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# Phylogenetic and molecular characteristics of Eurasian H9 avian influenza viruses and their detection by two different H9-specific RealTime reverse transcriptase polymerase chain reaction tests

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**Abstract**

Avian influenza viruses (AIVs) of the H9 haemagglutinin subtype are endemic in many Asian and Middle-East countries, causing mortality and morbidity in poultry. Consequently there is a need for accurate and sensitive detection of Eurasian H9 subtype viruses. Two H9 RealTime reverse transcriptase polymerase chain reaction (RRT-PCR) tests, developed by Monne *et al* (2008) and Ben Shabat *et al* (2010), were originally validated with a limited number of H9 specimens. In the present study, the two tests have been assessed using 66 diverse H9 isolates and 139 clinical specimens from six H9 poultry outbreaks in four geographically disparate Eurasian countries. The Monne *et al* (2008) test was modified and successfully detected all H9 viruses from all three Eurasian H9 lineages. Bayesian analysis of the clinical specimens' results revealed this test to be more sensitive (97%) than the Ben Shabat *et al* (2010) test (31%). The latter test detected most H9 isolates of the G1 lineage, but no isolates from other H9 lineages. Mismatches in the primer / probe binding sequences accounted for sensitivity differences between the two H9 RRT-PCRs. Genetic analysis of 34 sequenced H9 haemagglutinin genes showed the South Asian and Middle-East H9 isolates to belong to the H9 G1 lineage, and possessed residues that appear to preferably bind alpha 2,6-linked sialic acid receptors which indicate a potential for human infection. European H9s clustered phylogenetically in a broader geographical group that includes recent North American H9 wild bird isolates and contemporary Asian viruses in the Y439 H9 lineage.

**Key words:** avian influenza, H9, RRT-PCR, lineage, phylogeny, epidemiology

## Introduction

Avian influenza viruses (AIVs) are grouped into 16 subtypes (H1-H16) on the basis of the antigenic relatedness of the haemagglutinin (HA) surface glycoprotein (OIE, 2011). AIVs have a natural reservoir in wild birds (Alexander, 2007), and are primarily low pathogenicity (LP)AI viruses, although LPAIVs of H5 and H7 subtypes are important and cause notifiable AI (NAI) in poultry because they can mutate from LPAI to highly pathogenic (HP)AI in gallinaceous hosts (OIE, 2011).

H9N2 AIV infection was first reported in a turkey outbreak in the United States in 1966 (Homme and Easterday, 1970), but in Asia H9 viruses had been only reported in ducks until 1992 (Shortridge, 1992). Subsequently H9 AI infections in terrestrial poultry, mainly caused by H9N2 viruses, have been reported widely in China and other parts of Asia and the Middle-East (Alexander, 2007). Although H9 poultry infections are not classified as NAI, a decrease in egg laying may be observed in chickens, and coinfection with other pathogens can accentuate morbidity and result in mortality (Kishida *et al*, 2004). Widespread distribution of H9N2 viruses in poultry has been accompanied by reports of pig and human H9 virus infections in China and Hong Kong, and this potential zoonosis continues to cause public health concerns (Yu *et al* 2011).

Consequently, the epidemiology of H9N2 has attracted strong interest in Asia. Molecular phylogenetic studies of the HA gene have revealed three distinct lineages in the Far East, represented by the following prototype poultry isolates (i) A/quail/Hong Kong/G1/97 (G1-like), (ii) A/chicken/Beijing/1/94 or A/duck/Hong

Kong/Y280/97 (Y280-like) and (iii) A/duck/Hong Kong/Y439/97 (Y439-like or Korean-like) (Huang *et al* 2010, Lee *et al* 2007, Li *et al* 2003, Xu *et al* 2007). At the time of writing, the majority of full HA gene nucleotide sequences from Eurasian H9 AI virus isolates available in the sequence databases are of Chinese origin. One of the aims of this study was to sequence the full-length HA genes from a variety of different H9 isolates from other parts of Asia, including South Asia and the Middle-East. Full HA sequences were also obtained for European H9 isolates, which are presently underrepresented in the databases.

