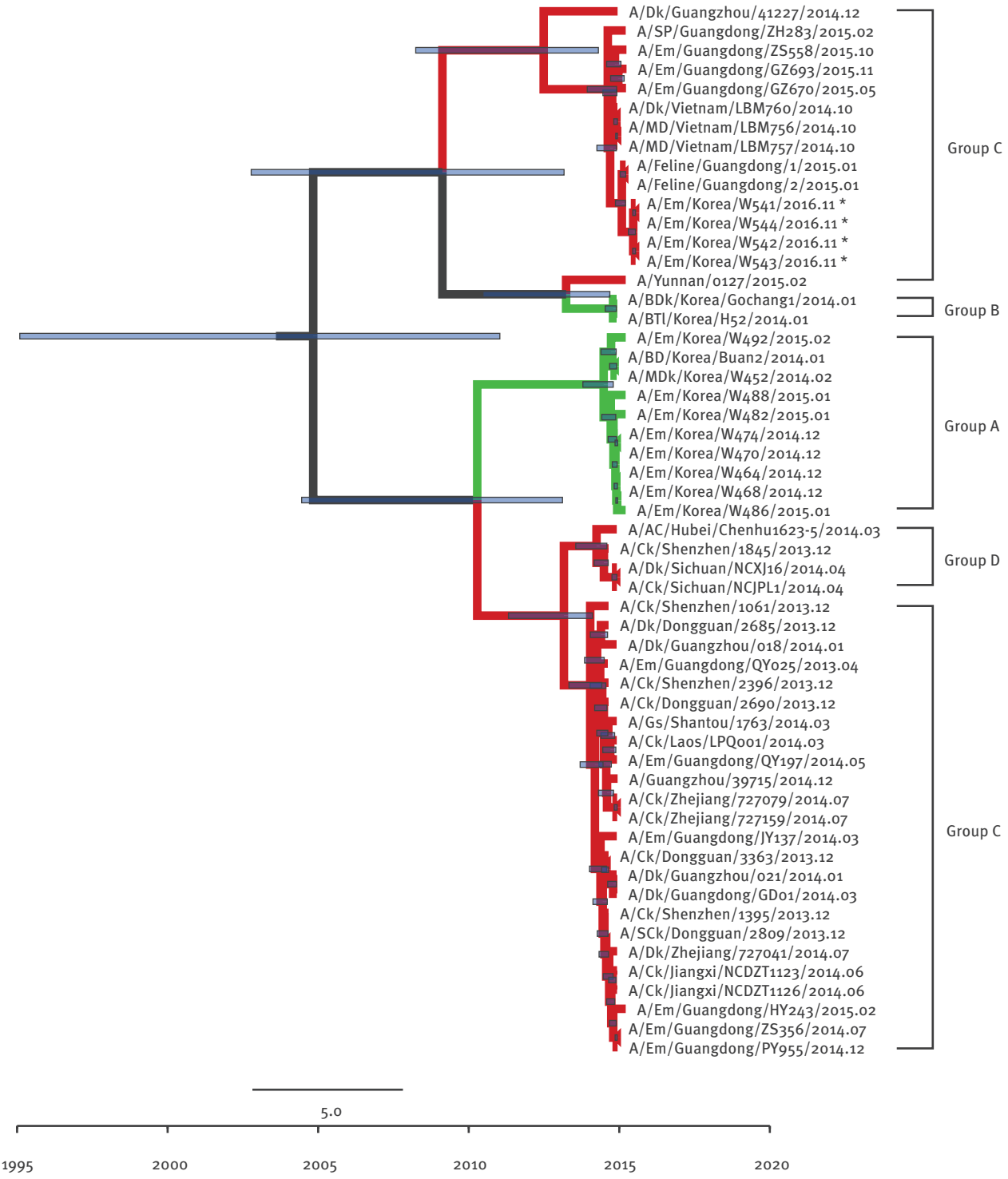


AC: anas crecca; BDk: breeder duck; BD: broiler duck; BTl: baikal teal; Ck: chicken; Dk: duck; Em: environment; GE, great egret; Gs, goose; MD, mallard; NA: neuraminidase; nt: nucleotides; SCk: silkie chicken; SP: syrrhaptes paradoxus.

To investigate the origins of novel H5N6 viruses (A/Em/Korea/W541/2016, A/Em/Korea/W542/2016, A/Em/Korea/W543/2016, and A/Em/Korea/W544/2016: marked with asterisks), full-length nt sequences of each segment were compared with available H5Nx and high blast scoring virus sequences from the GenBank. The deposited GenBank accession numbers of NA genes are KY273005-KY273008. Time-scaled phylogenies (dates shown on the horizontal axis) were inferred using strict-clock Bayesian Markov chain Monte Carlo analysis. Times of most recent common ancestors with 95% highest posterior density intervals are shown by the horizontal bars at each node (violet line). The month of isolation is indicated at the end of the viral nomenclature. The green line indicates the H5N8 subtype while the red line indicates the H5N6 subtype.

Figure 3

Phylogenetic tree of PB2 segment of novel H5N6 viruses, South Korea, November 2016

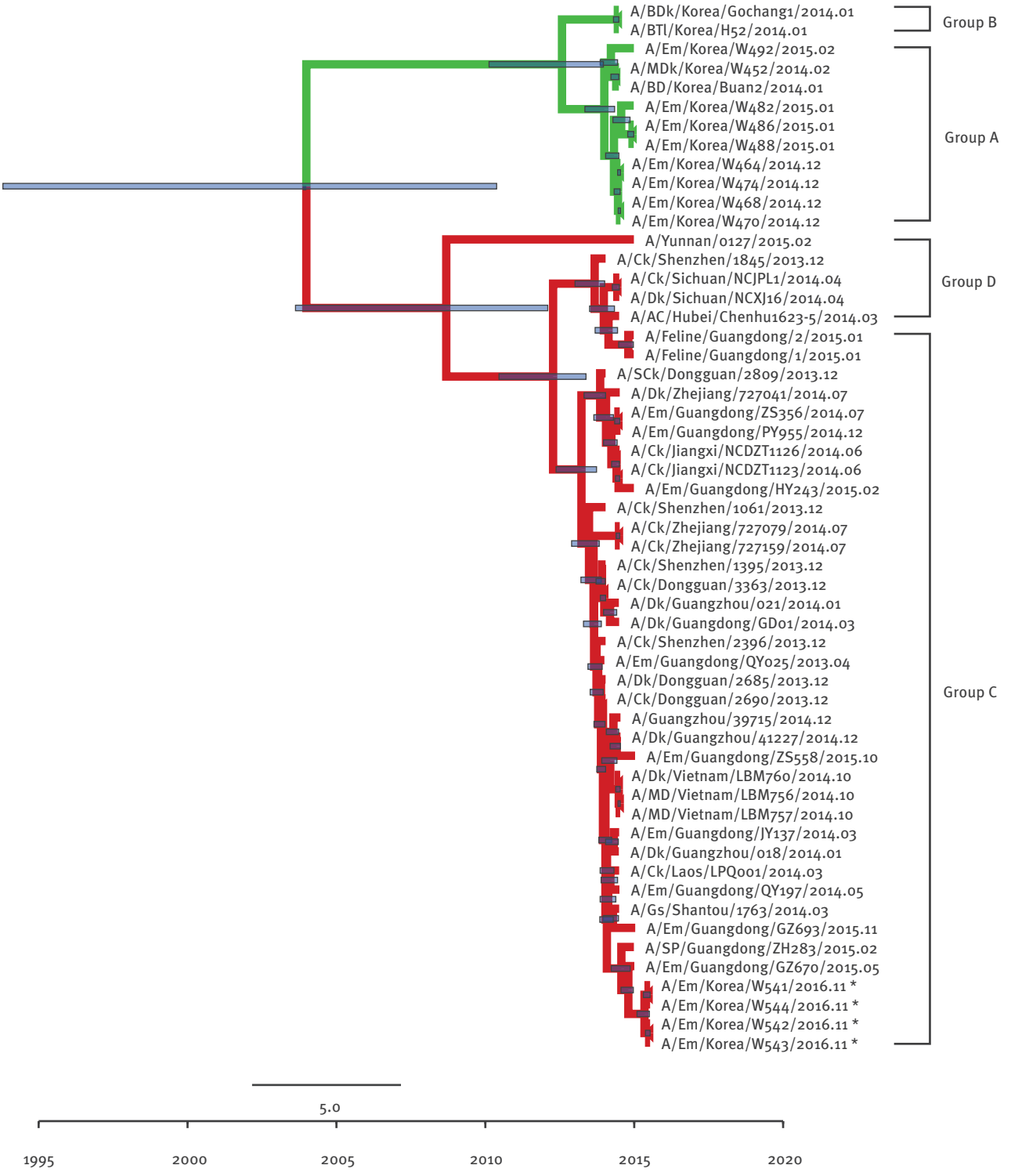


AC: anas crecca; BDk: breeder duck; BD: broiler duck; BTl: baikal teal; Ck: chicken; Dk: duck; Em: environment; GE, great egret; Gs, goose; MD, mallard; nt: nucleotide; PB: polymerase basic protein; SCk: silkie chicken; SP: syrrhaptes paradoxus.

To investigate the origins of novel H5N6 viruses (A/Em/Korea/W541/2016, A/Em/Korea/W542/2016, A/Em/Korea/W543/2016, and A/Em/Korea/W544/2016: marked with asterisks), full-length nt sequences of each segment were compared with available H5Nx and high blast scoring virus sequences from the GenBank. The deposited GenBank accession numbers of PB2 genes are KY273025-KY273028. Time-scaled phylogenies (dates shown on the horizontal axis) were inferred using strict-clock Bayesian Markov chain Monte Carlo analysis. Times of most recent common ancestors with 95% highest posterior density intervals are shown by the horizontal bars at each node (violet line). The month of isolation is indicated at the end of the viral nomenclature. The green line indicates the H5N8 subtype while the red line indicates the H5N6 subtype.

Figure 4

Phylogenetic tree of NP segment of novel H5N6 viruses, South Korea, November 2016

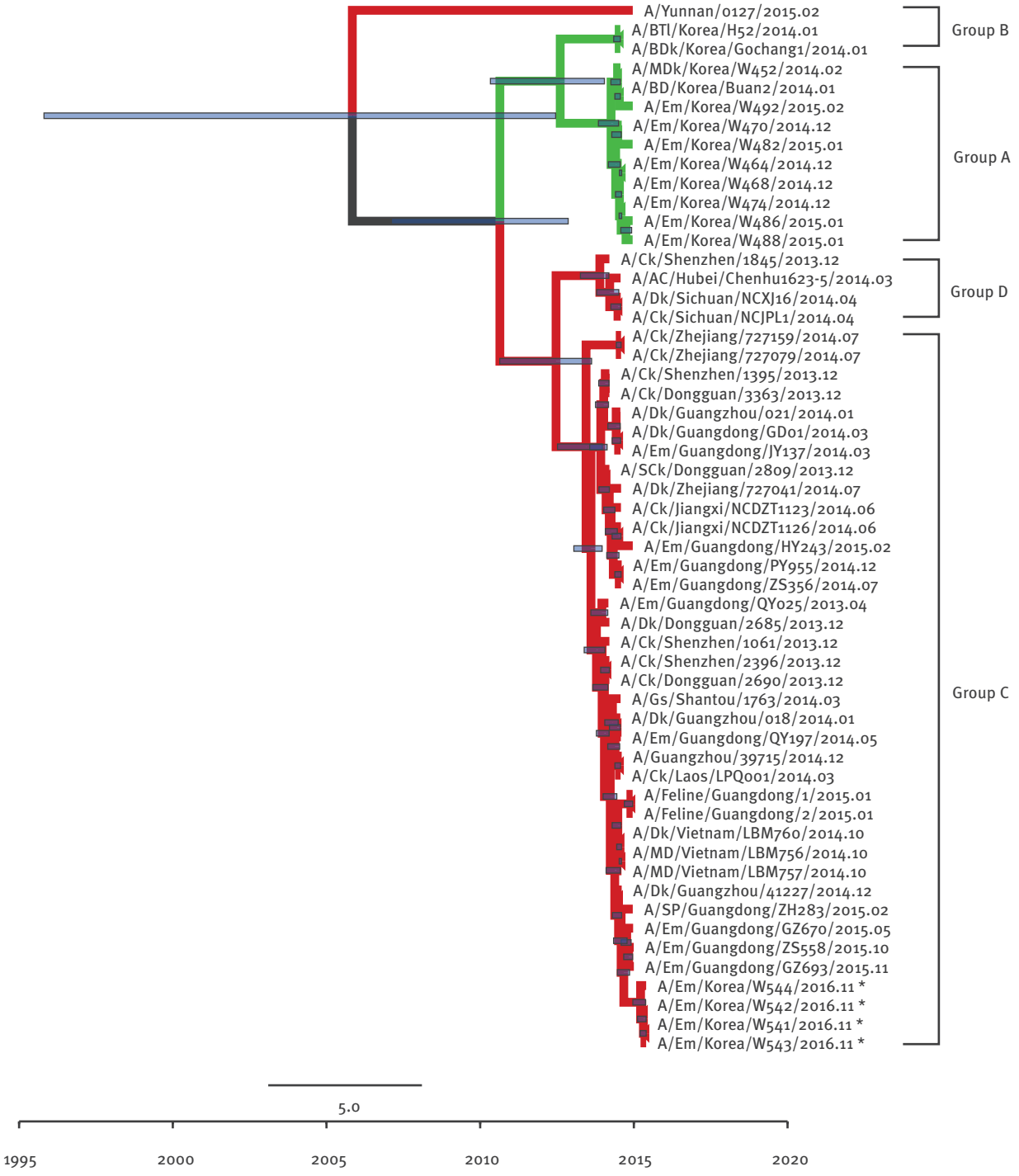


AC: anas crecca; BDk: breeder duck; BD: broiler duck; BTl: baikal teal; Ck: chicken; Dk: duck; Em: environment; GE, great egret; Gs, goose; MD, mallard; NP: nucleoprotein; nt: nucleotide; SCk: silkie chicken; SP: syrrhaptes paradoxus.

To investigate the origins of novel H5N6 viruses (A/Em/Korea/W541/2016, A/Em/Korea/W542/2016, A/Em/Korea/W543/2016, and A/Em/Korea/W544/2016: marked with asterisks), full-length nt sequences of each segment were compared with available H5Nx and high blast scoring virus sequences from the GenBank. The deposited GenBank accession numbers of NP genes are KY273009-KY273012. Time-scaled phylogenies (dates shown on the horizontal axis) were inferred using strict-clock Bayesian Markov chain Monte Carlo analysis. Times of most recent common ancestors with 95% highest posterior density intervals are shown by the horizontal bars at each node (violet line). The month of isolation is indicated at the end of the viral nomenclature. The green line indicates the H5N8 subtype while the red line indicates the H5N6 subtype.

Figure 5

Phylogenetic tree of M segment of novel H5N6 viruses, South Korea, November 2016

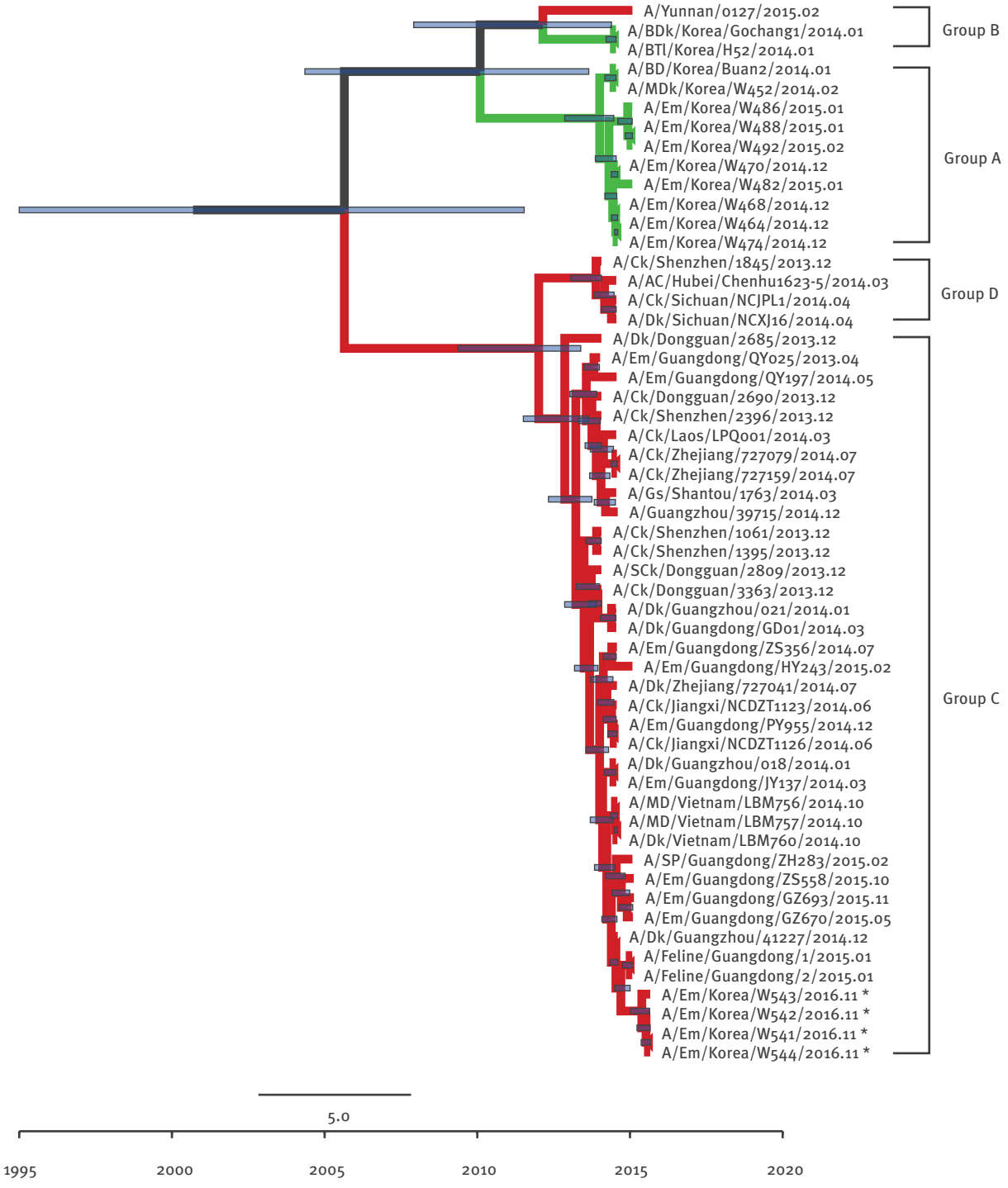


AC: anas crecca; BDk: breeder duck; BD: broiler duck; BTl: baikal teal; Ck: chicken; Dk: duck; Em: environment; GE, great egret; Gs, goose; M: matrix; MD, mallard; nt: nucleotide; SCk: silkie chicken; SP: syrrhaptes paradoxus.

To investigate the origins of novel H5N6 viruses (A/Em/Korea/W541/2016, A/Em/Korea/W542/2016, A/Em/Korea/W543/2016, and A/Em/Korea/W544/2016: marked with asterisks), full-length nt sequences of each segment were compared with available H5Nx and high blast scoring virus sequences from the GenBank. The deposited GenBank accession numbers of M genes are KY273001-KY273004. Time-scaled phylogenies (dates shown on the horizontal axis) were inferred using strict-clock Bayesian Markov chain Monte Carlo analysis. Times of most recent common ancestors with 95% highest posterior density intervals are shown by the horizontal bars at each node (violet line). The month of isolation is indicated at the end of the viral nomenclature. The green line indicates the H5N8 subtype while the red line indicates the H5N6 subtype.

Figure 6

Phylogenetic tree of NS segment of novel H5N6 viruses, South Korea, November 2016

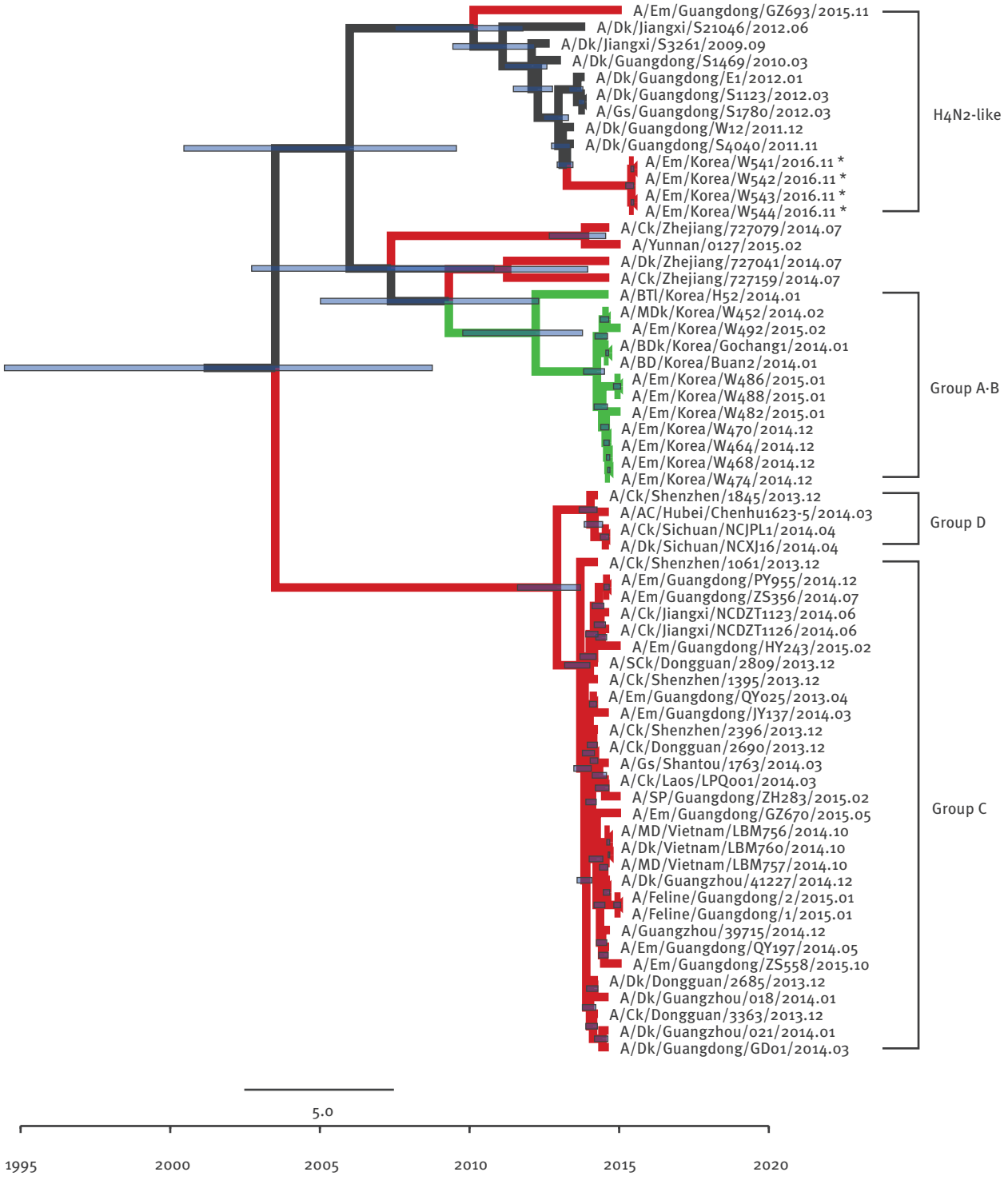


AC: anas crecca; BDk: breeder duck; BD: broiler duck; BTl: baikal teal; Ck: chicken; Dk: duck; Em: environment; GE, great egret; Gs, goose; MD, mallard; NS: nonstructural protein; nt: nucleotide; SCk: silkie chicken; SP: syrrhaptes paradoxus.