RealTime reverse transcription polymerase chain reaction (RRT-PCR) tests have gained acceptance for use in the diagnosis and investigation of notifiable veterinary viral diseases including AI outbreaks, where NAI caused by H5 and H7 subtypes are of greatest importance (Hoffmann *et al* 2009, Slomka *et al* 2010a). Provided that assay optimisation and validation have been carefully conducted, these RRT-PCRs are more rapid than and at least as sensitive as the "gold standard" of virus isolation (VI) in embryonated fowls' eggs (EFEs), and display a very high specificity. Monne *et al* (2008) described a RRT-PCR for the detection of H9 AI viruses, but validation was limited to four H9 laboratory isolates and 24 VI positive clinical specimens. Ben Shabat *et al* (2010) described an H9 RRT-PCR that was validated using recent Israeli H9 isolates, and claimed to be suitable for detection of other Eurasian H9 AIVs. The present study provided an opportunity to use additional Eurasian H9 isolates and clinical specimens to further validate these two H9 RRT-PCR tests.

## Methods

**Viruses.** Virus isolation (VI) was done by inoculating 9- to 10-day-old embryonated eggs from specific-pathogen free fowls according to standard procedures (OIE 2009). Sixty-six laboratory-grown H9 isolates that had been obtained between 1993 and 2011 from: China (n=7), South Asia (n=17), Middle-East (n=29) and Europe (n=13) (Table 1), and representatives of each of the other fifteen avian influenza HA subtypes (n=74) were grown in EFEs (Table S1).

**Clinical specimens from diseased chicken flocks.** Clinical specimens (104 swabs and 35 tissue specimens) were submitted to the EU and OIE Reference Laboratory at the Animal Health and Veterinary Laboratories Agency (AHVLA)-Weybridge, UK, from seven diseased chicken flocks following infection with H9 AIVs in four countries during 2010-2011 (Table S2): (i) Sixteen tracheal swabs from three premises in Nepal, two of which were submitted in November 2010 and one in March 2011. Each swab was stored in 1ml brain heart infusion broth containing antibiotics (BHIB). (ii) Thirty-two tracheal and 56 cloacal swabs available from two adjacent chicken sheds at a poultry premises in Norfolk, UK, in December 2010. (iii) Twenty-eight tissue samples (kidney, tracheal scrapings, lung and caecal tonsils) obtained from seven chickens in a diseased flock in Saudi Arabia during January 2010. These had been homogenised in BHIB and applied to FTA cards (Whatman, Maidstone, UK) for transport to AHVLA-Weybridge. (iv) Seven tissue pools from two flocks in the United Arab Emirates (UAE) submitted in 2011, each flock providing separate pools of trachea, caecal tonsils and viscera, with one flock providing an additional pool of viscera.

**Swabs obtained from healthy poultry and wild birds.** Clinical specimens (342 swabs and 18 feathers), which had tested negative by the matrix (M) gene RRT-PCR (below) were obtained from the following: (i) Turkeys: tracheal swabs (n=24), cloacal swabs (n=24) and feathers (n=15); (ii) chickens: tracheal swabs (n=23), cloacal swabs (n=23) and feathers (n=3); (iii) domestic ducks: tracheal swabs (n=88) and cloacal swabs (n=88); (iv) wild birds (mixed avian species, obtained from AI surveillance in the UK in 2010): tracheal swabs (n=36) and cloacal swabs (n=36). All swabs and feathers were placed in 1ml BHIB and agitated manually.

**RNA extraction.** RNA was extracted manually from 140µl volumes of infective allantoic fluid for laboratory grown AIV isolates (Slomka *et al* 2007). For swabs, feathers and 10% w/v homogenised tissues in BHIB, 140µl volumes of supernatant or homogenate were extracted robotically (Slomka *et al* 2009). RNA was extracted from tissues deposited on FTA cards by cutting a 2-3mm diameter section of each card, adding to 200µl DEPC-water (Ambion, Warrington, UK) and incubating at room temperature for 10 mins with occasional agitation, followed by robotic extraction of 140µl of the aqueous eluate.