To investigate the origins of novel H5N6 viruses (A/Em/Korea/W541/2016, A/Em/Korea/W542/2016, A/Em/Korea/W543/2016, and A/Em/Korea/W544/2016: marked with asterisks), full-length nt sequences of each segment were compared with available H5Nx and high blast scoring virus sequences from the GenBank. The deposited GenBank accession numbers of NS genes are KY273013-KY273016. Time-scaled phylogenies (dates shown on the horizontal axis) were inferred using strict-clock Bayesian Markov chain Monte Carlo analysis. Times of most recent common ancestors with 95% highest posterior density intervals are shown by the horizontal bars at each node (violet line). The month of isolation is indicated at the end of the viral nomenclature. The green line indicates the H5N8 subtype while the red line indicates the H5N6 subtype.

Figure 7

Phylogenetic tree of PB1 segment of novel H5N6 viruses, South Korea November 2016

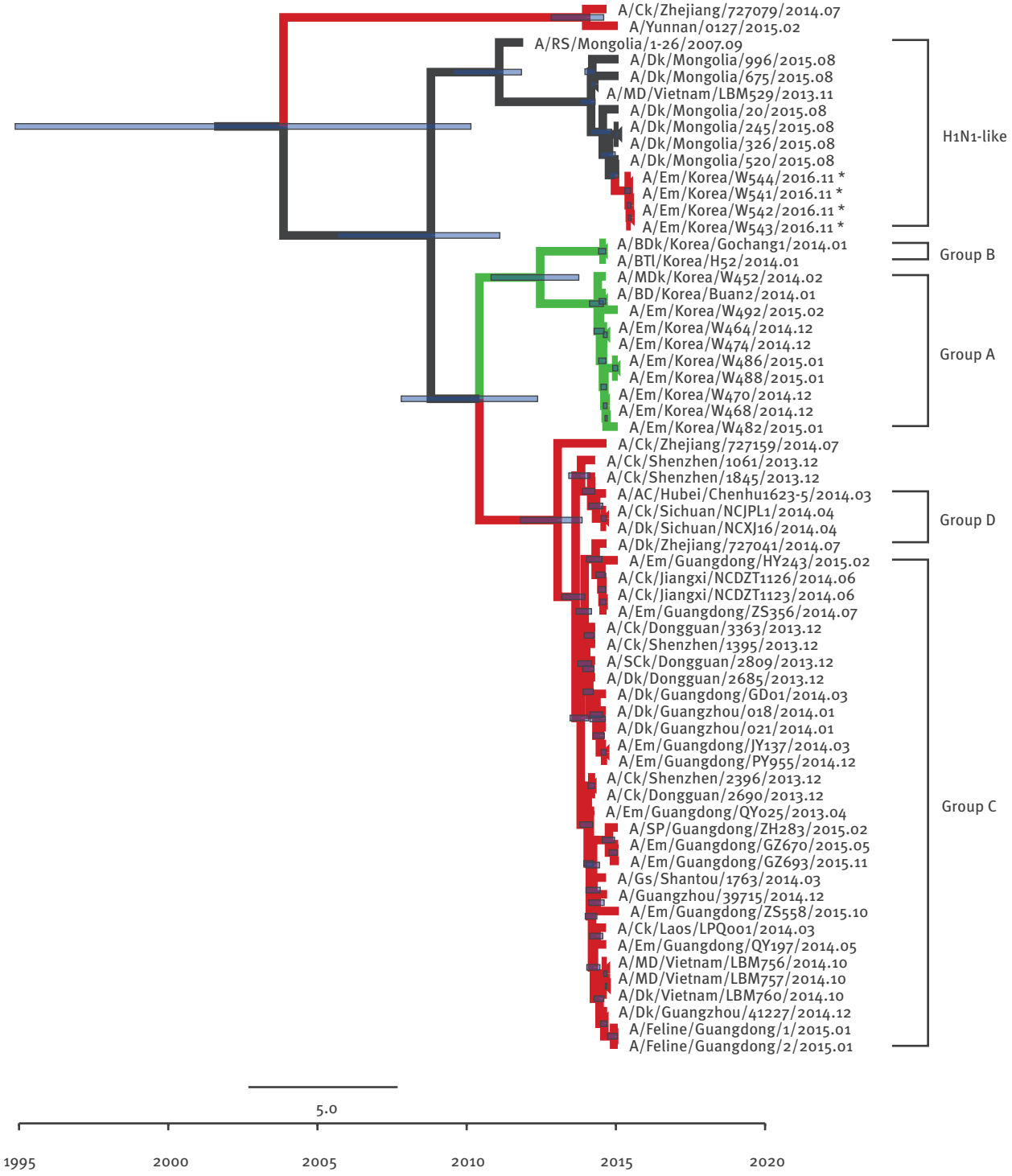


AC: anas crecca; BDk: breeder duck; BD: broiler duck; BTl: baikal teal; Ck: chicken; Dk: duck; Em: environment; GE, great egret; Gs, goose; MD, mallard; nt: nucleotide; PB: polymerase basic protein; SCk: silkie chicken; SP: syrrhaptes paradoxus.

To investigate the origins of novel H5N6 viruses (A/Em/Korea/W541/2016, A/Em/Korea/W542/2016, A/Em/Korea/W543/2016, and A/Em/Korea/W544/2016: marked with asterisks), full-length nt sequences of each segment were compared with available H5Nx and high blast scoring virus sequences from the GenBank. The deposited GenBank accession numbers of PB1 genes are KY273021-KY273024. Time-scaled phylogenies (dates shown on the horizontal axis) were inferred using strict-clock Bayesian Markov chain Monte Carlo analysis. Times of most recent common ancestors with 95% highest posterior density intervals are shown by the horizontal bars at each node (violet line). The month of isolation is indicated at the end of the viral nomenclature. The green line indicates the H5N8 subtype while the red line indicates the H5N6 subtype.

Figure 8

Phylogenetic tree of PA segment of novel H5N6 viruses, South Korea, November 2016

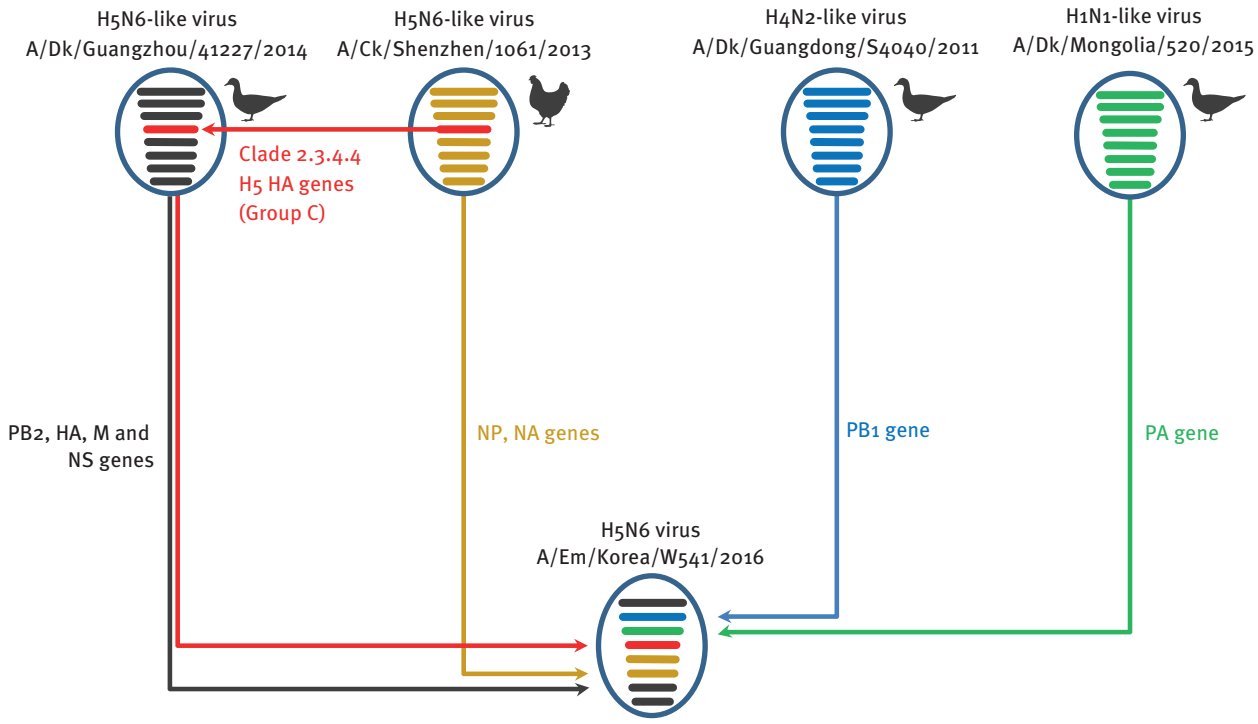


AC: anas crecca; BDk: breeder duck; BD: broiler duck; BTl: baikal teal; Ck: chicken; Dk: duck; Em: environment; GE, great egret; Gs, goose; M: matrix; MD, mallard; nt: nucleotide; PA: polymerase acidic; SCk: silkie chicken; SP: syrrhaptes paradoxus.

To investigate the origins of novel H5N6 viruses (A/Em/Korea/W541/2016, A/Em/Korea/W542/2016, A/Em/Korea/W543/2016, and A/Em/Korea/W544/2016: marked with asterisks), full-length nt sequences of each segment were compared with available H5Nx and high blast scoring virus sequences from the GenBank. The deposited GenBank accession numbers of PA genes are KY273017-KY273020. Time-scaled phylogenies (dates shown on the horizontal axis) were inferred using strict-clock Bayesian Markov chain Monte Carlo analysis. Times of most recent common ancestors with 95% highest posterior density intervals are shown by the horizontal bars at each node (violet line). The month of isolation is indicated at the end of the viral nomenclature. The green line indicates the H5N8 subtype while the red line indicates the H5N6 subtype.

Figure 9

Illustration of genotypes and reassortment events resulting in the novel avain influenza H5N6 virus isolated in South Korea, November 2016



Ck: chicken; Dk: duck; Em: environment; HA: haemagglutinin; HPAI: highly pathogenic avian influenza; M: matrix; NA: neuraminidase; NS: nonstructural; NP: nucleoprotein; PA: polymerase acidic; PB: polymerase basic.

The eight gene segments in each illustrative virus particle represent PB2, PB1, PA, HA, NP, NA, M, and NS genes (in order from top to bottom). The EM/W541 (H5N6) virus was the reassortant with at least three different subtypes (H5N6, H4N2, and H1N1) from the natural gene pool in Eurasia. Each colour represents a virus lineage (Black indicates origin from A/Dk/Guangzhou/41227/2014-like; Orange, A/Ck/Shenzhen/1061/13-like; Blue, A/Dk/Guangdong/S4040/11; Light Green, A/Dk/Mongolia/520/2015; Red, Clade 2.3.4.4 H5 HA genes).

Korea/W541/2016(H5N6), referred to as EM/W541 from here on, was selected for further study. Mitochondrial DNA sequence analysis of the faecal specimens revealed that *Anas Platyrhynchos* were the viral host [1]. Moreover, these 2016 H5N6 virus isolates belong to the A/Yunnan/0127/2015-like virus lineage (clade 2.3.4.4) detected in fatal human cases between 2014 and 2016 [12,13]. Molecular analysis demonstrated that the HA cleavage site of EM/W541 bears polybasic residues (RERRRKR/G) denoting a high-pathogenicity phenotype in chickens.

All four HPAI H5N6 virus isolates maintained the glutamine residue at position 226 (H3 numbering) and a glycine residue at position 228, which is suggestive of preferential binding to sialic acid receptors joined to sugar chains through an α-2,3 linkage, as is typical for avian influenza viruses. However, a characteristic of all H5N6 virus isolates was one amino acid deletion (133 site of HA1) relative to the other clade 2.3.4.4 HA genes (ex, MDk/Korea/W452/14), which is commonly found in avian influenza H5N6 viruses (Table).

The deletion at this position alters the 3D structure of the receptor binding unit causing an alteration of the HA receptor binding specificity and resulting in an increased affinity for the α-2,6 linkage [14,15]. A similar deletion has occurred and is maintained in 2.2.1.2 viruses in Egypt [14]. These viruses bear considerable zoonotic potential. In addition, the Korean H5N6 isolates had the characteristic 20 amino acid NA stalk deletion (49 to 68 sites) compared with the A/Ck/Sichuan/NCJPL1/2014 virus, whereas a substitution associated with resistance to NA inhibitors was not noted. The isolates also possess functional polymerase basic (PB)1-F2 proteins, which have been shown to impact on host defence mechanisms and enhance pathogenicity in vivo. However, no other mammalian-adaptive molecular determinants were observed in the viral genome [16]. The 2016 Korean virus isolates bear aspartic acid in place of glutamic acid at position 92 of the non-structural (NS)1 protein, which is responsible for attenuating anti-viral host interferon responses [17] and the C-terminal PDZ-binding motifs are both ESEV, which is typical for avian viruses and confers severe disease phenotype in mice [18].

Table

Molecular analysis of influenza A subtype H5 viruses emerging in November 2016 compared with previously isolated H5 viruses*

Viruses[a]	HA clade classification	HA sequence (aa)									HA deletion	NA stalk deletion	NS1			PB2 sequence at aa		Expression of PB1-F2 protein
		Cleavage site	Receptor binding sites										Deletion of aa 8084	Aa residue at				
		335-348[b]	158	193	222	224	226	227	228	318	133	49-68		92	C-term	627	701	
EM/Korea/ W541/16	2.3.4.4	RERRR_KR/G	N	N	Q	N	Q	Q	G	T	YES	YES	YES	E	ESEV	E	D	YES
Yunnan/ China/0127/15[c]	2.3.4.4	RERRR_KR/G	N	N	Q	N	Q	R	G	T	YES	YES	NO	D	KPEV	K	D	YES
Ck/Sichuan/ NCJPL1/2014	2.3.4.4	RERRR_KR/G	N	N	Q	N	Q	R	G	T	NO	NO	YES	E	ESEV	E	D	YES
MDk/Korea/ W452/14	2.3.4.4	RERRR_KR/G	N	N	Q	N	Q	R	G	T	NO	NO	NO	D	ESEV	E	D	YES
BDk/Korea/ Gochang1/14	2.3.4.4	RERRR_KR/G	N	N	Q	N	Q	R	G	T	NO	NO	NO	D	ESEV	E	D	YES
Em/Korea/ W149/06	2.2	GERRRKKR/G	N	K	K	N	Q	S	G	T	NO	YES	YES	D	ESKV	K	D	YES
MDk/Korea/ W401/11	2.3.2	RERRR_KR/G	D	R	K	N	Q	S	G	T	NO	YES	YES	D	ESEV	E	D	YES
Egypt/ MOH/7271/14 [c]	2.2.1.2	GERRRKKR/G	N	R	K	N	Q	S	G	T	YES	YES	YES	D	ESEV	K	D	YES

Aa: amino acid; BDk: Breeder duck; C-term: 4 amino acid sequence at the C-terminal end; Em: environment; HA: haemagglutinin; HPAI: highly pathogenic avian influenza; MDk: mallard duck; RBS: receptor binding site; MOH: Ministry of health; NA: neuraminidase; NS: nonstructural protein; PB: polymerase basic protein.

The accession numbers of each virus are followed: Yunnan/China/0127/15 : KT245143~KT245150, Ck/Sichuan/NCJPL1/2014: PB2-KM251533, PB1- KM251523, PA-KM251513, HA-KM251463, NP-KM251493, NA-KM251486, M-KM251473, and NS-KM251503, MDk/Korea/W452/14 : KJ746108~KJ746115, BDk/Korea/Gochang1/14 : KJ413831~KJ413838, EM/Korea/W149/06 : EU233731~EU233738, MDk/Korea/W401/11 : JN202558~JN202572, Egypt/MOH/7271/14 : KP702162~KP702169, and EM/Korea/W541/16 : KY272997~KY273025

[a] The isolates in boldface are the 2016 Korean HPAI H5N6 virus examined in this study.

[b] H3 numbering.

[c] Human isolates.

Phylogenetic analyses

To clarify the origins of EM/W541, phylogenetic analyses were conducted with available H5Nx virus sequences and other N6 viruses from the National Center for Biotechnology Information (NBCI) GenBank database. Phylogenetic analysis of the HA genes revealed that EM/W541 was evolutionarily close to the A/Yunnan/0127/2015-like H5N6 viruses isolated from poultry and environmental samples including fatal human cases in China during 2014–2016, and the HA genes belonged to the Group C of clade 2.3.4.4 HPAI H5 viruses (Figure 1).

The Group A and Group B of clade 2.3.4.4 viruses comprises H5N8 viruses identified in South Korea in 2013/14 and 2014/15 winter seasons (November to February), respectively. Group C comprises H5N6 viruses identified from China and Laos during 2013/14 and Group D comprises H5N6 viruses identified from China and Vietnam during 2013/14. The NA gene was also derived from Group C H5N6-like viruses persisting in China during 2013–2014 (Figure 2) and the most closely related strain was A/GE/Hong Kong/00032/2016.

Although the other internal genes (PB2 (Figure 3), NP (Figure 4), M (Figure 5), and NS (Figure 6) can also be traced back to the Group C H5N6-like viruses(with the exception of PB1 and PA), they were clustered with different ancestors, such as A/SP/Guangdong/ZH283/2015 and A/Dk/Guanzhou/41227/2014 (H5N6)-like viruses. In contrast, the PB1 gene was closely related to A/Dk/Guangdong/S4040/2011(H4N2) strains and the PA gene was closely related to A/Dk/Mongolia/20/2015(H1N1) strains (Figures 7 and 8). The genotype map demonstrates that the first Korean H5N6 viruses were reassorted from at least three different subtypes (H5N6, H4N2 and H1N1) present within the natural gene pool in Eurasian avian influenza viruses (Figure 9).

Virulence in chickens

To determine the pathogenicity of the EM/W541 in chickens, we initially measured the mean death times (MDT) and the intravenous pathogenicity index (IVPI) according to the recommendations outlined in the World Organisation for Animal Health (OIE) standards [19]. Briefly, 6.0 log10 egg infectious doses (EID50) /0.1 mL of the H5N6 virus were intravenously inoculated into ten 6-week-old chickens which were then monitored until death. The MDT was 36 hours and the IVPI was 2.66 in chickens, suggesting the EM/W541 virus should be classified as an HPAI virus according to OIE criteria [19].

Conclusions

Overall, we report the identification of a novel reassortant HPAI H5N6 virus that caused large outbreaks in

domestic poultry in the late 2016 winter in South Korea [11]. This H5N6 virus is a reassortant with multiple virus subtypes (H5N6, H4N2 and H1N1) from the gene pool in Eurasian avian influenza viruses. Initial animal studies revealed that this novel H5N6 virus is highly pathogenic in chickens.

At this moment, it is hard to determine whether the presented reassortment event of the H5N6 viruses occurred in 2016 during the wild bird migration into Korea or in a previous year and in another location before migration. Further detailed broad-range molecular studies are needed to elucidate when exactly the event occurred.

The first avian influenza H5N8 (clade 2.3.4.4) virus outbreak was reported in poultry in South Korea in 2014. It rapidly spread worldwide, including to Europe and North America, by migratory wild birds [20]. This rapid and wide spread underscores the need for continuous, intensive surveillance of avian influenza viruses in wild migratory birds as it can be envisaged that these viruses may be transmitted for example to Europe, or possibly worldwide, by any migratory birds that use the same migratory flyways as the birds in the previous 2014 poultry outbreak in South Korea.

*Erratum

The cells in the first row of the Table were erroneously shifted to the left. The table was corrected and replaced on 12 January 2017.

Acknowledgements

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Conflict of interest

None declared.

Authors' contributions

Wrote the manuscript: YK Choi, MS Song, EH Kim, YI Kim, HL Kwon, RJ Webby, and CJ Kim; performed laboratory investigations: YJ Si, IW Lee, EH Kim, SJ Park, HD Nguyen, SM Kim, JJ Kwon, WS Choi, YH Beak; performed phylogenetic analyses: YJ Si, YK Choi, HL Kwon, YI Kim; Animal experiment: EH Kim, HI Kwon, YI Kim, YJ Si, IW Lee. All authors reviewed the manuscript.

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Research article

Novel real-time PCR-based patho- and phylotyping of potentially zoonotic avian influenza A subtype H5 viruses at risk of incursion into Europe in 2017

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Since November 2016, Europe witnesses another wave of incursion of highly pathogenic avian influenza (HPAI) A(H5) viruses of the Asian origin goose/Guangdong (gs/GD) lineage. Infections with H5 viruses of clade 2.3.4.4b affect wild bird and poultry populations. H5 viruses of clades 2.2, 2.3.1.2c and 2.3.4.4a were detected previously in Europe in 2006, 2010 and 2014. Clades 2.2.1.2 and 2.3.2.1.c are endemic in Egypt and Western Africa, respectively and have caused human fatalities. Evidence exists of their co-circulation in the Middle East. Subtype H5 viruses of low pathogenicity (LPAI) are endemic in migratory wild bird populations. They potentially mutate into highly pathogenic phenotypes following transmission into poultry holdings. However, to date only the gs/GD H5 lineage had an impact on human health. Rapid and specific diagnosis marks the cornerstone for control and eradication of HPAI virus incursions. We present the development and validation of five real-time RT-PCR assays (RT-qPCR) that allow sequencing-independent pathotype and clade-specific distinction of major gs/GD HPAI H5 virus clades and of Eurasian LPAI viruses currently circulating. Together with an influenza A virus-generic RT-qPCR, the assays significantly speed up time-to-diagnosis and reduce reaction times in a OneHealth approach of curbing the spread of gs/GD HPAI viruses.

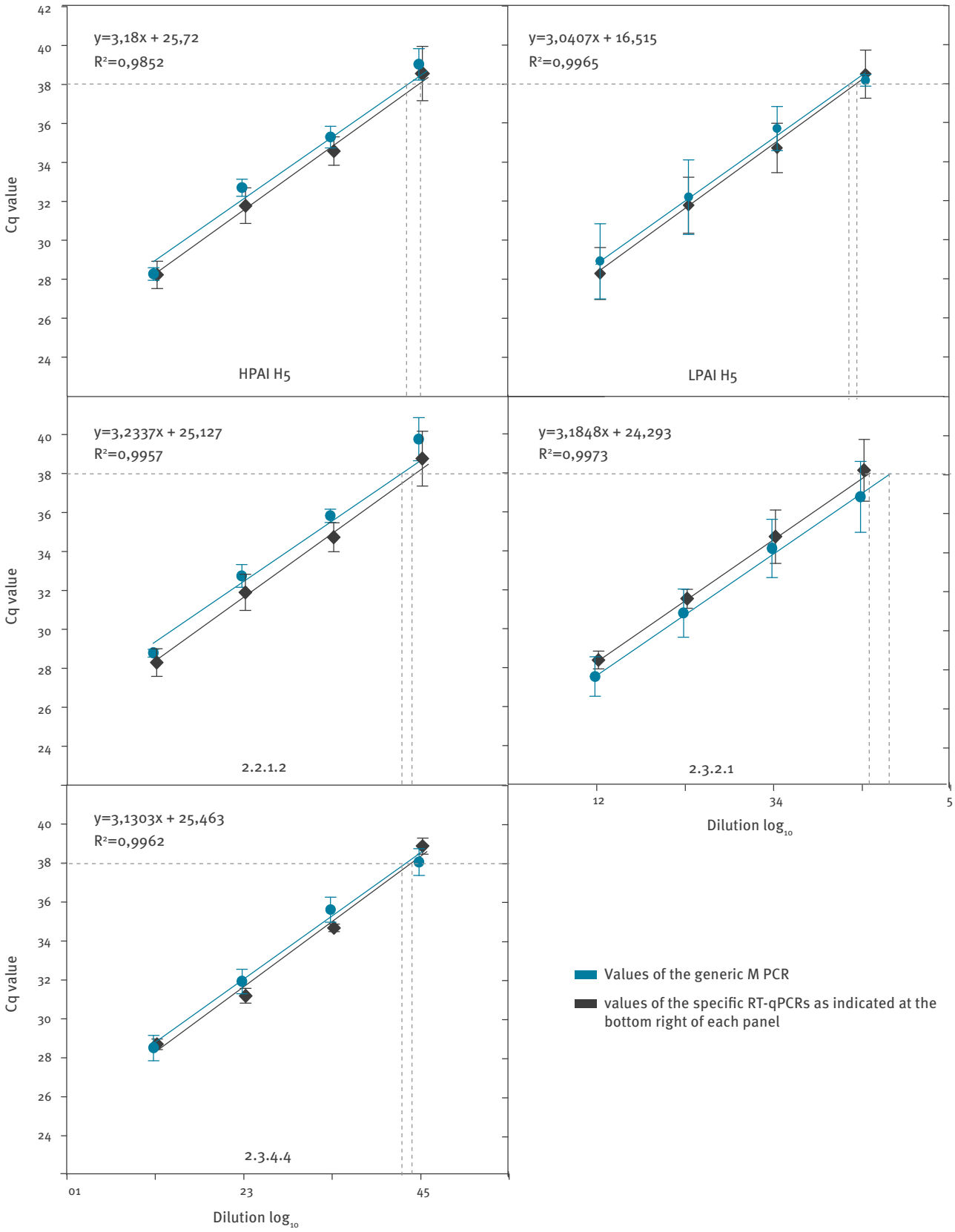
Introduction

Influenza A viruses constitute a virus species in the family *Orthomyxoviridae*. They harbour single-stranded negative-sense RNA arranged into eight genomic segments. Members of this species which infect avian hosts (avian influenza viruses, AIV) are grouped into 16 (H1 to H16) and 9 (N1 to N9) subtypes, respectively, based on phylogenetic and antigenic properties of their haemagglutinin (HA) and neuraminidase (NA) envelope glycoproteins [1]. Different species of aquatic wild birds are the natural reservoirs for all AIV subtypes. Novel subtypes and gene constellations continue to evolve in aquatic wild birds or in infected poultry populations by genetic reassortment during infection of a single host cell with two or more distinct AIV genotypes. In addition to reassortment, the intrinsically error-prone influenza virus genome replication machinery promotes the generation of quasi-species that can be shaped by directional selection pressures, e.g. following host species switches or by specific herd immunity. In the latter case, antigenic drift variants are selected that may escape immunity by very few amino acid substitutions in the HA [2].

Based on their virulence in galliform poultry (e.g. chicken, turkey), AIV are distinguished into groups of highly pathogenic (HP) and low pathogenic (LP) phenotypes [3]. Correct AI diagnosis includes determining the HA subtype and, in case of subtypes H5 or H7, also the pathotype. So far, HPAI phenotypes detected in the field (i.e. 'free' natural environment), were only described among AIV of subtypes H5 and H7 [4]. Some of these viruses including those of the HPAI H5 goose/Guangdong (gs/GD) lineage that emerged in southern China in 1996, have zoonotic potential and are sporadically transmitted from infected birds to humans [5,6]. HPAI viruses of the gs/GD lineage have continued to circulate and evolved into numerous clades. Viruses

Figure 1

Evaluation of detection limits and precision of pathotyping and phylotyping quantitative reverse transcription PCRs compared with a generic matrix (M) gene RT-qPCR[a]



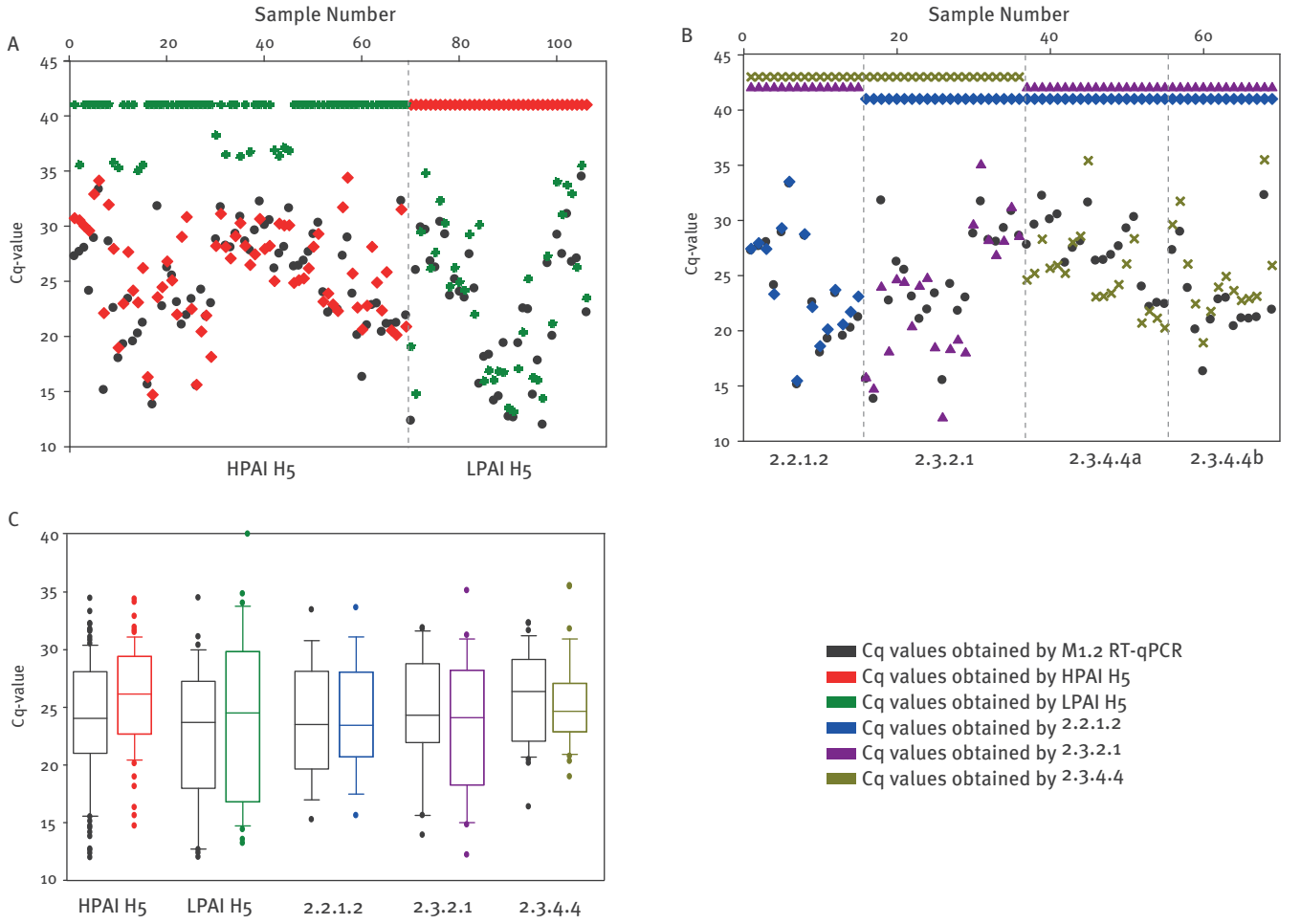
Cq: cycle of quantification; HPAI: highly pathogenic avian influenza; LPAI: low pathogenic avian influenza; M: matrix; RT-qPCR: quantitative reverse transcription PCR.

The detection limit was determined based on triplicate analyses of serial 10-fold dilutions of target RNA of reference viruses: HPAI H5: A/duck/Egypt/AR236-A3NLQP/2015 (H5N1); LPAI H5: A/teal-Foehr/Wv1378–79/2003 (H5N2) (upper panel); HPAI H5 clade 2.2.1.2: A/duck/Egypt/AR236-A3NLQP/2015 (H5N1); clade 2.3.2.1: A/quail/Dubai/AR3445–2504.3/2014 (H5N1) and clade 2.3.4.4: A/turkey/Germany-MV/R2472/2014 (H5N8) (lower panel). A cut-off value of Cq 38 was chosen to calculate limits of detection and confidence intervals thereof.

[a] Described in [29].

Figure 2

Pathotyping and phylotyping of virus isolates and clinical samples of potentially zoonotic Eurasian avian influenza A subtype H5 viruses by quantitative reverse transcription PCRs



Cq: cycle of quantification; HPAI: highly pathogenic avian influenza; LPAI: low pathogenic avian influenza; M: matrix; RT-qPCR: quantitative reverse transcription PCR.

Sample numbers in A and B refer to Table 3. Cq values obtained for each sample by M1.2 RT-qPCR are shown as black dots (panels A and B); Cq values obtained for each sample by the specific RT-qPCRs are depicted as follows: Panel A red lozenges – HPAI H5, green crosses - LPAI H5; panel B blue lozenges - clade 2.2.1.2, purple triangles - clade 2.3.2.1, ochre Xs - clade 2.3.4.4.; panel C compares categorised Cq values obtained for all samples by M1.2 RT-qPCR (black box-and-whiskers) and the specific RT-qPCRs (colours as described for panels A and B).

of three major phylogenetic clades (2.2.1.2, 2.3.2.1 and 2.3.4.4) as well as of three further minor clades (1.1.2, 2.1.3.2 and 7.2) have become endemic in poultry populations in several countries in Asia, Africa and the Middle East [7]. Occasionally, spillover transmission from infected poultry may cause infection and viral spread in wild birds with increased mortality in some species. Infected migratory wild birds may spread such viruses across wider distances and act as the source of transmission back to poultry [7,8].

Europe has experienced several incursions by viruses of the gs/GD lineage over the past decade; both wild birds and poultry were affected but no human cases were reported [9]. This is in sharp contrast to Egypt and Asian countries where the endemicity of HPAI H5 viruses in poultry is associated with repeated spillover transmission to and infection of humans. In fact, the majority of human HPAI H5 cases worldwide were registered in Egypt [10,11]. Moreover, a new major clade, designated 2.2.1.2, evolved along with transient spread of an escape mutant-based lineage, 2.2.1.1, in this country [12].

Further potentially zoonotic gs/GD viruses of clade 2.3.2.1c are widespread in Central and Southern Asia and they were sporadically detected along the European Black Sea coast as well as in the Middle East [13-15]. In addition, viruses of this clade have caused major outbreaks among poultry in several Western African countries with ongoing virus circulation to date [16]. Interestingly, 2.3.2.1c viruses have not (yet) been reported from Egypt. Since 2010, another gs/GD cluster, termed 2.3.4.4, has evolved in eastern China

Table 1

Primers and probes designed for differentiating pathotype and phylotype of Eurasian wild bird and goose/Guangdong origin potentially zoonotic avian influenza A subtype H5 viruses

Primer/Probe ID	Target	Sequence (5' to 3')	Location	Amplicon size	Accession number[a]
H5_HP_EA_F1	HPAI H5	CCTTGCDACTGGRCTCAG	984–1001	109	EPI647540
H5_HP_EA_F2		TCCTTGCAACAGGACTAAG	983–1001		
H5_HP_EA_probe		FAM- AAGAARAAARAGAGGACTRTTTGGAGCT-BHQ-1	1023–1050		
H5_HP_EA_R		GTCTACCATTCCYTGCCA	1092–1075		
H5LP-EA_F	LPAI H5	CCCAAATACGTGAAATCAGAT	955–975	133	EPI356413
H5LP1_EA_probe		FAM-CCAAATAGYCCTCTYGTYTCT-BHQ-1	1052–1072		
H5LP-EA_R		GCC ACC CTC CTT CTA TAA AG	1088–1069		
H5_2.2.1.2_Fw	Clade 2.2.1.2	CATTTTGAGAAAATTCAGATCATT	376–399	161	EPI573250
H5_2.2.1.2_probe		FAM-TCCATACCARGGAAGATCCTCCTTT-BHQ-1	451–474		
H5_2.2.1.2_Rev		GGTATGCATCGTTCTTTTTGG	537–517		
H5_2.3.2.1_F	Clade 2.3.2.1	GAGATTGGTACCAAAAATAGCC	669–690	146	EPI603577
H5_2.3.2.1_probe		FAM-ACGGGCAAAGTGGCAGGATAGATTTC-BHQ-1	707–732		
H5_2.3.2.1_R		CAATGAAATTTCCATTACTCTCG	815–793		
H5_2.3.4.4_F_A	Clade 2.3.4.4	ATACCAGGGAGCATCCTCA	484–502	114	EPI554605
H5_2.3.4.4_F_B		ATACCAGGGAACGCCCTCC	484–502		
H5_2.3.4.4_probe		FAM-TCGTTCTTTTTGATGAGCCATACCACA-BHQ-1	540–560		
H5_2.3.4.4_R_A		ATTATTGTAGCTTATCTTTATTGTC	598–574		
H5_2.3.4.4_R_B		ATTATTGTAGCTTATCTTTATTGTT	598–574		

gs/GD: goose/Guangdong; HA: haemagglutinin; ID: identity.

[a] Accession number used to describe the position of the oligonucleotide along the HA gene. Sequences were obtained from GenBank at the National Center for Biotechnology Information (NCBI) or the EpiFlu database of the Global Initiative on Sharing Avian Influenza Data (GISAID).

and on the Korean peninsula [17]. These viruses have revealed a strong propensity to reassort with other influenza subtypes giving rise to novel HPAI sub- and genotypes including influenza A(H5N6) and A(H5N8). The latter subtype has proven to be highly mobile and was carried by infected wild birds to Europe and the North American continent in late 2014 [8,18]. In November 2016, HPAI H5N8 viruses of the 2.3.4.4 clade re-emerged on a large scale in wild birds in several central European countries and caused considerable mortality especially among diving duck species; sporadic incursions into poultry holdings were documented as well [19]. At the same time, this lineage was also detected in poultry in Israel [20].

Eurasian-origin LPAI subtype H5 viruses distantly related to the gs/GD lineage are routinely detected in aquatic wild bird populations with peak incidences during the autumn migration period [21]. Spillover of LPAI virus into poultry may cause notifiable outbreaks and bears the risk of the de novo generation of HP phenotypes following spontaneous mutations [3]. No human LPAI H5 virus infections have been reported so far.

Continuous co-circulation in poultry and sporadic spillover into migratory wild bird populations of different endemic HPAI H5 virus lineages poses constant risks of new incursions into Europe by migrating wild birds or in association with (illegal) poultry trading practices [9]. Furthermore, co-circulation of various HPAI lineages with different antigenic properties potentiates problems of control and eradication. Given the zoonotic propensities of some of the H5 viruses, tight control of infections in poultry is essential to curtail risks of human infections and further spread [22,23]. Molecular diagnosis including patho- and phylotyping of the relevant AIV is an important prerequisite for effective control measures.

We developed rapid diagnostic solutions on the basis of quantitative reverse transcription real-time PCR assays (RT-qPCR), to pathotype, without sequencing, gs/GD lineage HPAI and Eurasian LPAI H5 subtype viruses, and to distinguish HPAI gs/GD viruses of clades 2.2.1.2, 2.3.2.1 and 2.3.4.4, including viruses of the ongoing 2016 epizootic in Europe.

Table 2

Reference viruses used to determine analytical specificity of five PCR assays to detect potentially zoonotic avian influenza subtype H5 viruses

Reference virus		Accession number of HA[a]	Patho- and Phylotype	PCR method [b]				
				HPAI H5	LPAI H5	Clade 2.2.1.2	Clade 2.3.2.1	Clade 2.3.4.4
1	A/turkey/Turkey/1/2005 (H5N1)	KF042153	HP Clade 2.2	**Pos**	**Neg**	**Pos**	**Neg**	**Neg**
2	A/chicken/Egypt/0879-NLQP/R737/2008 (H5N1)	GQ184238	HP Clade 2.2.1.1	**Pos**	**Neg**	**Neg**	**Neg**	**Neg**
3	A/chicken/Egypt/NLQP7FL-AR747/ 2013 (H5N1)	EPI557170	HP Clade 2.2.1.2	**Pos**	**Neg**	**Pos**	**Neg**	**Neg**
4	A/duck/Egypt/AR236-A3NLQP/2015 (H5N1)	EPI573260	HP Clade 2.2.1.2	**Pos**	**Neg**	**Pos**	**Neg**	**Neg**
5	A/turkey/Egypt/AR238-SD177NLQP/2014 (H5N1)	EPI573268	HP Clade 2.2.1.2	**Pos**	**Neg**	**Pos**	**Neg**	**Neg**
6	A/peregrine falcon/Dubai/AR3430/2014 (H5N1)	EPI603553	HP Clade 2.3.2.1c	**Pos**	**Neg**	**Neg**	**Pos**	**Neg**
7	A/quail/Dubai/AR3445–2504.3/2014 (H5N1)	EPI603577	HP Clade 2.3.2.1c	**Pos**	**Neg**	**Neg**	**Pos**	**Neg**
8	A/duck/Bangladesh/D3-AR2111/2013 (H5N1)	SA[c]	HP Clade 2.3.2.1a	**Pos**	**Neg**	**Neg**	**Pos**	**Neg**
9	A/turkey/Germany/AR2485–86/2014 (H5N8)	EPI552746	HP Clade 2.3.4.4a	**Pos**	**Neg**	**Neg**	**Neg**	**Pos**
10	A/turkey/Germany-MV/AR2472/2014 (H5N8)	EPI544756	HP Clade 2.3.4.4a	**Pos**	**Neg**	**Neg**	**Neg**	**Pos**
11	A/tufted duck/Germany/AR8444/2016 (H5N8)	EPI859212	HP Clade 2.3.4.4b	**Pos**	**Neg**	**Neg**	**Neg**	**Pos**
12	A/chicken/Indonesia/R132/2004 (H5N1)	EPI354072	HP Clade 2.1.1	**Pos**	**Neg**	**Neg**	**Neg**	**Neg**
13	A/chicken/Indonesia/R134/2003 (H5N1)	AM183669	HP Clade 2.1.1	**Pos**	**Neg**	**Neg**	**Neg**	**Neg**
14	A/chicken/Indonesia/R60/2005 (H5N1)	AM183670	HP Clade 2.1.1	**Pos**	**Neg**	**Neg**	**Neg**	**Neg**
15	A/Vietnam/1194/2004 (H5N1)	GQ149236	HP Clade 1.1	**Pos**	**Neg**	**Neg**	**Neg**	**Neg**
16	A/chicken/GXLA/1204/2004 (H5N1)	AM183671	HP Clade 2.4	**Pos**	**Neg**	**Neg**	**Neg**	**Neg**
17	A/chicken/Vietnam/P41/2005 (H5N1)	AM183672	HP Clade 1.1	**Pos**	**Neg**	**Neg**	**Neg**	**Neg**
18	A/chicken/Vietnam/P78/2005 (H5N1)	AM183673	HP Clade 1.1	**Pos**	**Neg**	**Neg**	**Neg**	**Neg**
19	A/common teal/Germany/Wv1378–79/2003 (H5N2)	HF563058	LP	**Neg**	**Pos**	**Neg**	**Neg**	**Neg**
20	A/duck/Germany/R1789/2008 (H5N3)	CY107849	LP	**Neg**	**Pos**	**Neg**	**Neg**	**Neg**
21	A/turkey/Germany/AR915/2015 (H7N7)	SA[c]	H7N7	**Neg**	**Neg**	**Neg**	**Neg**	**Neg**
22	A/chicken/Egypt/AR754–14/2013 (H9N2)	EPI557457	H9N2	**Neg**	**Neg**	**Neg**	**Neg**	**Neg**
23	A/chicken/Sudan/AR251–15/2014 (IBV)	KX272465	IBV	**Neg**	**Neg**	**Neg**	**Neg**	**Neg**
24	A/chicken/Egypt/AR254–15/2014 (NDV)	SA[c]	NDV	**Neg**	**Neg**	**Neg**	**Neg**	**Neg**

Cq: cycle of quantification; HA: haemagglutinin; HP: highly pathogenic; HPAI: highly pathogenic avian influenza; IBV: infectious bronchitis virus; LP: low pathogenic; LPAI: low pathogenic avian influenza; NDV: Newcastle disease virus; Neg: negative; Pos: positive; RT-qPCR: quantitative reverse transcription PCR; SA: sequences available.

[a] Sequences were obtained from GenBank at the National Center for Biotechnology Information (NCBI) or the EpiFlu database of the Global Initiative on Sharing Avian Influenza Data (GISAID).

[b] Positive results: Cq value in similar range as with influenza A virus generic M RT-qPCR; negative results: Cq>40. [c] Sequenced in the frame of the current study; sequences available from the authors upon request.

Methods

Virus isolates and clinical samples

A total of 24 reference virus isolates were obtained from the virus repositories at the Friedrich Loeffler Institute, Greifswald-Riems, Germany, or were provided by the National Laboratory for quality control on poultry production in Giza, Egypt, and by the Central Veterinary Research Laboratory (CVRL) in Dubai, United Arab Emirates (see also first table under Results).