**AI RRT-PCRs.** All RRT-PCRs were run on Mx3000 RealTime PCR instruments (Agilent Technologies, Wokingham, UK). M-gene RRT-PCR, originally described by Spackman *et al* (2002) was used for generic detection of all AI HA subtypes as described by Slomka *et al* (2009). The H9 RRT-PCR of Monne *et al* (2008) was modified by using the OneStep RT-PCR Kit (Qiagen, Crawley, UK) with magnesium chloride supplemented to 3.75mM and RNasin (Promega, Southampton, UK) at 4

units per reaction. However, the primer and probe sequences originally described by Monne *et al* (2008) remained unaltered. ROX passive reference dye (Agilent) was added as recommended by the manufacturer. The final concentration of each primer and probe was 0.40µM and 0.30µM respectively. The H9 RRT-PCR of Ben Shabat *et al* (2010) was as described using the AgPath-ID One-Step RT-PCR Kit (Applied Biosystems, Warrington, UK). Ct values were determined as positive (<36), indeterminate (36.01-39.99) or negative ("No Ct") (Slomka *et al* 2010a). The analytical sensitivity of each RRT-PCR was assessed by calculating the median egg infectious dose ($EID_{50}$) of isolate A/quail/Hong Kong/G1/97 (H9N2) according to recommended procedures (OIE 2009), and a ten-fold dilution series of extracted RNA tested by each AI RRT-PCR. The N1 RRT-PCR (Slomka *et al* 2012) was used as required.

**Statistical methods.** All 139 clinical specimens were tested by the M-gene and both H9 RRT-PCRs, and their Ct values compared by linear regression. These included all Ct values, including those in the indeterminate range. These results were then analysed by Bayesian modelling and for this purpose indeterminate Ct values were classed as negative together with "No Ct" results. This facilitated sensitivity estimations in the absence of a gold standard (Branscum *et al*., 2005), assuming conditional independence between the three RRT-PCRs and a specificity of 100% for each. For a Bayesian method priors need to be specified, these derive from prior knowledge of the tests in other experiments and will influence the final estimates of the unknown parameters. However, we assumed no previous knowledge of the test sensitivities, and so used non-informative priors, specified by beta distributions with both parameters equal to 1. The analysis was carried out in WinBUGS 3.1, using a

burn-in of 5,000 and 10,000 iterations to provide estimates of the sensitivity. The estimates of the sensitivity were provided from the median of the 5,000 iterations, and the uncertainty from the 2.5 and 97.5 percentiles, as is standard in Bayesian modelling.

**Conventional RT-PCRs, HA gene sequencing and phylogeny.** RNA was extracted extracted from undiluted H9 infective allantoic fluid of 34 AIV laboratory isolates (Table 1) plus one of the cloacal swabs from the UK 2010 H9N1 chicken outbreak (Table S2). cDNA was prepared using an influenza universal oligonucleotide primer, followed by conventional RT-PCR amplification using primers as outlined in Table S3. Sequencing was done using the Prism BigDye terminator v3.1 Cycle Sequencing Kit on the 3130 Genetic Analyser (Applied Biosystems, Warrington, UK) according to the manufacturer's instructions. The Lasergene package (DNAstar, Madison, Wisconsin, USA) was used for nucleotide sequence analysis. Full HA0 precursor glycoprotein sequences for the 34 H9 viruses are available with the GenBank Accession Numbers JQ609664, JX273537-JX273566, and JX273568-JX273570. Phylogenetic analyses were conducted as described (Slomka *et al* 2012). Putative N-glycosylation sites were identified using the Net Glycosylation software: http://www.cbs.dtu.dk/services/NetNGlyc/. Entropy plots were produced by using the BioEdit software (Hall 1999, available at: http://www.mbio.ncsu.edu/BioEdit/bioedit.html) to determine nucleotide sequence variability within the H9 RRT-PCR primer / probe binding sequences (van Boheemen *et al* (2012).

## Results

**HA sequencing and phylogeny.** The 34 H9 full-length gene sequences were trimmed to span 1525 nucleotides corresponding to positions 15-1539 inclusive in the pre-HA0 numbering of A/turkey/Wisconsin/1/66. This was to facilitate alignment with 36 H9 genes submitted previously to the public databases which included some incomplete HA sequences, and produced a phylogenetic tree which included prototype isolates of the three H9 lineages (Fig 1). The 25 Middle-East and South Asian H9 isolates clustered as G1 viruses. The one Chinese H9 isolate sequenced in this study, A/duck/Hunan/1/06, belonged to the Y280 group (Fig 1). The HA genes from the eight newly-sequenced European H9 viruses (1995-2011) clustered together in a broad phylogenetic grouping that included the Y439 viruses and North American H9 wild bird isolates (Fig 2).

**Modification to the H9 RRT-PCR of Monne *et al* (2008).** The H9 RRT-PCR as originally described by Monne *et al* (2008) was found to be insensitive. This was evident when testing a ten-fold dilution series of RNA extracted from A/turkey/Wisconsin/1/66, the prototype H9N2 isolate known to have perfect matching primer and probe binding sequences (Fig S1), for which detection by this H9 RRT-PCR was approximately 1000-fold less sensitive than detection by the M-gene RRT-PCR (data not shown). Sensitivity for this H9 RRT-PCR was restored by replacing the Qiagen "Quantitect" core chemistry described by Monne *et al* (2008) with the Qiagen "OneStep" RT-PCR kit, and adding additional magnesium chloride to a final concentration of 3.75mM (Spackman *et al* 2002, Slomka *et al* 2007, 2009 and 2010b). This modified H9 RRT-PCR then produced Ct values that were similar to those

generated by the M-gene RRT-PCR (Table 1), and will be described hereafter as the "Monne-mod H9 RRT-PCR".

**Range of Eurasian H9 AIVs detected by the two H9 RRT-PCRs.** Viral RNA extracted from sixty-six diverse Eurasian H9 isolates was diluted 100-1000 fold for testing by the two H9 RRT-PCRs and the generic M-gene RRT-PCR (Table 1). All were detected by the M-gene RRT-PCR and the Monne-mod H9 RRT-PCR (Table 1). However, the H9 RRT-PCR described by Ben Shabat *et al* (2010) (hereafter referred to as the "Ben Shabat H9 RRT-PCR") detected 16 of 17 H9 isolates from South Asia, 24 of 29 Middle-East H9 isolates and four of seven Chinese H9 isolates but none of the 13 European H9 isolates.

**Analytical sensitivity of the two H9 RRT-PCRs.** RNA was extracted from A/quail/Hong Kong/G1/97 (H9N2) infectious allantoic fluid of known infectivity titre ($EID_{50}$) and used to construct a ten-fold dilution series that was tested by the M-gene and the two H9 RRT-PCRs. Efficiencies in the range 90-110% were observed, which indicated optimal protocols, and the detection limit of the three RRT-PCRs was determined to be 10 $EID_{50}$/ml (prior to RNA extraction), which typically occurred at Ct 35-37, hence Ct values < 36 were considered to be positive for the three RRT-PCRs.

**Specificity of the two H9 RRT-PCRs:** Both H9 RRT-PCRs were used to test 74 non-H9 AIV isolates that included representatives of each of the other fifteen HA-subtypes that occur in avian species. None were detected by either of the H9 RRT-PCRs, but all were positive by M-gene RRT-PCR (Table S1). All AI-negative swabs

(n=342) and feathers (n=18) from turkeys, chickens, domestic ducks and wild birds gave "No Ct" results when tested by either of the H9 RRT-PCRs.