Moreover, 106 field samples were included. These were obtained from holdings of different poultry sectors and wild birds from countries in Western Europe (Germany), the Middle East (Egypt, Iraq, United Arab Emirates) and Western Africa (Burkina Faso, Cameroon, Ghana, Ivory Coast, Niger), for HPAI viruses in the period between

Table 3A

Pathotyping and phylotyping of different potentially zoonotic HPAI and LPAI influenza A subtype H5 virus isolates and field samples collected from poultry and wild bird species in different countries, 2013–2016

No.	Sample ID	Type of sample	Accession Number [a]	Clade	PCR results					
					M1.2	HPAI H5	LPAI H5	2.2.1.2	2.3.2.1	2.3.4.4
1	A/chicken/Egypt/NLQP33SD-AR748/2013	Isolate	EPI557178	HP 2.2.1.2	27.25	30.72	Neg	27.43	Neg	Neg
2	A/chicken/Egypt/NLQP2AL-AR749/2013	Isolate	EPI557186	HP 2.2.1.2	27.65	30.53	35.56	27.94	Neg	Neg
3	A/duck/Egypt/NLQP27SG-AR750/2013	Isolate	EPI557194	HP 2.2.1.2	28.01	30.01	Neg	27.41	Neg	Neg
4	A/chicken/Egypt/NLQP639V-AR752/2013	Isolate	EPI557202	HP 2.2.1.2	24.11	29.58	Neg	23.32	Neg	Neg
5	A/chicken/Egypt/NLQP20SL-AR751/2013	Isolate	EPI557210	HP 2.2.1.2	28.90	32.90	Neg	29.30	Neg	Neg
6	A/chicken/Egypt/NLQP139V-AR753/2013	Isolate	EPI557218	HP 2.2.1.2	33.32	34.13	Neg	33.51	Neg	Neg
7	A/quail/Egypt/BSU5514-AR2219/2014	Field sample	EPI557138	HP 2.2.1.2	15.12	22.12	Neg	15.47	Neg	Neg
8	A/chicken/Egypt/AR234-FAOF8NLQP/2014	Field sample	EPI573250	HP 2.2.1.2	28.60	31.95	Neg	28.75	Neg	Neg
9	A/turkey/Egypt/AR235-S240NLQP/2014	Field sample	EPI573252	HP 2.2.1.2	22.56	27.94	35.77	22.16	Neg	Neg
10	A/chicken/Egypt/AR3690A/2016	Field sample	SA[b]	HP 2.2.1.2	18.01	18.97	35.29	18.61	Neg	Neg
11	A/chicken/Egypt/AR3706/2016	Field sample	SA[b]	HP 2.2.1.2	19.27	22.98	Neg	20.13	Neg	Neg
12	A/chicken/Egypt/AR3707/2016	Field sample	SA[b]	HP 2.2.1.2	23.39	27.66	Neg	23.71	Neg	Neg
13	A/chicken/Egypt/AR3737/2016	Field sample	SA[b]	HP 2.2.1.2	19.53	24.16	Neg	20.58	Neg	Neg
14	A/chicken/Egypt/AR3741/2016	Field sample	SA[b]	HP 2.2.1.2	20.25	23.08	35.04	21.71	Neg	Neg
15	A/chicken/Egypt/AR3753/2016	Field sample	SA[b]	HP 2.2.1.2	21.22	26.21	35.55	23.10	Neg	Neg
16	A/seagull/Dubai/AR3443–2504.1/2014	Isolate	EPI603554	HP 2.3.2.1	15.62	16.32	Neg	Neg	15.72	Neg
17	A/stone curlew/Dubai/AR3444–2504.2/2014	Isolate	EPI603569	HP 2.3.2.1	13.81	14.72	Neg	Neg	14.70	Neg
18	A/duck/Ivory_Coast/15VIR2742–1/2015	Spleen and caecum	NA	HP 2.3.2.1	31.79	23.56	Neg	Neg	23.93	Neg
19	A/chicken/Ghana/15VIR2588–4/2015	Spleen	KU97137	HP 2.3.2.1	22.72	24.47	Neg	Neg	18.07	Neg
20	A/chicken/Ghana/15VIR2588–10/2015	Cloacal swab	KU971357	HP 2.3.2.1	26.24	26.80	Neg	Neg	24.61	Neg
21	A/chicken/Niger/15VIR2060–12/2015	Tracheal swab	KU971309	HP 2.3.2.1	25.50	25.08	Neg	Neg	24.37	Neg
22	A/chicken/Niger/15VIR2060–5/2015	Swab	KU971326	HP 2.3.2.1	23.08	21.99	Neg	Neg	20.35	Neg
23	A/domestic_bird/Burkina_Faso/15VIR1774–24/2015	Swab	KU971508	HP 2.3.2.1	21.05	29.03	Neg	Neg	24.01	Neg
24	A/domestic_bird/Burkina_Faso/15VIR1774–23/2015	Organ	KU971500	HP 2.3.2.1	21.91	30.83	Neg	Neg	24.72	Neg
25	A/chicken/Ghana/16VIR-4304–1/2016	Organ	SA[b]	HP 2.3.2.1	23.37	22.49	Neg	Neg	18.44	Neg
26	A/chicken/Ghana/16VIR-4304–25/2016	Organ	SA[b]	HP 2.3.2.1	15.51	15.62	Neg	Neg	12.09	Neg
27	A/chicken/Ghana/16VIR-4304–42/2016	Organ	SA[b]	HP 2.3.2.1	24.22	20.45	Neg	Neg	18.28	Neg
28	A/chicken/Ghana/16VIR-4304–9/2016	Organ	SA[b]	HP 2.3.2.1	21.79	21.90	Neg	Neg	19.13	Neg
29	A/duck/Cameroon/16VIR-3791–21/2016	Lung and trachea	SA[b]	HP 2.3.2.1	23.00	18.14	Neg	Neg	17.98	Neg
30	A/chicken/Iraq/AR5282/2016	Field sample	NA	HP 2.3.2.1	28.78	28.20	Neg	Neg	29.57	Neg
31	A/chicken/ Iraq/AR5283/2016	Field sample	NA	HP 2.3.2.1	31.70	31.12	Neg	Neg	35.02	Neg
32	A/chicken/Iraq/AR5286/2016	Field sample	SA[b]	HP 2.3.2.1	28.21	28.10	36.50	Neg	28.16	Neg
33	A/chicken/Iraq/AR5287/2016	Field sample	SA[b]	HP 2.3.2.1	28.05	27.08	Neg	Neg	26.80	Neg
34	A/chicken/Iraq/AR5291/2016	Field sample	SA[b]	HP 2.3.2.1	29.29	29.09	Neg	Neg	28.09	Neg
35	A/chicken/Iraq/AR5292/2016	Field sample	NA	HP 2.3.2.1	30.83	30.28	36.32	Neg	31.15	Neg
36	A/chicken/Iraq/AR5296/2016	Field sample	SA[b]	HP 2.3.2.1	28.60	28.21	Neg	Neg	28.53	Neg
37	A/turkey/Germany/AR2499/2014	Field sample	SA[b]	HP 2.3.4.4	27.78	26.48	36.71	Neg	Neg	24.61
38	A/turkey/Germany/AR2500/2014	Field sample	SA[b]	HP 2.3.4.4	29.59	27.44	Neg	Neg	Neg	25.20
39	A/turkey/Germany/AR2501/2014	Field sample	SA[b]	HP 2.3.4.4	32.21	30.65	Neg	Neg	Neg	28.30
40	A/turkey/Germany/AR2502/2014	Field sample	SA[b]	HP 2.3.4.4	30.08	27.92	Neg	Neg	Neg	25.67
41	A/turkey/Germany/AR2503/2014	Field sample	SA[b]	HP 2.3.4.4	30.52	28.21	Neg	Neg	Neg	25.92
42	A/turkey/Germany/AR2562/2014	Field sample	SA[b]	HP 2.3.4.4	26.15	25.02	36.88	Neg	Neg	25.21
43	A/turkey/Germany/AR2574/2014	Field sample	SA[b]	HP 2.3.4.4	27.49	30.23	36.36	Neg	Neg	28.01
44	A/turkey/Germany/AR2591/2014	Field sample	SA[b]	HP 2.3.4.4	28.09	30.06	37.13	Neg	Neg	28.57
45	A/teal/Germany/AR2917/2014	Field sample	SA[b]	HP 2.3.4.4	31.60	30.08	36.82	Neg	Neg	35.41
46	A/turkey/Germany/AR3372/2014	Field sample	EPI553172	HP 2.3.4.4	26.33	24.85	Neg	Neg	Neg	23.07
47	A/turkey/Germany/AR3376/2014	Field sample	SA[b]	HP 2.3.4.4	26.39	25.10	Neg	Neg	Neg	23.12
48	A/turkey/Germany/AR3381/2014	Field sample	SA[b]	HP 2.3.4.4	26.85	25.26	Neg	Neg	Neg	23.40
49	A/turkey/Germany/AR3382/2014	Field sample	SA[b]	HP 2.3.4.4	27.64	26.18	Neg	Neg	Neg	24.18
50	A/turkey/Germany/AR3383/2014	Field sample	SA[b]	HP 2.3.4.4	29.26	28.13	Neg	Neg	Neg	26.06
51	A/duck/Germany/AR3457/2014	Field sample	SA[b]	HP 2.3.4.4	30.29	29.30	Neg	Neg	Neg	28.34
52	A/duck/Germany/AR3465/2014	Field sample	SA[b]	HP 2.3.4.4	23.98	23.15	Neg	Neg	Neg	20.70
53	A/duck/Germany/AR3470/2014	Field sample	SA[b]	HP 2.3.4.4	22.15	23.89	Neg	Neg	Neg	21.78

HA: haemagglutinin; HP: highly pathogenic; HPAI: highly pathogenic avian influenza; ID: identity; LP: low pathogenic; LPAI: low pathogenic avian influenza; NA: sequence not available; Neg: negative; SA: sequence available.

[a] Sequences were obtained from GenBank at the National Center for Biotechnology Information (NCBI) or the EpiFlu database of the Global Initiative on Sharing Avian Influenza Data (GISAID).

[b] Sequenced in the frame of the current study; sequences available from the authors upon request.

2013 and 2016. Samples consisted mainly of oropharyngeal and/or cloacal swabs and tissues samples (n = 70) or AIV isolated from such samples (n = 36) (see also second table under Results).

A subsection of the 106 clinical samples (n = 13) was provided as dried material on Whatman FTA card (Sigma Aldrich, Germany). Samples from Western African countries were exclusively assayed at the Istituto Zooprofilattico Sperimentale delle Venezie, Padua, Italy.

Design of primers and probes

Primers were chosen based on alignments of the HA H5 gene of a selection of influenza A virus sequences submitted over the past 10 years to GenBank at the National Center for Biotechnology Information (NCBI) or to the EpiFlu database of the Global Initiative on Sharing Avian Influenza Data (GISAID). Selected sequences represented Eurasian LP viruses and HP isolates and clades of the gs/GD lineage that were detected in Europe, the Middle East and Western Africa during the past decade. Selection of primers to amplify a small fragment of the HA gene spanning the endoproteolytic cleavage site aimed at being broadly inclusive so as to target as many of the published LP Eurasian H5 HA sequences as possible and to distinguish them from HP viruses of the gs/GD lineage. The probes were placed directly onto the cleavage site in the attempt to specifically bind to sequences encoding either mono- or polybasic patterns that distinguish LP and HP pathotypes, respectively (Table 1).

At first, sets of primers and probes were designed to detect and discriminate between HP and LP biotypes, i.e. Eurasian H5 viruses encoding a monobasic or a polybasic HA cleavage site. In addition, four different sets of primers and probes were developed to differentiate between gs/GD clades 2.2.1.2, 2.3.2.1 and 2.3.4.4 (A and B). Pre-selected primers were then screened in silico for their specificity properties using Shannon entropy plots implemented in the Entropy One software (http://www.hiv.lanl.gov/content/sequence/-ENTROPY/entropy_one.html). Oligont (oligont) were selected so as to retain full specificity for the selected clade and to maximise entropy against all other clades. Basic physical properties of oligont were checked using the online web interface Oligo Calculator version 3.27. The finally chosen oligont are shown in Table 1. Detailed results of the in silico analyses are available on request from the authors.

One-step quantitative reverse transcription PCR assays

All reactions were performed using the AgPath-ID One-Step RT-qPCR kit (Thermofisher, scientific, United States) as follows: Reverse transcription at 45 °C for 10 min, initial denaturation at 95 °C for 10 min, 40 cycles of PCR amplification at 95 °C for 30 s, 58 °C for 15 s, and 72 °C for 15 s in a 25 µl reaction mixture using 15 pmol of each forward and reverse primers and 5 pmol probe per reaction. For each parameter a separate reaction was used. Cycling was performed on a Biorad CFX96 Real-Time cycler (BioRad, Germany). Fluorescent signals were collected during the annealing phase, and the amplification data were analysed using Bio-Rad CFX Manager 3 software accessing automated fluorescence drift correction for baseline adjustment.

Nucleotide sequencing and clade assignment

Patho- and phylotyping results obtained by newly developed RT-qPCRs were counter-checked by nt (nt) sequencing of the entire or parts of the HA gene of the respective isolates/clinical samples. Amplification of the HA gene was performed using primers published previously [24] and primers recommended in the European Union Diagnostic Manual for AI in a one-step RT-PCR [25]. In addition, amplificates of the HPAI H5 and LPAI H5 RT-qPCRs were used for sequencing purposes as well. Products were size-separated in agarose gels, excised and purified using the QIAquick Gel Extraction Kit (Qiagen, Hilden, Germany). Purified PCR products were used for cycle sequencing reactions (BigDye Terminator v1.1 Cycle Sequencing Kit, Applied Biosystems, California, United States) the products of which were purified using NucleoSEQ columns (Macherey-Nagel GmbH and Co, Düren, Germany) and sequenced on an ABI PRISM 3130 Genetic Analyzer (Life Technologies, Darmstadt, Germany).

For pathotyping, deduced amino acid sequences of the endoproteolytical cleavage site of the HA gene were inspected and compared with the molecular pathotyping database provided by OFFLU [26]. Assignment of nt sequences to the gs/GD HPAI H5 virus clade system was performed by use of clade prediction tool implemented in the Influenza Research Database [27].

Results

Analytical specificity of pathotyping and phylotyping quantitative reverse transcription PCR assays

The specificity of the assays was evaluated with viral RNA from representative influenza A subtype H5 viruses that had been phylotyped based on full-length HA nt sequence analysis (Table 2). Furthermore, non-H5 subtypes, i.e. H9N2 and H7N7, as well as non-influenza avian viruses i.e. avian infectious bronchitis virus (IBV) and Newcastle disease virus (NDV) were employed (Table 2), and none of them was detected by any of the specific PCRs.

In the initial evaluation of the specificity of the pathotyping RT-qPCR assays carried out using two reference viruses: HPAI A/chicken/Egypt/AR236/2015 (H5N1, clade 2.2.1.2) and LPAI A/turkey/Germany/R2025/2008 (H5N3), specific reactivity exclusively with the homopathotypic virus was evident. In a second step, assays were extended to the full range of 24 reference viruses yielding a similar sharp distinction between HP and LP cleavage sites (Table 2).

Table 3B

Pathotyping and phylotyping of different potentially zoonotic HPAI and LPAI influenza A subtype H5 virus isolates and field samples collected from poultry and wild bird species in different countries, 2013–2016

No.	Sample ID	Type of sample	Accession Number [a]	Clade	PCR results					
					M1.2	HPAI H5	LPAI H5	2.2.1.2	2.3.2.1	2.3.4.4
54	A/wild-duck/Germany/AR8603/2016	Field sample	SA[b]	HP 2.3.4.4b	22.51	22.90	Neg	Neg	Neg	21.14
55	A/greyleg goose /Germany/AR8604/2016	Field sample	SA[b]	HP 2.3.4.4b	22.41	22.30	Neg	Neg	Neg	20.26
56	A/greater scaup/Germany/AR9090/2016	Field sample	SA[b]	HP 2.3.4.4b	27.29	31.71	Neg	Neg	Neg	29.61
57	A/greater scaup/Germany/AR9091/2016	Field sample	SA[b]	HP 2.3.4.4b	28.95	34.40	Neg	Neg	Neg	31.74
58	A/greater scaup/Germany/AR9092/2016	Field sample	SA[b]	HP 2.3.4.4b	23.85	25.70	Neg	Neg	Neg	26.05
59	A/grey heron/Germany/AR9093/2016	Field sample	SA[b]	HP 2.3.4.4b	20.10	22.62	Neg	Neg	Neg	22.44
60	A/greater scaup/Germany/AR9094/2016	Field sample	SA[b]	HP 2.3.4.4b	16.31	20.62	Neg	Neg	Neg	18.92
61	A/greater scaup/Germany/AR9095/2016	Field sample	SA[b]	HP 2.3.4.4b	20.99	22.79	Neg	Neg	Neg	21.75
62	A/northern pintail /Germany/AR9096/2016	Field sample	SA[b]	HP 2.3.4.4b	22.83	28.12	Neg	Neg	Neg	23.95
63	A/bean goose/Germany/AR9097/2016	Field sample	SA[b]	HP 2.3.4.4b	22.97	24.88	Neg	Neg	Neg	24.92
64	A/herring gull /Germany/AR9098/2016	Field sample	SA[b]	HP 2.3.4.4b	20.40	22.35	Neg	Neg	Neg	23.64
65	A/mute swan/Germany/AR9099/2016	Field sample	SA[b]	HP 2.3.4.4b	21.12	25.83	Neg	Neg	Neg	22.75
66	A/chicken/Germany/AR9140/2016	Field sample	SA[b]	HP 2.3.4.4b	21.08	20.55	Neg	Neg	Neg	22.92
67	A/chicken/Germany/AR9141/2016	Field sample	SA[b]	HP 2.3.4.4b	21.21	20.13	Neg	Neg	Neg	23.12
68	A/chicken/Germany/AR9143/2016	Field sample	SA[b]	HP 2.3.4.4b	32.27	31.51	Neg	Neg	Neg	35.49
69	A/chicken/Germany/AR9144/2016	Field sample	SA[b]	HP 2.3.4.4b	21.89	20.89	Neg	Neg	Neg	25.92
70	A/chicken/Italy/22/1998	Isolate	CAP58165	LPAI H5N9	12.34	Neg	19.06	Neg	Neg	Neg
71	A/mallard/Germany/Wv1349–51K/2003	Isolate	CAP58164	LPAI H5N3	26.00	Neg	14.79	Neg	Neg	Neg
72	A/mallard/Germany/Wv476/2004	Isolate	NA	LPAI H5N2	29.87	Neg	29.5	Neg	Neg	Neg
73	A/mallard/Germany/Wv474–77K/2004	Isolate	NA	LPAI H5N2	29.64	Neg	34.81	Neg	Neg	Neg
74	A/ostrich/Germany/R5–10/2006	Isolate	HF563057	LPAI H5N3	26.80	Neg	26.19	Neg	Neg	Neg
75	A/mallard/Germany/R2557/2006	Isolate	NA	LPAI H5N3	26.24	Neg	27.61	Neg	Neg	Neg
76	A/mallard/Germany/R731/2008	Isolate	SA[b]	LPAI H5N3	30.36	Neg	32.30	Neg	Neg	Neg
77	A/mallard/Germany/R771/2008	Isolate	SA[b]	LPAI H5N3	29.24	Neg	30.28	Neg	Neg	Neg
78	A/mallard/Germany/R772/2008	Isolate	SA[b]	LPAI H5N3	23.68	Neg	24.50	Neg	Neg	Neg
79	A/turkey/Germany/R1550/2008	Isolate	NA	LPAI H5N3	25.17	Neg	26.22	Neg	Neg	Neg
80	A/turkey/Germany/R1551/2008	Isolate	NA	LPAI H5N3	24.03	Neg	24.91	Neg	Neg	Neg
81	A/turkey/Germany/R1557/2008	Isolate	SA[b a]	LPAI H5N3	23.50	Neg	24.16	Neg	Neg	Neg
82	A/turkey/Germany/R1612/2008	Isolate	NA	LPAI H5N3	27.43	Neg	29.25	Neg	Neg	Neg
83	A/turkey/Germany/R2014/2008	Isolate	SA[b]	LPAI H5N3	24.33	Neg	21.99	Neg	Neg	Neg
84	A/turkey/Germany/R2015/2008	Isolate	SA[b]	LPAI H5N3	15.69	Neg	30.13	Neg	Neg	Neg
85	A/turkey/Germany/R2016/2008	Isolate	SA[b]	LPAI H5N3	18.13	Neg	15.96	Neg	Neg	Neg
86	A/turkey/Germany/R2017/2008	Isolate	SA[b]	LPAI H5N3	18.32	Neg	16.92	Neg	Neg	Neg
87	A/turkey/Germany/R2018/2008	Isolate	SA[b]	LPAI H5N3	14.16	Neg	16.05	Neg	Neg	Neg
88	A/turkey/Germany/R2019/2008	Isolate	SA[b]	LPAI H5N3	14.55	Neg	16.84	Neg	Neg	Neg
89	A/turkey/Germany/R2020/2008	Isolate	SA[b]	LPAI H5N3	19.38	Neg	16.73	Neg	Neg	Neg
90	A/turkey/Germany/R2021/2008	Isolate	SA[b]	LPAI H5N3	12.71	Neg	13.51	Neg	Neg	Neg
91	A/turkey/Germany/R2022/2008	Isolate	SA[b]	LPAI H5N3	12.63	Neg	13.18	Neg	Neg	Neg
92	A/turkey/Germany/R2023/2008	Isolate	SA[b]	LPAI H5N3	19.37	Neg	17.07	Neg	Neg	Neg
93	A/turkey/Germany/R2024/2008	Isolate	SA[b]	LPAI H5N3	22.52	Neg	20.39	Neg	Neg	Neg
94	A/turkey/Germany/R2025/2008	Isolate	SA[b]	LPAI H5N3	22.44	Neg	25.22	Neg	Neg	Neg
95	A/turkey/Germany/R2026/2008	Isolate	SA[b]	LPAI H5N3	14.70	Neg	16.26	Neg	Neg	Neg
96	A/turkey/Germany/R2027/2008	Isolate	SA[b]	LPAI H5N3	17.80	Neg	16.06	Neg	Neg	Neg
97	A/mallard/Germany/R2892–94/2009	Isolate	EPI356412	LPAI H5N3	11.98	Neg	14.37	Neg	Neg	Neg
98	A/duck/Germany/AR1965/2013	Field sample	NA	LPAI H5N3	26.62	Neg	27.25	Neg	Neg	Neg
99	A/turkey/Germany/AR1892/1/2014	Field sample	SA[b]	LPAI H5N2	20.03	Neg	21.15	Neg	Neg	Neg
100	A/duck/Germany/AR1/2015	Field sample	SA[b]	LPAI H5N3	29.20	Neg	34.01	Neg	Neg	Neg
101	A/swan/Germany/AR111/2015	Field sample	SA[b]	LPAI H5N4	27.45	Neg	31.02	Neg	Neg	Neg
102	A/goose/Germany/AR398/2015	Field sample	SA[b]	LPAI	31.09	Neg	33.69	Neg	Neg	Neg
103	A/duck/Germany/AR1231/1/2015	Field sample	NA	LPAI H5N2	26.74	Neg	32.91	Neg	Neg	Neg
104	A/duck/Germany/AR2853/15–1/2015	Field sample	SA[b]	LPAI H5N3	27.06	Neg	26.25	Neg	Neg	Neg
105	A/goose/Germany/AR3264/1/2015	Field sample	SA[b]	LPAI H5N2	34.47	Neg	35.50	Neg	Neg	Neg
106	A/wild bird/Germany/AR221/2015	Field sample	SA[b]	LP H5N3	22.17	Neg	23.48	Neg	Neg	Neg

HA: haemagglutinin; HP: highly pathogenic; HPAI: highly pathogenic avian influenza; ID: identity; LP: low pathogenic; LPAI: low pathogenic avian influenza; NA: sequence not available; Neg: negative; SA: sequence available.

[a] Sequences were obtained from GenBank at the National Center for Biotechnology Information (NCBI) or the EpiFlu database of the Global Initiative on Sharing Avian Influenza Data (GISAID).

[b] Sequenced in the frame of the current study; sequences available from the authors upon request.