**Sensitivities of the H9 RRT-PCRs with clinical specimens from H9 chicken outbreaks.** Linear regression was used to compare each of the two H9 RRT-PCRs to the M-gene RRT PCR by plotting the Ct values obtained for the 139 clinical specimens (48 tracheal and 56 cloacal swabs, 28 individual and seven pooled tissue specimens, Table S2). The Monne-mod H9 test showed greater agreement with the M-gene RRT PCR than the Ben Shabat H9 test (Fig 2). Ct values were higher with the Ben Shabat H9 test in comparison to the M-gene test (Fig 2b). The linear regression was determined using clinical specimens that registered both positive and indeterminate Ct values, giving 88 and 22 Ct values for Figs 1a and 1b respectively. Fig 2 also includes negative RRT-PCR results shown as Ct=40, and these were excluded from the linear regression. Bayesian sensitivity estimates for the three RRT-PCRs showed that the M-gene and Monne-mod H9 RRT-PCRs with median values of 82% and 97% respectively had much higher sensitivities than the Ben Shabat H9 RRT-PCR for which the median value was 31% (Table 2). The Monne-mod H9 RRT-PCR was estimated to have a higher sensitivity than the M-gene RRT-PCR, although there was some overlap of the credible intervals (Table 2).

**Comparison of H9 RRT-PCRs with VI for the clinical specimens.** Extracted RNAs from 88 UK chicken swabs were the remaining available clinical specimens from an initial submission of 240 swabs from 120 sampled chickens. Initial testing included VI attempts using pools of five BHIB swab fluids, with a given pool restricted to five tracheal or cloacal swabs. None of the 48 pools gave a positive VI

result (Table S2). Successful VI for H9 AIVs were obtained from the Nepalese swabs (8/16) and the UAE tissue pools (7/7) (Table S2). The Monne-mod H9 RRT-PCR detected all 15 of the Nepalese and UAE VI positives plus two VI negatives, while one VI negative was indeterminate by this H9 RRT-PCR (Table S2). The Ben Shabat RRT-PCR detected all eight Nepalese VI positive swabs, but failed to detect any of the H9 seven UAE VI positive tissue pools. The 28 Saudi H9 specimens from 2010 were submitted on FTA cards and therefore were unsuitable for VI testing as application of AI infectious specimens to FTA cards appeared to eliminate infectivity.

**H9 RRT-PCRs: Mismatches in primer / probe binding regions.** For the Monne-mod H9 RRT-PCR, the primer and probe binding sequences are perfectly conserved in 23 of the 34 sequenced H9 viruses (Fig S1), and the low sequence variability in European and G1 H9 viruses is evident from the corresponding entropy plots (Fig 3). In contrast, the primer and probe binding sites for the Ben Shabat H9 RRT-PCR were perfectly conserved in only four of the 34 sequenced H9 viruses (Fig S2), with sequence variability more apparent than for the Monne-mod RRT-PCR (Fig 3). The mismatches in the primer / probe binding region for the Ben Shabat H9 RRT-PCR appeared to account for its failure to detect any of the 13 European H9 isolates (Table 1) and its poor sensitivity with the UK 2010 H9N1 swab specimens (Table S2). The 25 sequenced HA genes from the G1 H9 isolates plus the G1 prototype isolate A/quail/Hong Kong/G1/97 also demonstrated variability in the primer / probe binding sequences (Fig 3), but these may have occurred at less critical nucleotide positions (Fig S2). This was reflected in the successful detection of 24 of 29 Middle-East and 16 of 17 South Asian H9 isolates by the Ben Shabat H9 RRT-PCR, although several did display elevated Ct values compared to the other two RRT-PCRs (Table 1). Three

of seven Chinese H9 isolates were not detected by the Ben Shabat H9 RRT-PCR, but were positive by the Monne-mod H9 RRT-PCR (Table 1). Again, sequencing of the HA gene of one Chinese isolate (Y 280-like) and examination of the respective primer / probe binding regions appeared to account for the difference between the two H9 tests (Figs S1 and S2).