Primers and probes for phylotyping RT-qPCR assays distinguishing three clades of gs/GD origin HPAIV H5 were placed within the HA1-fragment of the HA gene. This region encodes the receptor binding unit and harbours a number of neutralisation-relevant epitopes that are targets of antigenic drift. Hence, the HA1 fragment harbours regions that are the least conserved within the influenza A virus genome. Primer selection aimed at the inclusion of as many as possible distinguishing nt that would define exclusivity at the five most 3' positions while probes were placed so as to accommodate distinguishing nt in the centre of the oligont. In order not to compromise amplification efficacy, amplicon size was limited to 130 nt wherever possible given the above mentioned constraints for primers and probes. The finally chosen oligont are listed in Table 1 and provided specific detection exclusively of the homologous clade. No cross-reactivity among the other gs/GD clades examined was evident on basis of the used panel of reference viruses (Table 2). Also, no cross-reactivity was detected for any of the five assays against other influenza A viruses or other avian viral respiratory pathogens (Table 2).

Validation of the analytical sensitivity, limit of detection and precision

Detection limits of the assays were determined by testing 10-fold serial dilutions of viral RNA extracted from representative viruses of each of the three HPAI virus clades (2.2.1.2, 2.3.2.1 and 2.3.4.4), and of Eurasian H5 LPAI virus. Cycle of quantification (Cq) values were compared with a standard RT-qPCR for the matrix (M) gene of these viruses with a reported detection limit of 2 to 20 RNA copies/5 µl [28]. Average values of three separate runs were computed and plotted using SigmaPlot V 11 software. Plotting these values revealed a linear relationship between the log of the viral RNA dilution and the Cq value for all assays and the kinetics of the assays and their sensitivity were determined to be very similar to the generic M gene RT-qPCR (M1.2 RT-qPCR [29]) (Figure 1).

The correlation coefficient of the standard curves was 0.99 for all assays, indicating a highly precise log–linear relationship between the viral RNA log dilution and the corresponding Cq-value (Figure 1). Based on these results the threshold distinguishing positive and negative was set at Cq = 38.

Pathotyping and phylotyping of clinical samples of potentially zoonotic Eurasian avian influenza A subtype H5 viruses by quantitative reverse transcription PCR

In order to evaluate the diagnostic performance capacity of the developed assays, field samples (RNA extracted from swabs, tissues or FTA cards) and clinical virus isolates obtained during the period 2013 to 2016 (HPAI viruses) or 2003 to 2015 (LPAI viruses) were examined. The sample set was preselected on basis of a positive generic M-specific RT-qPCR.

Among the final set of 106 samples, the pathotyping RT-qPCRs sharply discerned two groups of 69 samples reacting only in the new HPAI H5 RT-qPCR while 37 samples reacted positive in the LPAI H5 RT-qPCR (Figure 2a; Table 3).

All pathotyping results matched the results obtained by nt sequence analysis of the HA cleavage site. However, in a few samples (two isolates, 10 clinical samples) of HP viruses, the LPAI H5 RT-qPCR also gave a weak positive signal (Cq > 35). Compared with the LPAI H5 signal the HPAI H5 signal of these samples yielded Cq values 6–10 units lower on average ascertaining good diagnostic specificity. Depending on the clade, the HP phenotype was detected with equal (clade 2.3.2.1) or slightly reduced (clade 2.2.1.2) sensitivity; the LP H5 RT-qPCR appeared to be slightly less sensitive than the M PCR as far as clinical samples were concerned (Table 3; Figure 2a and c). Sequences across the cleavage sites of these samples are presented in a supplemental alignment (Figure 2).

In a next step, the samples that were designated HPAI H5-positive were subjected to the three phylotyping RT-qPCRs. Here, 15, 21 and 33 samples, respectively, were exclusively positive for either clade 2.2.1.2, 2.3.2.1 or 2.3.4.4 (Table 3). Thus, a clear cut clade assignment was possible for all gs/GD HP H5 samples. Results were counterchecked by feeding available HA sequences of these samples into the IRD clade prediction tool (www.fludb.org/brc/h5n1-Classifier.spg?method=ShowCleanInputPage&decorator=influenz): In all cases the same clade was assigned by sequence analysis and by PCR. In a final step also all LPAI H5 samples were tested in the phylotyping RT-qPCRs and none of them cross-reacted. Regarding the sensitivity of these PCRs, the Cq values were compared with those of the generic M1.2-specific RT-qPCR (Figure 2b). For clade 2.2.1.2 and 2.3.2.1 the sensitivity was almost identical to the M PCR; for clade 2.3.4.4a, the clade-specific PCR proved to be slightly more sensitive while viruses of clade 2.3.4.4b were detected at a slightly lower sensitivity; detection of clade 2.3.4.4b viruses was slightly less sensitive than the M PCR (Figure 2b and c; Table 3) as far as clinical samples were concerned.

Rank Sum tests implemented in the SigmaPlot software package were performed and no statistically significant difference between the median Cq values of each specific assay and the M1.2 RT-qPCR assay was found ($p > 0{,}50$) indicating that the newly developed RT-qPCRs display similar analytical sensitivity. Thus, the phylotyping RT-qPCRs allow a sensitive and highly specific detection and distinction of the three major gs/GD clades currently circulating in countries where the viruses were obtained from.

Discussion

Rapid molecular diagnosis including patho- and phylotyping is basis to enable measures aimed at repressing

the spread of potentially zoonotic HPAI viruses. The TaqMan PCR technology has proven reliable, versatile, and comparatively cost-effective in the generic detection and subtype differentiation of AIV [30]. Further differentiation of clades, lineages and pathotypes was previously nearly entirely based on nt sequencing approaches which require expensive equipment and are time consuming. In epidemiologically complex settings where different lineages and pathotypes of potentially zoonotic and notifiable infectious agents co-circulate, a more rapid and direct access to testing and results, e.g. by using RT-qPCRs, is desirable. Although RT-qPCRs are inferior to sequencing techniques in terms of retrievable data details, they are superior with respect to time-to-diagnosis and ease-of-use. This concept which we used earlier for pathotyping of H5N1 [31], was here further extended and refined for the identification and discrimination of avian influenza A subtype H5 viruses of different patho- and phylotypes. The focus was put on those clades of H5 viruses (2.2.1.2, 2.3.2.1, 2.3.4.4) that had previously 'escaped' from Asia and were detected in western parts of Eurasia and in Africa.

Pathotyping of avian influenza A subtype H5 viruses is mandatory from an animal health perspective. The pathotyping RT-qPCRs presented here reduce time-to-diagnosis to just three hours following sample receipt. To our knowledge this is the broadest and most detailed attempt of AIV pathotyping using RT-qPCR. The availability of highly sensitive pathotyping PCRs would also allow to detect mixtures of HP and LP H5 viruses in the same sample; in fact, some of our HP-positive field samples also gave weak LP signals (Table 3, sample numbers 2, 9, 10, 14, 15, 30, 32, 35, 37, 42–5). Yet, LPAI pathotypes in these samples were detected at distinctly higher Cq values indicating either a minor population in a quasispecies of different pathotypes or expressing some cross-reactivity of LPAI primers and probe; in any case, the detection of HPAI genotypes as a major population in a set of field samples was always unequivocal. Further insight into the true nature of these mixtures would only be unravelled by deep sequencing approaches of those samples.

Rapid pathotyping enables rapid implementation of appropriate measures to prevent further spread of virus such as closure of poultry holdings and/or live poultry markets, culling of infected flocks etc. This impedes accumulation of potentially zoonotic AIV at the poultry-human interface which in turn lowers the risks of human infection.

Phylotyping of gs/GD HPAI H5 virus clades is important since each clade, and often also sublineages thereof, display distinct antigenic and pathogenetic properties. This has direct implications, as by assigning the matching clade, appropriate vaccines that ensure the closest antigenic match with the circulating viruses can be selected [32,33]. In particular, countries where gs/GD viruses have become endemic in poultry populations, rely on vaccination of poultry on a broad scale to suppress circulating viruses and to limit risks of human exposure [23]. However, it should be noted that mutant escape variants within these clades selected by vaccine-induced population immunity will not be detected as such by the assays, and in fact, such mutants may also be detected at lower sensitivity if primer and/or probe binding sites are affected by mutations. Detection of variants will still depend on either nt sequencing or virus isolation/antigenic characterisation approaches but the newly developed assays will aid in selection of meaningful samples in this respect. In particular, samples that do not give conclusively similar Cq values in the generic and the specific assays should prompt in-depth analysis by nt sequencing.

It should be clearly stated that the assays presented here have limitations owed to the restricted geographical distribution of the targeted clades. The use of the newly developed PCRs in regions where viruses belonging to the targeted clades (2.2.1.2, 2.3.2.1c and 2.3.4.4) are reportedly absent is only recommended if immediate incursions with any of these clades are apprehended. Phylotyping indirectly may point towards zoonotic potential since different gs/GD lineages vary in their zoonotic propensity: Egyptian 2.2.1.2 viruses are characterised by increased affinity to human-like sialic acid receptors and have caused by far the largest number of human influenza A(H5N1) virus infections over the past decade [12]. For clade 2.3.2.1c viruses, repeatedly detected in the Middle East (excluding Egypt) and endemic in Western African countries, only few human cases have been recorded. The 2.3.4.4 viruses currently present in various parts of Europe have not provoked human infection so far [34].

Extended co-circulation of more than one gs/GD lineage in poultry and/or wild birds in a wider geographic region was repeatedly reported [35,36]. It is pivotal, for the above mentioned reasons, to detect incursions of distinct HPAI virus lineages in a timely manner. In this respect, the newly developed RT-qPCR assays were shown to be useful tools for an improved rapid and simple characterisation of patho- and phylotypes of Eurasian origin avian influenza A subtype H5 viruses. The assays aid in speeding up diagnosis on clinical samples because the time consuming (initial) need of virus isolation and nt sequencing is avoided. Given the high substitution rate of HP H5 influenza viruses frequent checks and, if required, updates of the primers and probes are recommended to ensure full specificity and sensitivity of the patho- and phylotyping RT-qPCRs. These PCRs are advantageous in particular for wild bird samples, especially those that contain LPAI viruses, often with low viral loads and therefore fail to yield replication-competent virus. With respect to HPAI virus, the renouncement from initial virus isolation improves biosecurity. However, the presented assays are not intended to replace virus isolation and antigenic characterisation as a means to detect emerging antigenic drift mutants. Nevertheless, they may aid in selection

of appropriate samples for such tasks. Accurate phylotyping also facilitates selection of appropriate vaccines as it serves as an early warning for the incursion of new and antigenically possibly distinct phylotypes.

Conclusions

The assays reported here are primarily intended for screening purposes of avian samples; confirmatory assays, including nt sequence analyses and antigenic characterisation, are still required for new incursions and outbreak scenarios that feature an expansion of the geographic area and/or the range of affected species or poultry sectors. When used in the frame of on-going outbreaks, in particular in regions where vaccination is not used as a preventive measure, results of the patho- and phylotyping PCRs are deemed solid enough for reporting purposes and to justify the implementation of restriction measures. In such settings, similar to the current outbreaks of clade 2.3.4.4b HP H5N8 in Europe, the assays can be prioritised to running the HP and only one (i.e. the fitting) of the phylotyping PCRs on M1.2- and H5 PCR-positive samples. This significantly speeds up time-to-diagnosis and reduces reaction times in a OneHealth approach of repressing the spread of gs/GD HP AIV. Sequencing facilities, classically required for patho- and phylotyping, may not be available, and even not logistically accessible in many regions severely affected by H5 HPAI incursions. The prospect of having sequencing-independent, TaqMan-based specific and sensitive typing assays, as described here, available in developing regions is expected to boost regional diagnostic capacities eventually leading to improved disease control.

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Conflict of interest

None declared. One of the authors (N.A.) works for Boehringer Ingelheim, Dubai, United Arab Emirates.

Authors' contributions

Mahmoud M. Naguib, Annika Graaf and Timm Harder conceived the study. Andrea Fortin, Ulrich Wernery, Nadim Amarin, Hussein A. Hussein, Hesham Sultan and Basem Al Adhadh were involved in the collection, initial analysis and provision of viruses and field samples. Christine Luttermann conducted the Sanger sequencing analyses. Mahmoud M. Naguib, Annika Graaf and Andrea Fortin produced, analysed and interpreted data. Timm Harder and Mahmoud M. Naguib drafted the manuscript. Isabella Monne, Martin Beer and all co-authors critically analysed and revised the manuscript and provided final approval.

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Research article

Trends in antimicrobial susceptibility for azithromycin and ceftriaxone in *Neisseria gonorrhoeae* isolates in Amsterdam, the Netherlands, between 2012 and 2015

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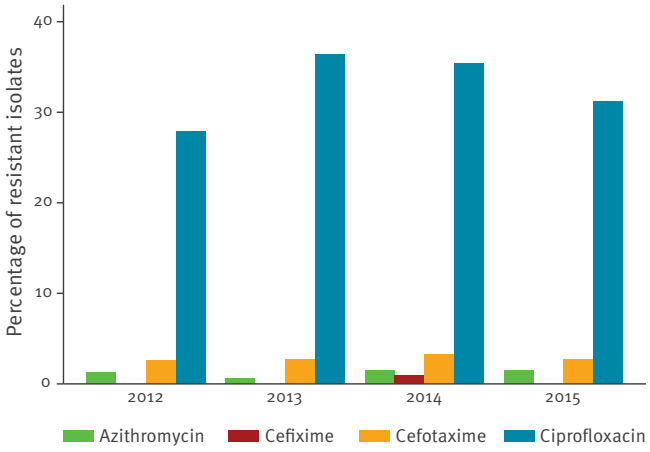
Resistance of *Neisseria gonorrhoeae* to azithromycin and ceftriaxone has been increasing in the past years. This is of concern since the combination of these antimicrobials is recommended as the first-line treatment option in most guidelines. To analyse trends in antimicrobial resistance, we retrospectively selected all consultations with a positive *N. gonorrhoeae* culture at the sexually transmitted infection clinic, Amsterdam, the Netherlands, from January 2012 through September 2015. Minimum inhibitory concentrations (MICs) for azithromycin and ceftriaxone were analysed per year, and determinants associated with decreased susceptibility to azithromycin (MIC > 0.25 mg/L) or ceftriaxone (MIC > 0.032 mg/L) were assessed. Between 2012 and 2015 azithromycin resistance (MIC > 0.5 mg/L) was around 1.2%, the percentage of isolates with intermediate MICs (> 0.25 and ≤ 0.5 mg/L) increased from 3.7% in 2012, to 8.6% in 2015. Determinants associated with decreased azithromycin susceptibility were, for men who have sex with men (MSM), infections diagnosed in the year 2014, two infected sites, and HIV status (HIV; associated with less decreased susceptibility); for heterosexuals this was having ≥ 10 sex partners (in previous six months). Although no ceftriaxone resistance (MIC > 0.125 mg/L) was observed during the study period, the proportion of isolates with decreased ceftriaxone susceptibility increased from 3.6% in 2012, to 8.4% in 2015. Determinants associated with decreased ceftriaxone susceptibility were, for MSM, infections diagnosed in 2014, and pharyngeal infections; and for heterosexuals, infections diagnosed in 2014 or 2015, being of female sex, and having ≥ 10 sex partners. Continued decrease of azithromycin and ceftriaxone susceptibility will threaten future treatment of gonorrhoea. Therefore, new treatment strategies are warranted.

Introduction

Since penicillin became available in the 1940s, *Neisseria gonorrhoeae* infection has become a treatable sexually transmitted infection (STI) [1]. Yet successful eradication is hampered by emerging resistance to all first-line antibiotics used so far. Latest in this trend are resistance and treatment failures to extended-spectrum cephalosporins (ESC) [1,2]. We reported an increase in ESC-resistant *N. gonorrhoeae* among men who have sex with men (MSM) in Amsterdam, the Netherlands, between 2006 and 2008 [3]. To halt the development and spread of resistance, international gonorrhoea guidelines recommend dual therapy consisting of ceftriaxone (an ESC) and azithromycin [4-6]. Dual therapy is also effective against *Chlamydia trachomatis*, which frequently coincides with gonorrhoea [4]. However, resistance and treatment failures have been documented for both drugs [7-13]. Taking the historical course of emerging antimicrobial-resistant gonorrhoea strains into account, without additional measures, a further decrease in ceftriaxone and azithromycin susceptibility is to be expected [1]. Moreover, high level azithromycin-resistant gonorrhoea has been reported in the United Kingdom (UK) since 2015 [9]. In addition, the first treatment failure on dual therapy of azithromycin and ceftriaxone was reported in 2016 [14]. The World Health Organization (WHO) recommends abandoning an antibiotic as first-line treatment once the prevalence of resistant strains in the population exceeds 5% [15]. Surveillance is essential to monitor this development. Therefore, we analysed the susceptibility to azithromycin and ceftriaxone of *N. gonorrhoeae* isolates among

Figure 1

Percentage of resistant *Neisseria gonorrhoeae* isolates[a] per year, at the STI Outpatient Clinic Amsterdam, the Netherlands, January 2012–September 2015 (n = 3,151 isolates)



EUCAST: European committee on antimicrobial susceptibility testing; MIC: minimum inhibitory concentration; STI: sexually transmitted infection.

Azithromycin resistance: MIC > 0.5 mg/L, cefixime resistance: MIC > 0.125 mg/L, cefotaxime resistance: MIC > 0.125 mg/L, ciprofloxacin resistance: MIC > 0.06 mg/L.

[a] According to EUCAST breakpoints.

attendees of the STI Outpatient Clinic in Amsterdam, the Netherlands, between 2012 and 2015. We also assessed which determinants were associated with decreased susceptibility.

Methods

Study population

The STI Outpatient Clinic in Amsterdam, is the largest centre for STI care in the Netherlands, with up to 40,000 consultations each year [16]. We test and treat (free of charge) patients who: are younger than 25 years-old, commercial sex workers, clients of commercial sex workers, MSM, have ≥ 3 sex partners in the previous six months, were notified of an STI by a sex partner, have STI-related complaints, are of non-West-European origin, or are of non-North-American origin.

Dual therapy for gonorrhoea is not recommended in the Netherlands, instead ceftriaxone 500 mg is used, and azithromycin is added only in case of a suspected or proven coinfection with *C. trachomatis* [17]. This single treatment alternative is supported by the 2016 WHO gonorrhoea treatment guideline [6].

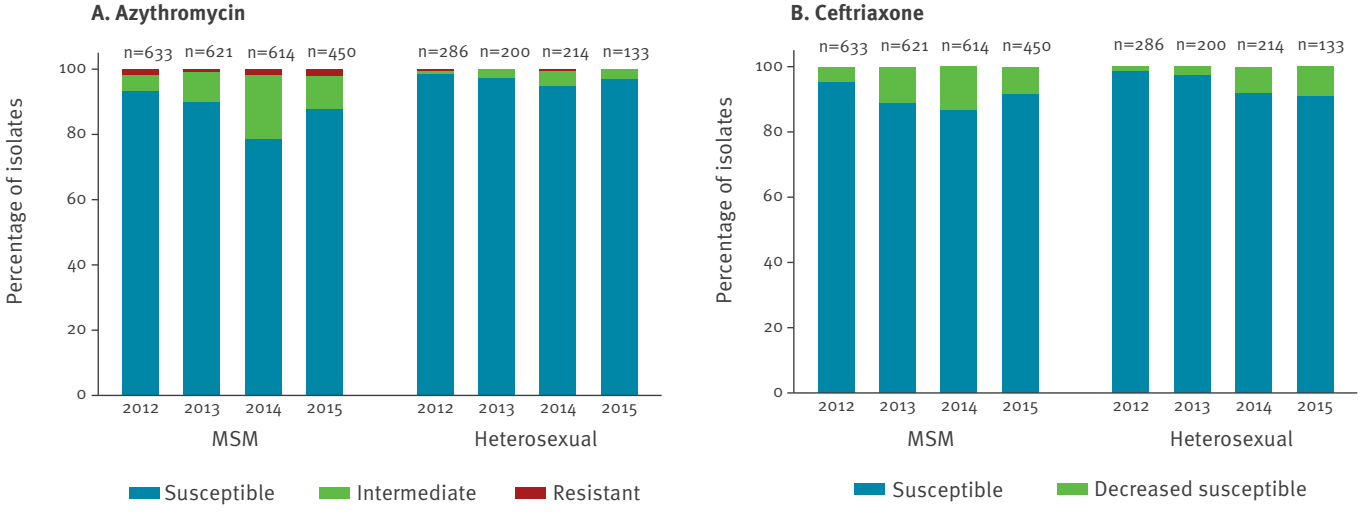
For this study, we included consultations from January 2012 through September 2015, with a positive *N. gonorrhoeae* culture, and available minimum inhibitory concentrations (MICs) for azithromycin and ceftriaxone. Per consultation, defined as all visits that are part of a new request for healthcare, a patient could be infected at up to four anatomical sites (cervix/vagina, pharynx, rectum, and urethra). Samples were collected from any site upon risk assessment; rectal and pharyngeal samples were not obtained from heterosexual males. When more than one culture was obtained during a single consultation, we included the one with the highest MIC for either azithromycin or ceftriaxone. In case of equal MICs at different anatomical sites, we gave priority in the following order: pharynx, cervix/vagina, rectum, and urethra. All analyses were performed using isolates collected during individual consultations, therefore some patients were included more than once. Patient and clinical characteristics were obtained from the electronic patient file. Syphilis status (past and active) was based on *Treponema pallidum* particle agglutination (TPPA) and rapid plasma reagin (RPR) testing, human immunodeficiency virus (HIV)-positivity was based on HIV-antibodies, and coinfection with *C. trachomatis* was diagnosed using a nucleic acid amplification test (NAAT) [3]. As this was a retrospective cohort study using only routinely obtained data, no ethical clearance or informed consent was required.

Antimicrobial susceptibility testing

Up to May 2014, direct *N. gonorrhoeae* cultures instead of NAATs, were routinely obtained from urogenital and rectal sites, if patients met at least one of the following criteria: being MSM, having STI-related symptoms, being notified of gonorrhoea by a sex partner, or performing sex work. In addition, cultures were obtained from patients, who did not have any of the prior-described criteria for culture but had a positive NAAT for *N. gonorrhoeae*. Pharyngeal sites were primarily tested using NAAT, and cultures were obtained in case of positive results. From May 2014 onward this policy was changed, and NAAT was used as the routine test for gonorrhoea diagnosis in all patients and all anatomical sites. Cultures were obtained if a patient had symptoms suggestive of gonorrhoea, and intracellular Gram-negative diplococci had been identified in a Gram-stained smear, or if the NAAT was positive for gonorrhoea. In case of a positive culture for *N. gonorrhoeae*, antimicrobial susceptibility testing was routinely performed at the Public Health Laboratory in Amsterdam, the Netherlands [18]. MICs for azithromycin, cefixime, cefotaxime, ceftriaxone and ciprofloxacin were determined using Etests according to the manufacturer's instructions (bioMérieux SA, Marcy-l'Étoile, France). For this study MIC data were obtained as recorded in the electronic laboratory patient files. To determine resistance we used the European committee on antimicrobial susceptibility testing (EUCAST) breakpoints [19]. For azithromycin we categorised MIC values into susceptible (MIC ≤ 0.25 mg/L), intermediate (MIC > 0.25 and ≤ 0.5 mg/L), and resistant (MIC > 0.5 mg/L). For ceftriaxone, cefixime and cefotaxime we categorised MICs into susceptible (MIC ≤ 0.125 mg/L) and resistant (MIC > 0.125 mg/L). For ciprofloxacin we

Figure 2

Susceptibility to azithromycin and ceftriaxone of *Neisseria gonorrhoeae* isolates, according to year and sexual orientation, STI Outpatient Clinic Amsterdam, the Netherlands, January 2012–September 2015 (n = 3,151 isolates)



MIC: minimum inhibitory concentration, MSM: men who have sex with men, STI: sexually transmitted infection.