**Amino acid residues at key positions in the H9 gene.** The cleavage site (CS) connecting peptide between the HA1 and HA2 domains (326-329, H3 numbering) was RSSR in 18 and KSSR in five G1 isolates (Table 3). The RSSR motif occurred in 11 poultry isolates from the Middle-East and seven from South Asia, while the KSSR motif occurred in one Middle-East isolate and four obtained from Bangladesh and Nepal between 2007 and 2011 (Table 3). The one newly-sequenced Y280 isolate, A/duck/Hunan/1/06, also possessed the RSSR CS. The eight European H9 AIVs, however, possessed quite different CS motifs with only one basic amino acid. These were ASDR, present in four of the more recent viruses from 2002-2010, and similar motifs were observed in the other sequenced European H9 AIVs (Table 3).

Amino acid positions that have been shown to influence binding of the influenza HA to cell receptors were examined in the 34 newly-sequenced H9 isolates. The Q226 residue in the receptor binding site (RBS) was observed in all eight European H9 AIVs and in one of the Middle-East G1 viruses (Table 3). Thirteen Middle-East and nine South Asian G1 viruses plus the Y280 H9 AIV differed in possessing L226. Two G1 isolates from Nepal (2010) possessed the chemically similar I226 residue (Table 3). Amino acid variation was observed at other residues associated with the

RBS (Table 3), in particular there was a high degree of heterogeneity at position 190 of the G1 viruses.

Thirty-one of the newly sequenced H9 AIVs possessed seven predicted N-glycosylation sites, and three possessed eight. All 25 G1 isolates possessed seven conserved N-glycosylation sites (Table 3). For the eight European H9 AIVs, six possessed a different pattern of seven glycosylation sites, while an eighth N-glycosylation site occurred at residue 273 in A/duck/Italy/260-V04/04 and residue 95 in A/chicken/England/51184/10. The Y280 isolate A/duck/Hunan/1/06 had a distinct pattern of eight N-glycosylation sites (Table 3).

## Discussion

H9 AIV is endemic in poultry in many parts of Asia and the Middle-East where outbreaks can include a considerable degree of morbidity, sometimes with mortality. Consequently, there is a need for optimised and validated molecular tests for the sensitive, specific and rapid diagnosis of H9 outbreaks in poultry. This study included a detailed validation of two H9 RRT-PCRs, which had been described previously and validated with a limited number of specimens (Monne *et al* 2008, Ben Shabat *et al* 2010). The Monne-mod H9 RRT-PCR was shown to be able to detect all the 66 H9 Eurasian isolates obtained during 1993-2011, whereas the Ben Shabat H9 RRT-PCR was unable to detect any of the 13 European H9 isolates and failed to detect five Middle-East H9 isolates and one South Asian isolate (Table 1). Linear regression analysis (Fig 1) and Bayesian modelling were employed to evaluate each of the H9 RRT-PCRs alongside the previously validated M-gene RRT-PCR in testing 139 clinical specimens from H9 outbreaks in four countries submitted in 2010 and 2011. This showed the Monne-mod H9 RRT-PCR to be more sensitive (97%) than the Ben Shabat H9 RRT-PCR (31%) (Table 2).

Testing of 360 known AI negative clinical specimens (*ie* negative by the M-gene RRT-PCR) had demonstrated 100% specificity for both H9 RRT-PCRs, and thereby provided the known specificity prior that is necessary for the Bayesian estimate of sensitivity (Table 2). The Bayesian model was previously used to estimate the sensitivity of three AI RRT-PCRs by testing clinical specimens from H5N1 HPAI infected chickens and ducks in Vietnam (Slomka *et al* 2012), and has the advantage of not requiring comparison to a defined gold standard. Earlier validations have

compared AI RRT-PCR performance to VI on populations of clinical specimens to determine relative sensitivity and specificity values, but the superior sensitivity of an optimised AI RRT-PCR compared to VI may give the erroneous impression that the former has a low specificity (Slomka *et al* 2009). Bayesian modelling circumvents the need for calculating sensitivity relative to VI, and indeed in the present study only 23 clinical specimens were available for VI testing (Table S2).