Azithromycin susceptible: MIC ≤ 0.25 mg/L, intermediate: MIC 0.38–0.5 mg/L, resistant: MIC > 0.5 mg/L. Ceftriaxone susceptible: MIC ≤ 0.032 mg/L, decreased susceptible: MIC > 0.032 mg/L.

categorised MICs into susceptible (MIC ≤ 0.06 mg/L) and resistant (MIC > 0.06 mg/L).

Statistical analyses

Baseline characteristics were compared for MSM and heterosexuals using $X2$, Fisher exact, or Kruskal–Wallis tests. The prevalence of antimicrobial resistance in our population is still very low, and we could not determine associations with resistance. Therefore, we used not resistance, but decreased susceptibility as endpoint in the analyses. Decreased susceptibility was determined for azithromycin as MIC > 0.25 mg/L, and for ceftriaxone as MIC > 0.032 mg/L (the epidemiological cut-off as reported by EUCAST) [19]. Mean MICs were calculated as geometric means. To assess determinants associated with decreased susceptibility we performed logistic regression analyses. Since sexual orientation is highly correlated with many other variables, such as anatomical site, origin, age, and coinfections like HIV, syphilis and *C. trachomatis*, we performed separate analyses for MSM and heterosexuals. All determinants that were associated in the univariable analysis ($p < 0.1$) were included in the multivariable analysis, using backward selection. As our main category of interest for trend analysis, year of infection was always included in the model. Also sex (for heterosexuals only) and age were always included in the model. In the multivariable analysis statistical significance was determined as $p < 0.05$. All analyses were performed using Stata (version 13; StataCorp, College Station, Texas).

Results

Gonorrhoea was diagnosed at our STI Clinic in 5,431 consultations from January 2012 through September 2015. We excluded 2,280 consultations in which a gonorrhoea diagnosis was based on results of a NAAT or a Gram-stained smear, but a *N. gonorrhoeae* culture was not performed (n = 653), was not positive (n = 1,590), or because no susceptibility data were available (n = 37). This resulted in 3,151 included consultations, from 2,573 individual patients. The majority of patients (n = 2,573) were included only once; 408 patients (13.0%) were included twice, 109 patients (3.5%) were included three times, and 61 patients (1.9%) were included with four to eight episodes. Of the 578 patients who were included more than once, 522 (90.3%) were MSM.

Baseline characteristics of patients

Of the 3,151 included isolates, 2,318 (73.6%) were from MSM, and 833 (26.4%) were from heterosexual patients, of which 436 (52.3%) were from males and 397 (47.7%) were from females (Table 1). The median age was 34 years (interquartile range (IQR): 26–43) for MSM, and 23 years (IQR: 20–28) for heterosexuals. The majority of MSM were of Dutch origin (n = 1,347 isolates, 58.1%), while among heterosexuals the largest group was of Surinamese origin (n = 342 isolates, 41.1%), followed by 158 isolates of Dutch origin (19.0%). Heterosexuals were more likely to be symptomatic (n = 559, 67.1%) compared with MSM (n = 1,249, 53.9%, $p < 0.001$). The median number of sex partners in the previous six months was eight for MSM (IQR: 4–15), and three for heterosexuals (IQR: 2–5). While MSM were more likely to be HIV-positive (n = 900, 38.8%), or have (ever had)

Table 1a

Baseline characteristics of included consultations with culture positive *Neisseria gonorrhoeae*, at the STI Outpatient Clinic Amsterdam, the Netherlands, January 2012–September 2015 (n = 3,151 consultations)

Characteristic	MSM n (%)a	Heterosexual n (%)a	P
Isolates	2,318 (73.6)	833 (26.4)	NA
Year of diagnosis			
2012	633 (27.3)	286 (34.3)	0.001
2013	621 (26.8)	200 (24.0)	
2014	614 (26.5)	214 (25.7)	
2015[b]	450 (19.4)	133 (16.0)	
Sex			
Male	2,318 (100.0)	436 (52.3)	NA
Female	0 (0.0)	397 (47.7)	NA
Median age, years (IQR)	34 (26–43)	23 (20–28)	<0.001
Origin			
Dutch	1,347 (58.1)	158 (19.0)	<0.001
Asian	158 (6.8)	25 (3.0)	
Dutch-Antillean	56 (2.4)	68 (8.2)	
Eastern European	70 (3.0)	50 (6.0)	
European	251 (10.8)	36 (4.3)	
Latin American	146 (6.3)	32 (3.8)	
North African	37 (1.6)	43 (5.2)	
Sub-Sahara African	40 (1.7)	50 (6.0)	
Surinamese	114 (4.9)	342 (41.1)	
Turkish	46 (2.0)	18 (2.2)	
Other	47 (2.0)	10 (1.2)	
Unknown	6 (0.3)	1 (0.12)	
Symptoms at triage	1,249 (53.9)[c]	559 (67.1)	<0.001
Notified by sex partner	683 (29.5)[d]	218 (26.2)	0.09
Sex worker (MSM or women)	68 (2.9)[e]	73 (18.4)[f]	<0.001
Median number of sex partners in the previous six months (IQR)	8 (4–15)	3 (2–5)	<0.001
HIV status			
Negative	1,377 (59.4)	805 (96.6)	<0.001
Positive	900 (38.8)	7 (0.84)	
Unknown	41 (1.8)	21 (2.5)	
Previous or active syphilis			
No	1,566 (67.6)	819 (98.3)	<0.001
Yes	752 (32.4)	14 (1.7)	
Chlamydia trachomatis co-infection			
No	1,816 (78.3)	460 (55.2)	<0.001
Yes	502 (21.7)	373 (44.8)	

HIV: human immunodeficiency virus; IQR: interquartile range; MIC: minimum inhibitory concentration; MSM: men who have sex with men; NA: not applicable; STI: sexually transmitted infection.

[a] n (%) unless otherwise indicated.

[b] Inclusion up to and including September 2015.

[c] For two patients the information in question was not available.

[d] For four patients the information in question was not available.

[e] For 25 patients the information in question was not available.

[f] The number (n=73) and percentage (18.4%) are only presented for women, as, within the study, only one heterosexual male reported sex work.

[g] In case of multiple infected sites per patient, the isolate with the highest MIC for was selected. Therefore the included anatomical sites differ per antimicrobial drug.

[h] Including cervical and vaginal samples.

Table 1B

Baseline characteristics of included consultations with culture positive *Neisseria gonorrhoeae*, at the STI Outpatient Clinic Amsterdam, the Netherlands, January 2012–September 2015 (n = 3,151 consultations)

Characteristic	MSM n (%)a	Heterosexual n (%)a	P
Included anatomical site, azithromycin analysis[g]			
Urethra	752 (32.4)	451 (54.1)	<0.001
Rectum	1,301 (56.1)	64 (7.7)	
Cervix[h]	NA	263 (31.6)	
Pharynx	265 (11.4)	55 (6.6)	
Included anatomical site, ceftriaxone analysis[g]			
Urethra	740 (31.9)	451 (54.1)	<0.001
Rectum	1,305 (56.3)	80 (9.6)	
Cervix[h]	NA	252 (30.3)	
Pharynx	273 (11.8)	50 (6.0)	
Number of culture positive sites			
1	2,098 (90.5)	704 (84.5)	<0.001
2	218 (9.4)	109 (13.1)	
3	2 (0.1)	18 (2.2)	
4	NA	2 (0.2)	

HIV: human immunodeficiency virus; IQR: interquartile range; MIC: minimum inhibitory concentration; MSM: men who have sex with men; NA: not applicable; STI: sexually transmitted infection.

[a] n (%) unless otherwise indicated.

[g] In case of multiple infected sites per patient, the isolate with the highest MIC for was selected. Therefore the included anatomical sites differ per antimicrobial drug.

[h] Including cervical and vaginal samples.

syphilis (n=752, 32.4%) compared with heterosexuals (n=7, 0.8%, and n=14, 1.7%; p<0.001 for both), they were less likely to be coinfected with *C. trachomatis* (n=502, 21.7% for MSM, and n=373, 44.8%, for heterosexuals, p<0.001). Among the 2,318 MSM, the majority of isolates were from the rectum (56.2%), while 11.6% were from the pharynx, and 90.5% (n = 2,098) had only one culture positive site. Among heterosexuals the majority of isolates were from the urethra (54.1%) or the cervix/vagina (31.0%).

Antimicrobial resistance according to European committee on antimicrobial susceptibility testing

Figure 1 shows the percentage of the 3,151 isolates that were resistant to azithromycin, cefixime, cefotaxime, and ciprofloxacin, according to EUCAST breakpoints [19]. No resistance to ceftriaxone was observed. Resistance to cefixime was rare (8 isolates in 2014, 0.3%). Overall resistance was highest for ciprofloxacin (n=1,030, 32.7%), followed by cefotaxime (n=89, 2.8%), and azithromycin (n=38, 1.2%).

Azithromycin susceptibility

The mean azithromycin MIC was 0.12 mg/L, with a range of<0.016 to>256 mg/L (Table 2). When categorising according to EUCAST breakpoints, overall 2,838 of the 3,151 isolates (90.1%) were susceptible, 275 (8.7%) were intermediate, and 38 (1.2%) were resistant [19]. Over time the mean MIC increased from 0.09 mg/L in 2012 to 0.13 mg/L in 2015, and the percentage of resistant strains increased slightly from 1.3% (12/919) in 2012 to 1.5% (9/583) in 2015. However, the percentage of intermediate MICs increased from 3.7% (34/919) to 8.6% (50/583), especially among MSM (Figure 2).

Determinants of decreased azithromycin susceptibility (MIC > 0.25 mg/L)

Men who have sex with men

Decreased susceptibility to azithromycin was 12.5% (289/2,318). Univariable logistic regression analysis (Table 3) showed an association (p<0.1) between decreased susceptibility and year of infection, anatomical site, number of infected anatomical sites, and HIV-status. In the multivariable analysis decreased susceptibility was significantly associated with infections diagnosed in 2014 (odds ratio (OR): 3.83; 95%-confidence interval (CI): 2.64–5.55, compared with 2012), and two infected sites (OR: 1.56; 95% CI: 1.05–2.30), and was less frequent in HIV-positive patients (OR: 0.72; 95% CI: 0.54–0.96).

Heterosexuals

The percentage of isolates with decreased susceptibility to azithromycin in heterosexuals was 2.9% (24/833), which was significantly lower compared with MSM (p<0.001). Univariable logistic regression analysis (Table 4) showed an association (p<0.1) with sex, age, origin, and number of sex partners. Higher ORs were observed for calendar years after 2012 (p=0.11). In the multivariable regression only≥10 sex partners in

Table 2

Susceptibility to azithromycin and ceftriaxone by year of infection, of *Neisseria gonorrhoeae* isolates from the STI Outpatient Clinic Amsterdam, the Netherlands, January 2012–September 2015 (n = 3,151 isolates)

Antibiotic and characteristics of the isolates	Total 3,151	Year and number of isolates			
		2012 919	2013 821	2014 828	2015 583
Azithromycin					
Mean[a] MIC in mg/L (range)	0.12 (<0.016 to>256)	0.09 (<0.016 to>256)	0.12 (<0.016–4)	0.15 (<0.016 to>256)	0.13 (<0.016–64)
Susceptible: MIC ≤0.25 mg/L; n(%)	2,838 (90.1)	873 (95.0)	754 (91.8)	687 (83.0)	524 (89.9)
Intermediate: MIC >0.25 to≤0.5 mg/L; n (%)	275 (8.7)	34 (3.7)	62 (7.6)	129 (15.6)	50 (8.6)
Resistant: MIC >0.5 mg/L; n (%)	38 (1.2)	12 (1.3)	5 (0.6)	12 (1.5)	9 (1.5)
Ceftriaxone					
Mean MIC[a] in mg/L (range)	0.005 (<0.002–0.125)	0.004 (<0.002–0.094)	0.006 (<0.002–0.125)	0.007 (<0.002–0.125)	0.005 (<0.002–0.125)
Susceptible: MIC ≤0.032 mg/L; n (%)	2,898 (92.0)	886 (96.4)	748 (91.1)	730 (88.2)	534 (91.6)
Decreased susceptible: MIC >0.032 mg/L; n(%)	253 (8.0)	33 (3.6)	73 (8.9)	98 (11.8)	49 (8.4)

MIC: minimum inhibitory concentration, STI: sexually transmitted infection.

[a] Mean was calculated as geometric mean.

the previous six months was significantly associated with decreased susceptibility (OR: 5.65; 95% CI: 1.49–21.39, compared with 0–1 sex partners).

Ceftriaxone susceptibility

The mean MIC was 0.005 mg/L, the range was<0.002–0.125 mg/L (Table 2). We categorised 2,898 of the 3,151 isolates (92.0%) as susceptible (MIC≤0.032 mg/L), and 253 isolates (8.0%) as decreased susceptible (MIC>0.032 mg/L). The mean MIC increased slightly from 0.004 mg/L in 2012, to 0.005 mg/L in 2015. The percentage of decreased susceptible isolates increased from 3.6% (33/919) in 2012 to 8.4% (49/583) in 2015. This increase was noted among both MSM and heterosexuals (Figure 2).

Determinants of ceftriaxone decreased susceptibility (MIC>0.032 mg/L)

Men who have sex with men

The percentage of isolates with decreased susceptibility to ceftriaxone in MSM was 9.3% (215/2,318). Univariable logistic regression analysis (Table 3) showed an association (p<0.1) between decreased susceptibility and calendar year, anatomical site of infection, HIV-status, and previous or active syphilis. In the multivariable analysis decreased susceptibility was significantly associated with infections diagnosed in 2014 (OR: 3.00, 95% CI: 1.92–4.66, compared with 2012), and pharyngeal infection (OR: 2.52, 95% CI: 1.64–3.89, compared with urethral infection).

Heterosexuals

The percentage of isolates with decreased susceptibility to ceftriaxone in heterosexuals was 4.5% (38/833), which was significantly lower compared with MSM (p<0.001). Univariable logistic regression analysis (Table 4) showed an association (p<0.1) with year of infection, sex, age, origin, anatomical site of infection, number of sex partners, and number of infected anatomical sites. In the multivariable analysis infections diagnosed in 2014 (OR: 5.44; 95% CI: 1.71–17.23, compared with 2012), female sex (OR: 3.14; 95% CI: 1.32–7.45), and≥10 sex partners (OR: 6.16; 95% CI: 1.92–19.79, compared with 0–1 sex partners) were significantly associated with decreased susceptibility.

Decreased susceptibility to azithromycin or ceftriaxone, and resistance to other drugs

Among the 313 isolates with decreased susceptibility for azithromycin, 110 isolates (35.1%) were resistant to ciprofloxacin, 20 (6.4%) to cefotaxime and two (0.6%) to cefixime. In addition, 18 isolates (5.8%) were resistant to at least two antibiotics (apart from azithromycin). Among the 253 isolates with decreased susceptibility to ceftriaxone, 242 (95.7%) were resistant to ciprofloxacin, 80 (31.6%) to cefotaxime, six (2.4%) to azithromycin, and six (2.4%) to cefixime. Also 72 isolates (28.5%) were resistant to at least two, and eight (3.2%) to at least three antibiotics (apart from ceftriaxone).

Discussion

This study shows trends in antimicrobial resistance, and determinants of decreased susceptibility for azithromycin and ceftriaxone in *N. gonorrhoeae* at the STI Clinic Amsterdam, the Netherlands, from January 2012 through September 2015. Resistance to azithromycin remained stable around 1.2%, although the percentage of isolates with intermediate MICs increased from 3.7% in 2012 to 15.6% in 2014, and then decreased to 8.6% in the first nine months of 2015. Resistance to ceftriaxone has not yet been documented in our population.

Table 3a

Determinants, according to logistic regression analysis, of decreased susceptibility for azithromycin (MIC > 0.25 mg/L) and ceftriaxone (MIC > 0.032 mg/L) in *Neisseria gonorrhoeae* isolates from men who have sex with men at the STI Outpatient Clinic Amsterdam, the Netherlands, 2012–2015 (n = 2,318 isolates)

Characteristics	Azithromycin					Ceftriaxone				
	N (%)	OR (95% CI)	P	aOR (95% CI)	P	N (%)	OR (95% CI)	P	aOR (95% CI)	P
Year of diagnosis										
2012	42 (6.6)	1.00		1.00		29 (4.6)	1.00		1.00	
2013	62 (10.0)	1.56 (1.04–2.35)		1.57 (1.04–2.37)		68 (11.0)	2.56 (1.63–4.02)		2.56 (1.63–4.02)	
2014	130 (21.2)	3.78 (2.62–5.46)	< 0.001	3.83 (2.64–5.55)	< 0.001	81 (13.2)	3.17 (2.04–4.91)	< 0.001	3.00 (1.92–4.66)	< 0.001
2015[a]	55 (12.2)	1.96 (1.29–2.99)		1.93 (1.26–2.95)		37 (8.2)	1.87 (1.13–3.08)		1.71 (1.03–2.83)	
Age in years										
≤ 24	55 (12.9)	1.00		1.00		49 (11.5)	1.00		1.00	
25–34	106 (13.1)	1.02 (0.72–1.44)		1.18 (0.82–1.68)		69 (8.5)	0.72 (0.49–1.06)		0.73 (0.49–1.08)	
35–44	72 (11.6)	0.88 (0.61–1.28)	0.82	1.15 (0.78–1.71)	0.74	53 (8.5)	0.72 (0.47–1.08)	0.34	0.79 (0.52–1.19)	0.35
≥ 45	56 (12.2)	0.93 (0.63–1.39)		1.26 (0.83–1.92)		44 (9.5)	0.81 (0.53–1.25)		0.95 (0.61–1.47)	
Origin										
Dutch	155 (11.5)	1.00		Excluded[b]		121 (9.0)	1.00		Excluded[b]	
Non-Dutch	133 (13.8)	1.23 (0.96–1.58)	0.25	Excluded[b]	NA	93 (9.6)	1.08 (0.81–1.44)	0.74	Excluded[b]	NA
Unknown	1 (16.7)	1.54 (0.18–13.3)		Excluded[b]		1 (16.7)	2.03 (0.23–17.45)		Excluded[b]	
Anatomical site										
Urethra	95 (12.6)	1.00		Excluded[b]		53 (7.2)	1.00		1.00	
Rectum	147 (11.3)	0.88 (0.67–1.16)	0.02	Excluded[b]	NA	117 (9.0)	1.28 (0.91–1.79)	< 0.001	1.29 (0.92–1.82)	< 0.001
Pharynx	47 (17.7)	1.49 (1.02–2.18)		Excluded[b]		45 (16.5)	2.56 (1.67–3.91)		2.52 (1.64–3.89)	
Number of sex partners[c,d]										
0–2	35 (12.3)	1.00		Excluded[b]		25 (8.8)	1.00		Excluded[b]	
3–6	98 (12.5)	1.02 (0.67–1.54)		Excluded[b]		71 (9.1)	1.03 (0.64–1.67)		Excluded[b]	
7–15	84 (11.7)	0.95 (0.62–1.44)	0.82	Excluded[b]	NA	73 (10.2)	1.18 (0.73–1.89)	0.79	Excluded[b]	NA
≥ 16	72 (13.5)	1.11 (0.72–1.72)		Excluded[b]		46 (8.7)	0.98 (0.59–1.63)		Excluded[b]	
HIV status										
Negative	191 (13.9)	1.00		1.00		144 (10.5)	1.00		Excluded[b]	
Positive	91 (10.1)	0.70 (0.54–0.91)	0.02	0.72 (0.54–0.96)	0.04	67 (7.4)	0.69 (0.51–0.93)	0.05	Excluded[b]	NA
Missing	7 (17.1)	1.28 (0.56–2.93)		1.43 (0.62–3.33)		4 (9.8)	0.93 (0.33–2.63)		Excluded[b]	

aOR: adjusted odds ratio; CI: confidence interval; HIV: human immunodeficiency virus; MIC: minimum inhibitory concentration; n: number of isolates with decreased susceptibility; NA: not applicable; OR: odds ratio; STI: sexually transmitted infection.

[a] Inclusion up to and including September 2015.

[b] Variable that was excluded by backward selection.

[c] In previous six months.

[d] Four patients with information on number of sexual partners missing.

Table 3b

Determinants, according to logistic regression analysis, of decreased susceptibility for azithromycin (MIC > 0.25 mg/L) and ceftriaxone (MIC > 0.032 mg/L) in *Neisseria gonorrhoeae* isolates from men who have sex with men at the STI Outpatient Clinic Amsterdam, the Netherlands, 2012–2015 (n = 2,318 isolates)

Characteristics	Azithromycin					Ceftriaxone				
	N (%)	OR (95% CI)	P	aOR (95% CI)	P	N (%)	OR (95% CI)	P	aOR (95% CI)	P
Previous or active syphilis										
No	196 (12.5)	1.00	0.92	Excluded[b]	NA	158 (10.1)	1.00	0.05	Excluded[b]	NA
Yes	93 (12.4)	0.99 (0.76–1.28)		Excluded[b]		57 (7.6)	0.73 (0.53–1.00)		Excluded[b]	
Chlamydia trachomatis										
No	225 (12.4)	1.00	0.83	Excluded[b]	NA	172 (9.5)	1.00	0.53	Excluded[b]	NA
Yes	64 (12.8)	1.03 (0.77–1.39)		Excluded[b]		43 (8.6)	0.90 (0.63–1.27)		Excluded[b]	
Number of anatomical sites with gonorrhoea										
1	253 (12.1)	1.00	0.07	1.00	0.03	193 (9.2)	1.00	0.67	Excluded[b]	NA
2	36 (16.5)	1.44 (0.99–2.11)		1.56 (1.05–2.30)		22 (10.1)	1.11 (0.70–1.76)		Excluded[b]	
3	0 (0.0)	NA		NA		0 (0.0)	NA		Excluded[b]	

aOR: adjusted odds ratio; CI: confidence interval; HIV: human immunodeficiency virus; MIC: minimum inhibitory concentration; n: number of isolates with decreased susceptibility; NA: not applicable; OR: odds ratio; STI: sexually transmitted infection.

[b] Variable that was excluded by backward selection.

Decreased susceptibility to ceftriaxone (defined as MIC›0.032 mg/L) increased from 3.6% in 2012 to 11.8% in 2014, and then decreased to 8.4% in the first nine months of 2015. Future surveillance will demonstrate if these small decreases in reduced susceptibility continue, and if so may provide reasons for this. Like we published previously in 2009, decreased susceptibility or resistance to more than one drug remains common [3]. Among isolates with decreased susceptibility to azithromycin or ceftriaxone, 35.1% and 95.7% respectively were also resistant to ciprofloxacin.

Compared with data of various other European countries as reported by the European Centre for Disease Prevention and Control (ECDC), overall resistance in Amsterdam is lower [20]. Although overall resistance was highest for ciprofloxacin (32.7%), it is lower than the overall European prevalence of ciprofloxacin resistance (53%) reported from 2012 to 2013 [20,21]. An explanation could be the large inter-country variability, and the large number of MSM in our population, as in Europe ciprofloxacin resistance was most common among heterosexual males [20]. Cefixime resistance across Europe is 5% [20-22]. Our results show lower cefixime resistance in Amsterdam (0.3%; only noted in 2014), which is comparable to that in the United States from 2006 to 2014 [23]. Cefixime has never been used as first-line treatment of gonorrhoea in the Netherlands, which could explain the lower prevalence of cefixime resistance in our population. Due to unavailability of ceftriaxone in required dosages, cefotaxime was the first-line treatment in the Netherlands for several years up to 2006, which may have caused the relatively high overall resistance for cefotaxime (2.8%) in Amsterdam [3,24]. Since cefotaxime was abandoned as first-line treatment, resistance has decreased again from 12% at the end of 2008, to 2.7% in 2015 [3,25]. Ceftriaxone resistance has been reported in the WHO Western Pacific Region, Asia, the United States and also in several European countries [20,22,23,26,27]. Despite the concurrent increase of ceftriaxone resistance, no resistant isolates have been documented in the Netherlands yet [20,22,23,26,27]. European azithromycin resistance is reported at 5% in 2013 [20,21,28]. In our population azithromycin resistance has not been above 1.5% since 2012, which is lower than the overall European prevalence. Although both the mean MIC and the percentage of resistance have increased slightly during our study period, the high increase reported elsewhere in Europe, was not seen in our population [9,20]. The outbreak of azithromycin high-resistant isolates in England in 2015 occurred despite the use of dual therapy, as recommended by European and United States Centers for Disease Control and Prevention (CDC) guidelines [4,5]. Dutch guidelines do not recommend dual therapy, but advise a single intramuscular dose of 500 mg ceftriaxone [17]. Azithromycin is only added if a *C. trachomatis* coinfection is suspected or diagnosed. The strict adherence to the Dutch guidelines at our clinic will have resulted in lower exposure of our population to azithromycin. In addition, over the counter antibiotics are not available in the Netherlands, and self administration of azithromycin will have been very limited. As exposure to antibiotics is the most important risk factor for antimicrobial resistance, the lower exposure to azithromycin in our population could account for the absence of increased azithromycin resistance in Amsterdam

Table 4a

Determinants, according to logistic regression analysis, of decreased susceptibility to azithromycin (MIC > 0.25 mg/L) and ceftriaxone (MIC > 0.032 mg\L) in *Neisseria gonorrhoeae* isolates from heterosexual males and females at the STI Outpatient Clinic Amsterdam, the Netherlands, 2012–2015 (n = 833 isolates)

Characteristics	Azithromycin					Ceftriaxone				
	n (%)	OR (95% CI)	p	aOR (95% CI)	p	n (%)	OR (95% CI)	p	aOR (95% CI)	p
Year of diagnosis										
2012	4 (1.4)	1.00	0.11	1.00	0.35	4 (1.4)	1.00	< 0.001	1.00	< 0.001
2013	5 (2.5)	1.81 (0.48–6.82)		1.44 (0.37–5.61)		5 (2.5)	1.81 (0.48–6.82)		1.12 (0.28–4.44)	
2014	11 (5.1)	3.82 (1.20–12.17)		2.74 (0.83–9.11)		17 (7.9)	6.08 (2.02–18.36)		5.44 (1.71–17.23)	
2015[a]	4 (3.0)	2.19 (0.54–8.88)		1.65 (0.38–7.15)		12 (9.0)	6.99 (2.21–22.11)		5.54 (1.65–18.65)	
Sex										
Male	8 (1.8)	1.00	0.06	1.00	0.16	10 (2.3)	1.00	< 0.001	1.00	0.007
Female	16 (4.0)	2.25 (0.95–5.31)		1.95 (0.76–5.01)		28 (7.1)	3.23 (1.55–6.74)		3.14 (1.32–7.45)	
Age in years										
≤ 19	3 (1.8)	1.00	0.02	1.00	0.08	2 (1.2)	0.14 (0.03–0.65)	0.02	0.23 (0.05–1.17)	0.26
20–24	8 (2.5)	1.40 (0.37–5.34)		1.23 (0.31–4.84)		14 (4.4)	0.54 (0.25–1.19)		0.69 (0.28–1.70)	
25–29	2 (1.1)	0.61 (0.10–3.68)		0.51 (0.08–3.28)		9 (5.0)	0.62 (0.26–1.49)		0.77 (0.29–2.08)	
≥ 30	11 (6.6)	3.83 (1.05–13.99)		2.86 (0.71–11.60)		13 (7.8)	1.00		1.00	
Origin										
Dutch	7 (4.4)	1.00	0.09	Excluded[b]	NA	8 (5.1)	1.00	0.002	1.00	0.05
Surinamese	5 (1.5)	0.32 (0.10–1.02)		Excluded[b]		6 (1.8)	0.33 (0.11–0.98)		0.96 (0.29–3.14)	
Other	12 (3.6)	0.81 (0.31–2.10)		Excluded[b]		24 (7.2)	1.46 (0.64–3.33)		2.46 (0.98–6.21)	
Unknown	0 (0.0)	NA		Excluded[b]		0 (0.0)	NA		Excluded[b]	
Anatomical site										
Urethra	9 (2.0)	1.00	0.19	Excluded[b]	NA	11 (2.4)	1.00	< 0.001	Excluded[b]	NA
Rectum	3 (4.7)	2.42 (0.64–9.17)		Excluded[b]		10 (12.5)	5.71 (2.34–13.95)		Excluded[b]	
Cervix	8 (3.0)	1.54 (0.59–4.04)		Excluded[b]		8 (3.2)	1.31 (0.52–3.30)		Excluded[b]	
Pharynx	4 (7.3)	3.85 (1.15–12.96)		Excluded[b]		9 (18.0)	8.78 (3.44–22.42)		Excluded[b]	
Number of sex partners[c]										
0–1	3 (1.6)	1.00	< 0.001	1.00	0.01	4 (2.2)	1.00	< 0.001	1.00	0.001
2	4 (2.0)	1.21 (0.27–5.47)		1.44 (0.31–6.66)		3 (1.5)	0.67 (0.15–3.04)		0.85 (0.18–3.95)	
3–9	5 (1.6)	0.95 (0.23–4.04)		1.12 (0.26–4.84)		11 (3.4)	1.60 (0.50–5.08)		1.98 (0.59–6.66)	
≥ 10	12 (10.0)	6.74 (1.86–24.42)		5.65 (1.49–21.39)		20 (16.7)	9.05 (3.01–27.21)		6.16 (1.92–19.79)	

HIV: human immunodeficiency virus; MIC: minimum inhibitory concentration; n: number of isolates with decreased susceptibility; NA: not applicable; OR: odds ratio; aOR: adjusted odds ratio; 95%-CI: 95% confidence interval; STI: sexually transmitted infection.

[a] Inclusion up to and including September 2015.

[b] Variable that was excluded by backward selection.

[c] In the previous six months.

Table 4b

Determinants, according to logistic regression analysis, of decreased susceptibility to azithromycin (MIC > 0.25 mg/L) and ceftriaxone (MIC > 0.032 mg\L) in *Neisseria gonorrhoeae* isolates from heterosexual males and females at the STI Outpatient Clinic Amsterdam, the Netherlands, 2012–2015 (n = 833 isolates)

Characteristics	Azithromycin					Ceftriaxone				
	n (%)	OR (95% CI)	p	aOR (95% CI)	p	n (%)	OR (95% CI)	p	aOR (95% CI)	p
HIV status										
Negative	23 (2.9)	1.00	0.19	Excluded[b]	NA	38 (4.7)	NA	NA	Excluded[b]	NA
Positive	1 (14.3)	5.67 (0.66–49.00)		Excluded[b]		0 (0.0)	NA		Excluded[b]	
Missing	0 (0.0)	NA		Excluded[b]		0 (0.0)	NA		Excluded[b]	
Previous or active syphilis										
No	23 (2.8)	1.00	0.41	Excluded[b]	NA	37 (4.5)	1.00	0.66	Excluded[b]	NA
Yes	1 (7.1)	2.66 (0.33–21.22)		Excluded[b]		1 (7.1)	1.63 (0.21–12.76)		Excluded[b]	
Chlamydia trachomatis										
No	17 (3.7)	1.00	0.11	Excluded[b]	NA	25 (5.4)	1.00	0.18	Excluded[b]	NA
Yes	7 (1.9)	0.50 (0.20–1.21)		Excluded[b]		13 (3.5)	0.63 (0.32–1.25)		Excluded[b]	
Number of anatomical sites with gonorrhoea										
1	18 (2.6)	1.00	0.45	Excluded[b]	NA	27 (3.8)	1.00	0.07	Excluded[b]	NA
2	5 (4.6)	1.83 (0.67–5.04)		Excluded[b]		9 (8.3)	2.26 (1.03–4.94)		Excluded[b]	
3	1 (5.6)	2.24 (0.28–17.78)		Excluded[b]		1 (5.6)	1.47 (0.19–11.49)		Excluded[b]	
4	0 (0.0)	NA		Excluded[b]		1 (50.0)	25.07 (1.53–411.66)		Excluded[b]	

HIV: human immunodeficiency virus; MIC: minimum inhibitory concentration; n: number of isolates with decreased susceptibility; NA: not applicable; OR: odds ratio; aOR: adjusted odds ratio; 95%-CI: 95% confidence interval; STI: sexually transmitted infection.

[b] Variable that was excluded by backward selection.

[29,30]. However, the larger increase in isolates with an intermediate MIC during our study period suggests that an increase in resistant strains is possible in the future.

Strains with decreased susceptibility, for either azithromycin or ceftriaxone, were significantly more often isolated from MSM compared with heterosexuals (both p<0.001). This suggest that sexual orientation (or risk behaviour) is associated with decreased susceptibility to both azithromycin and ceftriaxone. However, because of correlation with other variables, we had to stratify for sexual orientation, and could not correct this possible association for confounders. Among MSM we noted a significant association between more recent year of infection (more recent than 2012) and decreased susceptibility to both azithromycin and ceftriaxone. These results confirm the reported decrease in azithromycin and ceftriaxone susceptibility in Europe [20,26,31]. For heterosexuals being diagnosed in 2014 or 2015 compared to 2012 was only significantly associated with decreased susceptibility to ceftriaxone. Unlike in other countries, this association was not significant for azithromycin, possibly due to a lower number of samples with decreased susceptibility in this group (n = 24) [20]. In addition to time, decreased ceftriaxone susceptibility among MSM was associated with pharyngeal infections. We did not find an association with anatomical site for azithromycin, in either MSM or heterosexuals. Although studies combining antimicrobial resistance and epidemiology are few, previous studies in the UK and France also report higher ceftriaxone MICs in pharyngeal infections [26,31,32]. It is of concern that many cases of pharyngeal gonorrhoea are culture negative, resulting in no diagnosis or diagnosis by NAAT only (which is the recommended routine diagnostic test) [33]. Pharyngeal infections due to strains with decreased susceptibility or even resistance could therefore be missed by routine diagnosis. This is especially worrisome because it is assumed that ceftriaxone resistance in *N. gonorrhoeae* originates from commensal *Neisseria* species in the pharynx [14,34]. Unlike Trecker et al. and Town et al. we found not male, but female sex to be significantly associated with decreased ceftriaxone susceptibility [26,35]. This association might have been caused

by the substantial number of sex workers (18%) among women in our study. However, when adjusting for the number of sex partners (a very good proxy for sex work), female sex remained significantly associated. Also, in a sensitivity analysis adjusting for sex work, female sex still remained significantly associated with decreased ceftriaxone susceptibility (data not shown). In addition, like Town et al. our study shows no significant association with age, in contrast to what was previously reported by Trecker et al. [26,35]. However, among heterosexuals, we did find a significant association between a high number of sex partners (≥ 10; this category consisted mainly of female sex workers) and decreased susceptibility to both azithromycin and ceftriaxone. This adds to the limited evidence that high risk-behaviour and the associated sexual networks are important factors for the spread of resistance among heterosexuals [22,35]. To improve surveillance in populations at high risk of resistant gonorrhoea more studies combining susceptibility and epidemiological data are needed.

There are some limitations to this study. We selected isolates based on new consultations, and some patients were included multiple times. If patients were reinfected by an untreated partner, the same strain could have been included more than once. Depending on the susceptibility of such a strain this could have influenced our analysis of determinants for decreased susceptibility. The change in policy to obtain cultures at the STI clinic in May 2014 may have changed the composition of patients in our study population, and thus could have influenced our results. MSM and commercial sex workers were no longer primarily tested using culture, but with NAAT. In addition, cultures were mainly obtained from patients returning to the STI clinic for treatment after a positive NAAT. Therefore, cultures from patients who did not return to the STI clinic, or did not consent to sampling for culture may have been missed after May 2014. Lastly, as we did not have information on the use of alcohol or drugs, or travel history from our population. Therefore, we were unable to take these possible determinants of decreased susceptibility into account [35,36].

In conclusion, between 2012 and 2015 antimicrobial resistance to azithromycin was less prevalent in Amsterdam compared with other European countries. However, we did note a rise in decreased susceptibility, particularly among MSM. Resistance to ceftriaxone has not been documented in the Netherlands yet, but we also noted a rise in decreased ceftriaxone susceptibility among both MSM and heterosexuals. Given the higher resistance in other countries and increasing globalisation, standardised surveillance of antimicrobial resistance in *N. gonorrhoeae* will remain indispensable. A continued and combined increase of azithromycin and ceftriaxone resistance will likely impede the effectivity of the current dual therapy. Because there is very limited development of new antibiotics, this could lead to severe public health consequences, such as hospital admittance for intravenous treatment in patients with gonorrhoea. Therefore, urgency in the development of novel treatment strategies and reassessment of older antimicrobial agents is warranted. Funding for this research is essential on both national and European levels.

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Conflict of interest

None declared.

Authors' contributions

CW, MSvdL, AvD, HdV and JvdH initiated and designed the study. CW, MSvdL and JvdH analysed and interpreted the data. CW wrote the first draft of the manuscript. All authors read, commented and approved the final manuscript.

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Sentinel surveillance of imported dengue via travellers to Europe 2012 to 2014: TropNet data from the DengueTools Research Initiative

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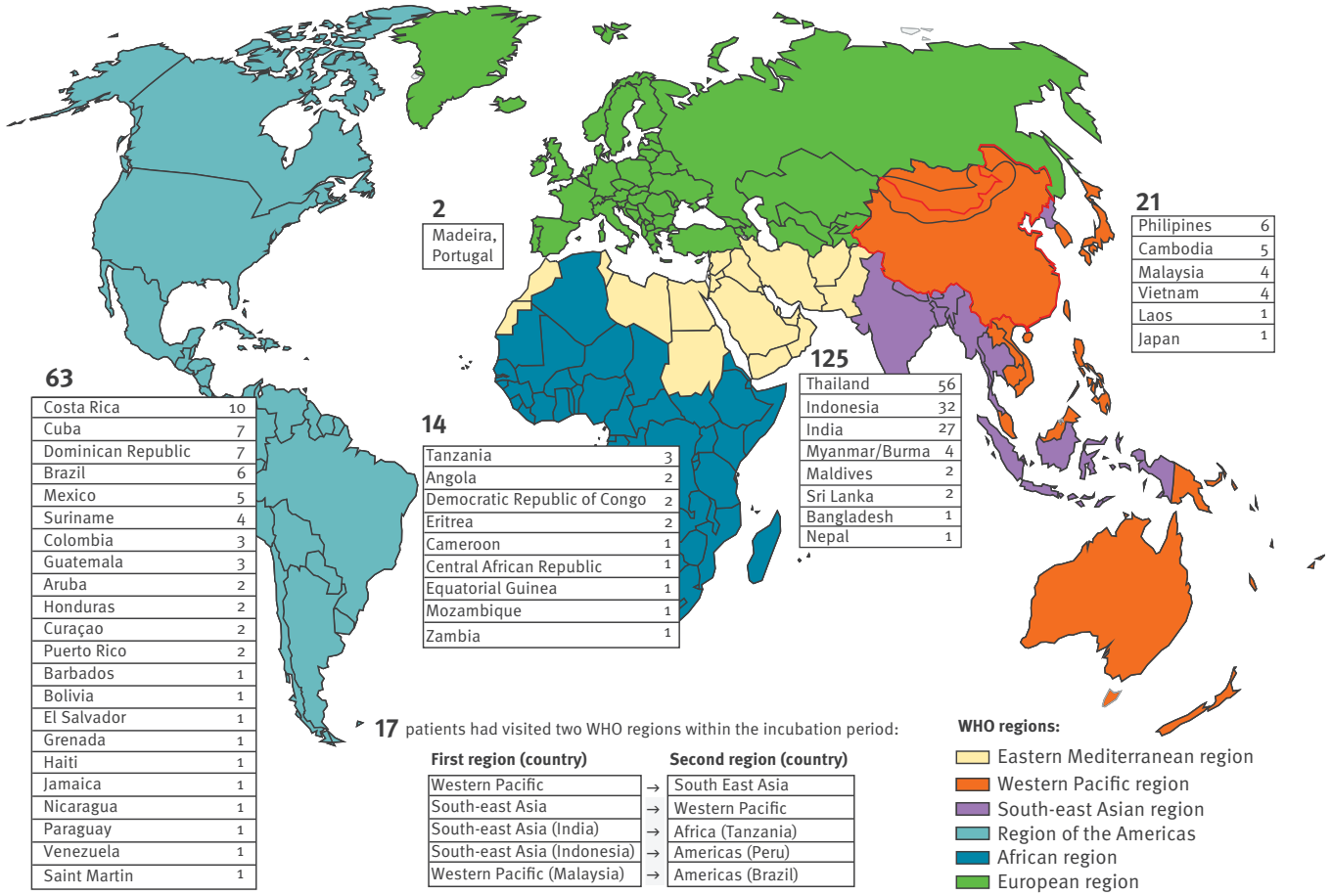
We describe the epidemiological pattern and genetic characteristics of 242 acute dengue infections imported to Europe by returning travellers from 2012 to 2014. The overall geographical pattern of imported dengue (South-east Asia›Americas›western Pacific region›Africa) remained stable compared with 1999 to 2010. We isolated the majority of dengue virus genotypes and epidemic lineages causing outbreaks and epidemics in Asia, America and Africa during the study period. Travellers acted as sentinels for four unusual dengue outbreaks (Madeira, 2012–13; Luanda, 2013; Dar es Salaam, 2014; Tokyo, 2014). We were able to characterise dengue viruses imported from regions where currently no virological surveillance data are available. Up to 36% of travellers infected with dengue while travelling returned during the acute phase of the infection (up to 7 days after symptom onset) or became symptomatic after returning to Europe, and 58% of the patients with acute dengue infection were viraemic when seeking medical care. Epidemiological and virological data from dengue-infected international travellers can add an important layer to global surveillance efforts. A considerable number of dengue-infected travellers are viraemic after arrival back home, which poses a risk for dengue introduction and autochthonous transmission in European regions where suitable mosquito vectors are prevalent.

Background

Over the last decades, dengue has emerged as the most important arthropod-borne viral disease globally. Currently, almost half of the world's population lives in

Figure 1

Geographical distribution of imported dengue cases, by WHO region, 2012–14 (n = 242)



WHO: World Health Organization.

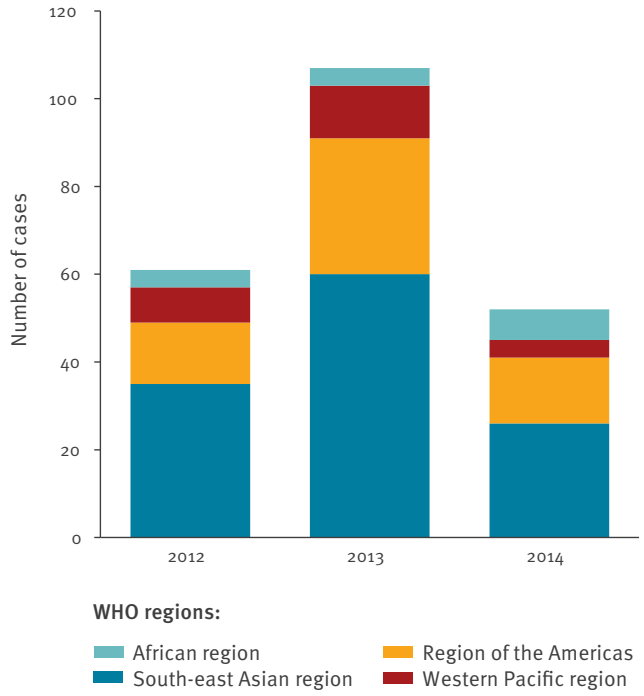
Note: The figure shows absolute numbers and not incidence rates and thus reflects the popularity of travel destinations rather than the risk.

endemic regions, and it is estimated that ca 390 million infections occur annually, of which 96 million cases manifest clinically. In the absence of a vaccine and due to the limited efficacy of vector control strategies, dengue has seen a 30-fold increase in disease burden over the last half century, primarily in tropical and subtropical regions of South-east Asia, the Pacific region and the Americas [1]. With increasing international tourism, dengue has also emerged as an important cause of fever in travellers returning from endemic regions, and the frequency of dengue importation to non-endemic regions such as Europe continues to increase [2,3]. This trend is paralleled by the introduction, or presence and rapid expansion, of potential mosquito vectors such as *Aedes (Stegomyia) albopictus*, which is currently present in at least 15 European countries [4]. While *A. albopictus* is present in the Mediterranean region, *A. aegypti*, the primary vector of dengue in most endemic regions of the world, is found on Madeira (where *A. albopictus* is absent) and in the Black Sea region of Russia's Southern Federal District (Sochi region) and the neighbouring Abkhazia region of Georgia (where *A. albopictus* is also absent) [5].

Therefore, dengue does not only pose a risk to the health of the individual traveller but is also a public health problem as travellers contribute to the spread of the disease [6]. The potential threat from dengue importation to non-endemic, but vector-infested, regions has been highlighted in the recent years by cases of autochthonous dengue transmission in southern Europe [7-9] and a major outbreak with more than 2,000 autochthonous dengue cases in Madeira, Portugal from 2012 to 2013 [10]. Although a great deal of effort is made to prevent the spread of dengue viruses via infected mosquitoes by implementing mosquito abatement programmes at international airports and spraying adulticides in passenger cabins of arriving aircraft, mosquitoes as agents of spread are probably overrated and viraemic travellers are a more likely source of importation of dengue viruses [6]. Therefore, when assessing the risk of introducing dengue to

Figure 2

Geographical pattern of imported dengue cases, by WHO region, 2012–14 (n = 242)



WHO: World Health Organization.

Note: The figure shows absolute numbers and not incidence rates and thus reflects the popularity of travel destinations rather than the risk.

non-endemic regions like Europe, the key focus of surveillance is, besides evaluation of the local prevalence and distribution of potential mosquito vector species, to evaluate the extent of imported dengue via travellers. This task was covered by the European Network for Tropical Medicine and Travel Health (*TropNet*) in the past and is currently covered by the European Travel Medicine Network (*EuroTravNet*).

In addition, sentinel surveillance of travellers represents an additional important layer in the currently still fragmentary global surveillance situation. Especially travellers returning from regions where surveillance capacities are limited can uncover outbreaks that would otherwise go unnoticed. The detection of dengue fever in 10 travellers returning from Luanda, Angola to five countries on four continents in 2013 highlights this aspect [11]. Genetic characterisation of dengue virus strains collected from different geographical locations over time via returning travellers offers the opportunity to understand the global distribution and evolution of dengue sero- and genotypes and may allow us to identify and trace virus strains with epidemic potential that pose an increased risk of introduction to non-endemic regions like Europe [12].

The aims of this study were to report the phylogeny and genetic characteristics of dengue viruses imported to Europe by returning travellers and to describe the epidemiological trends of dengue infections imported to Europe by returning travellers.

Methods

Study objectives, patient recruitment and sample collection

The presented data were collected within the framework of the *DengueTools* research initiative (funded by the 7th Framework Programme for Research and Technological Development of the European Commission) as part of a study conducted in research area 3, 'Risk of dengue spreading to uninfected regions' work package 6, 'sentinel surveillance of imported dengue in returning travellers: trends and virus evolution' [13]. The study was conducted as a prospective observational multi-centre study by major *TropNet* centres, enrolling patients with acute dengue infections between September 2011 and December 2014. The participating *TropNet* sites were: Antwerp (Belgium), Munich (Germany), Berlin (Germany), Hamburg (Germany), Negrar (Italy), Turin (Italy), Brescia (Italy), Leiden (the Netherlands), Madrid (Spain), Barcelona (Spain), Basel (Switzerland) and Lausanne (Switzerland). The study was approved by the responsible ethics committees at all participating study sites.

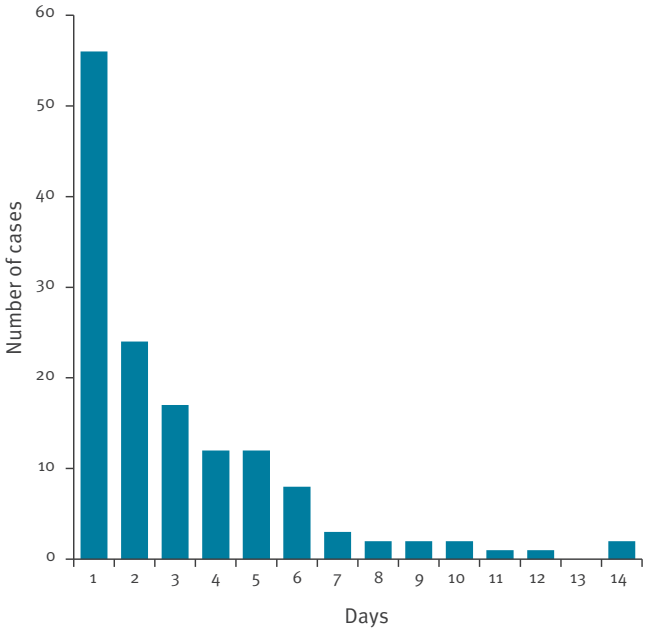
Between September 2011 and December 2014, all European residents (all age groups) returning from dengue-endemic regions and presenting with an acute dengue infection (confirmed by PCR, NS1 antigen detection or positive IgM serology at the participating study sites) no later than 7 days after onset of fever, were eligible for study inclusion. The cut-off at 7 days of illness was chosen because virus isolation after this time point becomes unlikely due to declining viraemia. After signing the informed consent form, the participants completed a questionnaire on demographic data, travel history, clinical and paraclinical data and blood serum was obtained and stored at −80 °C for latter shipment on dry ice to the National Centre of Microbiology at the Instituto de Salud Carlos III (ISCIII) in Madrid, Spain, where all samples (tested positive at the participating study sites) were processed for virus sequencing and virus isolation.

Virus isolation, sequencing and phylogenetic analysis

Sero- and genotyping was performed by RT-PCR targeting the junction between the envelope (E) and non-structural 1 (NS1) protein genes with subsequent sequencing of 400–500 bp of the E/NS1 region for each serotype as described previously [14]. The sequences were edited and analysed in Mega 6 software [15] using maximum likelihood or neighbour-joining methods. All samples were subjected to virus isolation in C6/36 *A. (S.) albopictus* mosquito cells.

Figure 3

Time between onset of symptoms and returning back home in returning travellers developing dengue fever, 2012–14 (data available from 143 travellers)



Results

Demographic data of enrolled cases

Between September 2011 and December 2014, a total of 673 laboratory-confirmed imported dengue cases were seen at the participating study sites, of whom 244 (36%) presented during the acute phase of the infection (≤7 days after onset of symptoms). Of the 244 patients, 242 consented to participate in the study and were enrolled. Table 1 shows the number of cases enrolled per country and their travel profile. Some 128 (53%) of enrolled patients were male (median age: 41 years; range: 17–73 years) and 114 (47%) were female (median age: 32 years; range: 17–73 years).

Geographical origin of imported dengue cases

Figure 1 shows the geographical background of the imported dengue cases by World Health Organization (WHO) region: 125 cases (52%) were imported from the South-east Asian region, 63 cases (26%) from the Americas, 21 cases (8%) from the Western Pacific region, 14 cases (6%) from the African region, two cases (0.8%) from Madeira (Portugal) and 17 cases (7%) had visited two different WHO regions in the incubation period. Of the 17 cases who had visited two WHO regions, 14 travellers had visited countries of the neighbouring WHO regions South-east Asia and Western Pacific and three cases had visited two non-neighbouring WHO regions in the incubation period. Virus isolation and sequencing was successful in two of the three cases who had visited two non-neighbouring WHO regions in the incubation period.

Pattern of imported dengue cases during the study period

Because ethical clearance was obtained by the study sites at different time points, the cases collected in 2011 (n = 21) were not homogeneously enrolled into the study and therefore excluded from the trend analysis. In 2012, 2013 and 2014, all participating study sites recruited patients. Figure 2 depicts the overall importation pattern of acute dengue fever from January 2012 to December 2014. No seasonal importation pattern was observed from any endemic region (data not shown).

Proportion of travellers presenting with acute/viraemic dengue infection

To assess the overall risk of dengue importation by potentially infectious/viraemic travellers (who may introduce the virus to regions of Europe where suitable mosquito vectors are present) we assessed the proportion of acutely ill/viraemic travellers among all imported dengue cases seen at the participating study sites. Of 673 imported dengue cases seen during the study period, 244 (36%) presented during the acute phase of the infection (≤7 days after onset of symptoms). The remaining 64% of patients presented later than 7 days after onset of illness (mainly for follow-up or confirmation of the diagnosis) but we have no further details on these patients as our aim was to include acutely ill, potentially viraemic travellers. Among the 242 study participants presenting with acute dengue (symptoms ≤7 days), 87 (36%) already developed symptoms while travelling and 155 (64%) became symptomatic after returning home. Figure 3 shows the delay between onset of symptoms and returning home. Of the 242 acute dengue cases, 160 (66%) were positive by PCR and virus isolation followed by sero- and genotyping was successful in 141 (58%) cases.

Virus isolation, sequencing and phylogenetic analysis

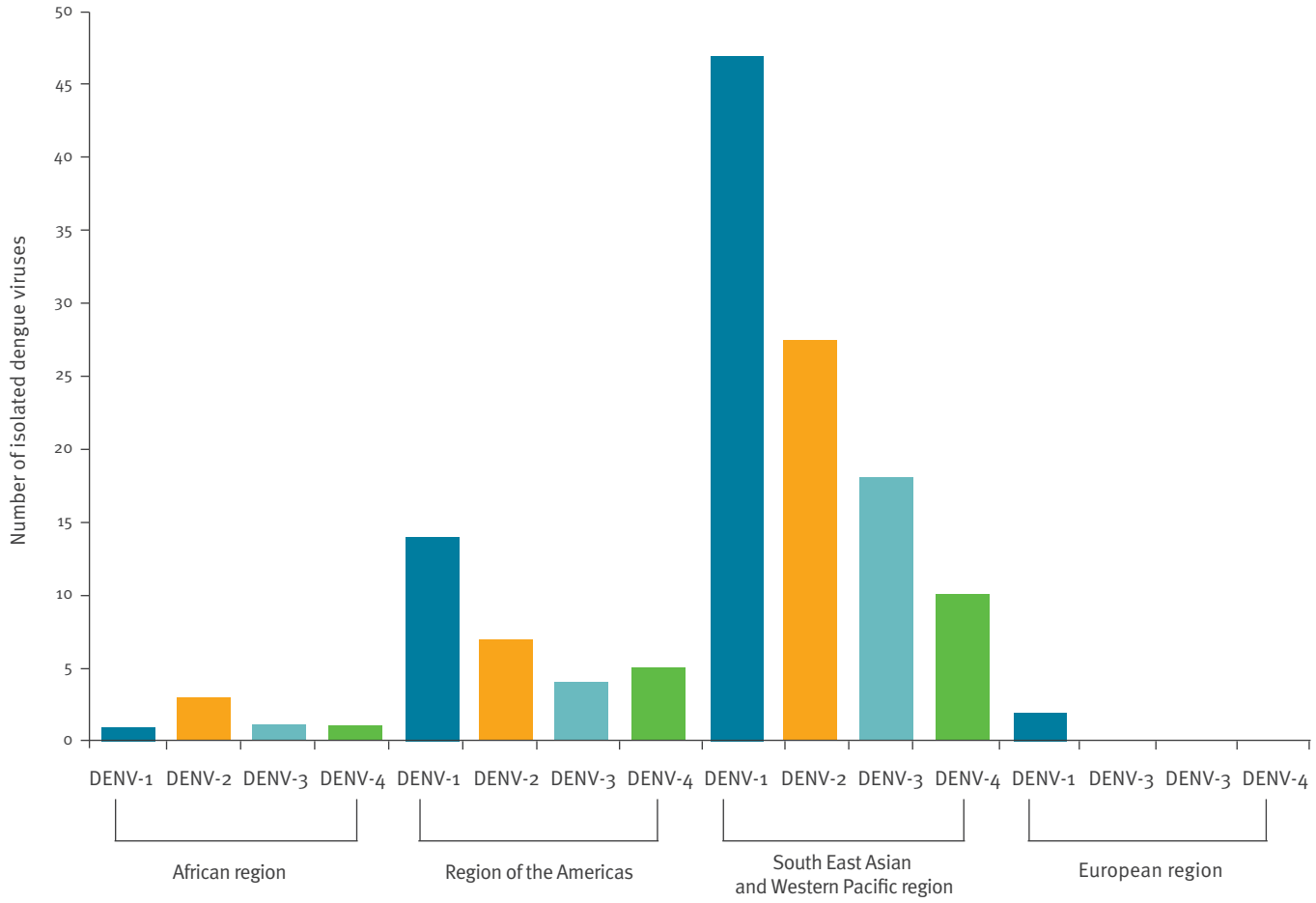
Of the 141 virus isolates that could be typed, DENV-1 was identified in 46% (n = 65), DENV-2 in 26% (n = 37), DENV-3 in 16% (n = 23) and DENV-4 in 11% (n = 16) of cases (Tables 2 and 3). All four dengue serotypes were imported by travellers, irrespective of which region they had visited (Figure 4). The two dengue infections acquired within the European region were diagnosed in travellers returning from Madeira during the local dengue outbreak in 2012–13 and were due to DENV-1 (Figure 4).

Overall, DENV-1 (n = 65) was the serotype most frequently detected in the study population. All isolated DENV-1 strains belonged to two of the five genotypes previously described [16-18]: genotype I (Asian) and genotype V (America-Africa). The most notable findings were:

Genotype I (Asian): All genotype I (Asian) virus strains (n = 42) were isolated from travellers returning from Asia. One virus was isolated from a traveller returning from Japan in September 2014; the case was linked to

Figure 4

Distribution of imported dengue virus serotypes, by WHO region, 2012–14 (n = 141)



a local outbreak with ca. 160 reported autochthonous cases affecting Tokyo from August to September 2014 [19].

Genotype V (America-Africa): As reported previously [14], genotype V (America-Africa) strains (n = 23) show a vast geographical distribution and we isolated virus strains from travellers returning from the Americas, Africa and Asia. Three cases were notable: two virus strains from travellers returning from Madeira during the dengue epidemic in 2012 and 2013 and one virus strain from a traveller returning from Angola during the dengue outbreak in 2013 [20].

The isolated DENV-2 strains (n = 37) grouped into three different genotypes that currently are of high epidemiological interest: genotypes America-Asia (n = 9), Cosmopolitan (n = 23) and Asian I (n = 5).

Genotype America-Asia: All strains were isolated from travellers returning from the Americas.
Genotype Cosmopolitan: We isolated two virus strains from travellers returning from Tanzania in 2014 at the time of an ongoing dengue outbreak in Dar es Salaam and neighbouring regions (personal communication: Boillat N, Sep 2015). The isolated virus strains clustered in a lineage different from the strains introduced to Africa in the early 1980s.

Four different genotypes of DENV-3 (n = 23) were detected during the study period: genotypes I (n = 7), II (n = 5), III (n = 8) and IV (n = 23), suggesting a broad expansion and diversity of circulating DENV-3 strains. The most notable findings were the isolation of two genotype III strains from travellers returning from Cuba in 2013 and 2014, suggesting epidemic circulation of these strains, and the isolation of a genotype III strain from a traveller returning from Burkina Faso in 2013, a region for which data on dengue endemicity are not available.

DENV-4 strains were the least frequently isolated virus strains in our study. All DENV-4 strains imported from the Americas (n = 16) belonged to genotype II, the main genotype circulating in the region since its introduction in 1982 [14]. All detected strains of DENV-4 from Asia (n = 16) were genotypes I (n = 7) and II (n = 9). Most notably, we isolated a genotype II strain from a traveller returning from Angola which showed 98% homology to strains currently circulating in Brazil, confirming previous data suggesting that the 2013 DENV-4 outbreak

Table 1

European imported dengue cases enrolled in the study, by country and travel profile, 2012–14 (n = 242)

	Number of cases	%
Enrolled cases by country		
Belgium	25	10.3
Germany	73	30.2
Italy	37	15.3
The Netherlands	13	5.4
Spain	74	30.6
Switzerland	20	8.3
Travel profile of cases		
Individual tourists	132	54.5
Package tourists	50	20.7
Visiting friends and relatives	29	12.0
Business travellers	23	9.5
European overseas residents/ expatriates	8	3.3

in Luanda was caused by a virus strain introduced from Brazil [21].

Discussion

The observed geographical pattern of the origin of imported dengue by international travellers was in line with previous reports from *TropNet* (1999–2001: South-east Asian/Western Pacific region: 53.4%, American region 36.5%, African region: 10.3%) [22] and data from the *GeoSentinel* network (2000–10: South-east Asian/ western Pacific region: 67%, American region: 28%; African region: 5%) [23].

The peak of imported dengue cases observed in 2013, compared with 2012 and 2014, was mainly attributable to the increase in cases imported from the Americas and South-east Asia and is in line with the isochronal epidemiological trend observed in these regions: The Pan American Health Organisation (PAHO) reported 1,120,902 cases in 2012, 2,386,836 cases in 2013 and 1,176,529 cases in 2014 which occurred in the American region [24]. Although neither the WHO figures for the South-east Asian region nor the official figures from Thailand (which accounts for the majority of dengue cases imported from the South-east Asian region to Europe) were traceable, accessible online media sources reported that in 2013, Thailand experienced its worst dengue epidemic in more than two decades [25], followed by a significant decline in cases in 2014 [26]. From 2012 to 2014, the overall geographical pattern of the origin of imported dengue to Europe remained unchanged and the importation pattern over the years appears to match the epidemiological situation in endemic regions. However, it should be kept in mind that the absolute numbers of dengue infections in travellers returning from different destinations primarily reflect the popularity of travel destinations and cannot provide incidence rates or an assessment of infection risk, as data on the exact number of travellers to the different regions (denominator) are not available.

Among the imported dengue cases, three travellers (1.2%) had visited two non-neighbouring WHO regions during the possible incubation period, highlighting the potential role of international travellers in transcontinental spread of DENV strains. This is corroborated by the DENV strains isolated from two of these three travellers: The virus isolated from a patient who travelled from Indonesia to Peru points to Indonesia as the most likely place of acquisition and the identified genotype has not yet been known to circulate in Peru. The virus isolate from the patient who travelled from Malaysia to Brazil points to Brazil as the most likely place of acquisition. (Note: the detailed phylogenetic analysis of all DENV isolated within the framework of this study is envisaged but currently pending).

When looking at the number of dengue cases enrolled into the study over time, no seasonal pattern of importation was detected. However, the number of enrolled cases may have been insufficient to see a seasonal trend. The European Centre for Disease Prevention and Control (ECDC) reports a seasonal trend of imported dengue cases in Europe, increasing during the summer and autumn months (June–October) and peaking in August [27]. This may be explained by the European summer holiday season with the corresponding increase of international travel during this time of the year as well as the epidemiological peak of dengue cases at the major holiday destination South-east Asia from June to September [28]. Mathematical modelling of the likelihood of dengue importation to Europe (taking into account dengue seasonality in the countries from which dengue could be imported, the number of reported dengue cases imported into Europe and the volume of airline travellers arriving from dengue-affected areas internationally) concluded that the risk of dengue importation is greatest in August, September and October [29]. Entomological monitoring in the Mediterranean region indicated that the development period for *A. albopictus* starts in April and closes in October/November with activity peaks from June/July to September [30,31]. The peak activity of *A. albopictus* populations in the south of Europe thus coincides with the seasonal peak of imported dengue cases in Europe which increases the risk of autochthonous transmission [29,32]. Case reports of autochthonously acquired dengue in Croatia and the south of France in August and September in the past years support this prediction [7-9]. We found that more than a third of travellers who are infected with dengue in endemic regions either return to Europe during the acute phase of the infection or become symptomatic after returning back home. More than half of the patients presenting with acute dengue infection were viraemic when seeking medical care. If we equate viraemia with risk of transmission, we can conclude that at least 58% (141/242) of dengue patients presenting during the acute phase of infection

Table 2

Study participants with imported dengue virus infection (n = 673) and PCR-positive cases wit available virus sequence (n = 141), 2012–14

Recruitment of study participants and processing of samples	Number of cases
All dengue cases seen at the participating study sites during the study period	673
Laboratory-confirmed acute dengue cases presenting within ≤7 days after onset of symptoms to one of the study sites (=enrolled patients according to inclusion criteria)	242
PCR-positive acute dengue cases	160
Acute dengue cases where sequencing and virus isolation was successful	141

may pose a potential risk to initiate autochthonous transmission in vector-infested regions of Europe. Travellers returning to regions where no *A. albopictus* is prevalent will, even if viraemic, not pose any relevant risk for authochtonous transmission. The used cut-off of 7 days for study enrolment was a pragmatic decision and does not exclude that some patients may be viraemic beyond that period. Thus, the observed proportion of viraemic cases should be seen as a minimum. Of note, almost half of the included 242 cases were enrolled by *TropNet* sites in Spain (Barcelona) and Italy (Brescia, Torino and Verona) where *A. albopictus* is prevalent.

Worldwide surveillance of circulating DENV strains is crucial for the understanding of transmission patterns and for tracking the emergence and spread of virus strains (especially those with high epidemic potential) around the world. However, currently global surveillance data remain fragmentary. This is especially true for resource-poor regions where no or only limited local surveillance data are available. Sentinel surveillance of international travellers returning with DENV infections from such regions has been suggested as a valuable tool for filling these current data gaps [6,14] and the phylogenetic analysis of our isolated DENV strains confirms this: We detected all four DENV serotypes in travellers returning from Asia, the Americas and Africa and identified the main genotypes and epidemic lineages causing outbreaks and epidemics during the study period (the DENV-1 genotypes America-Africa and Asian, the DENV-2 genotypes Cosmopolitan and Asian I and the DENV-3 genotype III). We also picked up changes in DENV strain circulation, e.g. we isolated a genotype I Asia strain in a traveller returning from Indonesia in 2013 at the same time as local reports describing a shift from the predominantly circulating cosmopolitan genotype strains to Asian genotype strains [33].

For regions where currently only scarce or no regional surveillance data are available, virus characterisation revealed several interesting findings:

Firstly, although 1,430 (clinical) dengue cases were officially reported in Cuba in 2013 and 2,522 in 2014, no dengue serotypes have been reported to PAHO/WHO for those years [24]. In our study, more than 10 years later, we detected circulation of dengue serotype 3 (genotype III) in Cuba in 2013 and 2014. According to PAHO, other Hispanic Caribbean countries did not report this serotype in those years. The last detection of dengue 3 (genotype III) in Cuba was during a big epidemic in 2001 and 2002 [14,34].

Secondly, we identified dengue virus circulating in countries in Africa that have so far rarely reported dengue. We isolated a genotype III DENV-3 strain from a traveller returning from Togo and Burkina Faso, a region where, besides one recent case report of a genotype III DENV-3 infection in a returning German traveller [35], circulation of dengue had been unknown. We also identified a dengue virus strain imported during the dengue outbreak in Angola in 2013. In a previous report, the outbreak was thought to be due to an endemic virus strain that had been circulating in West Africa for many years [20]. However, our analysis points towards an importation of a dengue virus from Brazil, consistent with a report by researchers from Portugal [21]. Furthermore, we isolated DENV from travellers returning from Tanzania during the dengue outbreak affecting Dar es Salaam and neighbouring regions in 2014 [36]. The isolated virus strains clustered in a lineage different from the strains introduced in Africa in the early 1980s, suggesting recent introduction from Asia.

Thirdly, our sentinel surveillance picked up a dengue outbreak in Europe: the 2012/13 outbreak in Madeira, Portugal [10].

Finally, the genotype I (Asian) DENV-1 isolate we isolated from a traveller returning from Japan in September 2014 was linked to a local outbreak with ca. 160 reported autochthonous cases affecting Tokyo in August and September 2014 [19]. The only previous autochthonous transmission of dengue in Japan was reported in 2013, when a DENV-2 strain was isolated in a returning German traveller [37], although no concurrent local cases were reported at that time and autochthonous transmission of dengue had not been reported in Japan for 70 years [19]. Despite Japan's temperate

Table 3

Virological results, returning travellers with dengue virus infection, 2012–14 (n = 141)

Serotypes (n = 141)	n	%	Genotypes
DENV 1	65	46	I (Asian) and V (America-Africa)
DENV 2	37	26	America-Africa, Cosmopolitan and Asian I
DENV 3	23	16	I, II, III and IV
DENV 4	16	11	I and II

climate, increasing travel between Japan and dengue-endemic areas, combined with more suitable climate and environmental drivers for dengue transmission, have made such an outbreak possible [38].

In conclusion, our data demonstrate that epidemiological and virological data obtained from dengue-infected international travellers can add an important layer to global dengue surveillance efforts.

Conflict of interest

None declared.

Authors' contributions

Data analysis and writing of manuscript: Andreas Neumayr; Recruitment of study subjects: Andreas Neumayr, Jose Muñoz, Mirjam Schunk, Emmanuel Bottieau, Jakob Cramer, Guido Calleri, Rogelio López-Vélez, Andrea Angheben, Thomas Zoller, Leo Visser, Nuria Serre Delcor, Blaise Genton, Lina Tomasoni, Alberto Matteelli, Laurence Rochat, Elena Sulleiro, Florian Kurth, Federico Gobbi, Francesca Norman, Ilaria Torta, Jan Clerinx, David Poluda, Antonia Calvo-Cano, Miguel Martinez, Laboratory investigations: Leticia Franco, Maria Paz Sanchez-Seco, Marjan Van Esbroeck, Elena Sulleiro. Study design: Annelies Wilder-Smith, Christoph Hatz, Andreas Neumayr. Review of manucript: all authors.

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