Entropy plots showed the primer / probe binding sites of the Monne-mod H9 RRT-PCR to be highly conserved among both European and G1 H9 viruses, while noteable sequence variation was observed in the corresponding regions of the Ben Shabat H9 RRT-PCR (Fig 3). The Monne-mod H9 RRT-PCR amplifies within the highly-conserved HA2 portion of the HA gene, whereas the Ben Shabat H9 RRT-PCR uses the more variable HA1 region, and this appears to have compromised the latter's performance in testing the diverse range of H9 specimens.

The M-gene RRT-PCR has been validated for global detection of all AI subtypes (Spackman *et al* 2002) and was included as part of the validation of the two H9 RRT-PCRs. However, using Bayesian analysis it was estimated that the sensitivity for the M-gene RRT-PCR in testing the 139 clinical specimens was 82% (Table 2). Amongst the 139 clinical specimens were a number of low titre samples that registered Ct values close to either side of the Ct 36 cut-off value (Fig 1a). Seven of these were positive by Monne-mod H9 and indeterminate by M-gene RRT-PCRs, and were called negative by the latter test in the Bayesian analysis. These seven specimens may have affected the sensitivity estimate for the M-gene RRT-PCR.

Three Eurasian H9 phylogenetic lineages, based on HA gene sequences, have been previously identified in China, namely the G1, Y280 and Y439 lineages. Withdrawl of quail from live poultry markets in Hong Kong followed the first isolation of the prototype A/quail/Hong Kong/G1/97 H9N2 G1 virus, and may have contributed to this lineage almost disappearing from this part of China within 5 years (Choi *et al* 2004). However, G1 viruses continue to be identified in South Asia and the Middle-East (Aamir *et al* 2007, Iqbal *et al* 2009, Nagarajan *et al* 2009, Perk *et al* 2009, Fusaro *et al* 2011). Full-length HA sequencing and phylogeny in the present study confirmed the presence of G1 viruses in these regions.

H9 AIVs have been reported in European wild birds and occasionally in poultry (Alexander 2007), but little information is available concerning their epidemiology and phylogeny (Banks *et al.* 2000). A novel aspect of the present study was the sequencing of the full HA gene from eight European H9 isolates from poultry and wild birds spanning the period 1995-2011, as well as the UK 2010 H9N1 specimen from a chicken outbreak (Parker *et al.* 2012). Interestingly, these eight European H9 HA genes clustered within a broad grouping that included Asian H9 sequences from the Y439 lineage (Fig 1). These encompassed recent H9 isolates from Korea and Vietnam, as well as the HA genes of recent North American H9 virus isolates from wild birds. This grouping was distinct from the small number of North American H9 poultry isolates (Fig 1).

The deduced amino acid sequences from the HA gene of 21 of the G1 H9 viruses from the Middle-East and South Asia and the Y280 virus possessed an RSSR CS sequence. This motif has been reported for many other G1 and Y280 viruses (Table

3) and is believed to have evolved as a feature of H9 viruses that are adapted to terrestrial poultry (Choi *et al* 2004). For one Middle-East and four South Asian isolates the CS slightly differed with a KSSR motif. These included three from Nepal isolated during 2010-2011, which differed from the RSSR CS observed in two earlier 2009 Nepalese H9N2 isolates (Table 3). Recent H9N2 poultry viruses in Pakistan (2007 and 2008) also possessed the KSSR CS, while earlier H9 CS motifs in Pakistan (2005 and 2006) were exclusively RSSR (Iqbal *et al* 2009). Historically, KSSR CS motifs have been reported occasionally in Chinese H9N2 isolates (Liu *et al* 2003, Xu *et al* 2007, Zhang *et al* 2009). It is speculated that the KSSR CS motif may be becoming more frequent in South Asia as part of the continuing evolution of H9N2 G1 viruses infecting terrestrial poultry.

Four sequenced European H9 viruses from 1995-2011 contained an ASDR CS sequence (Table 3). The same CS motif was observed in a Y439-like H9N1 isolate A/duck/Shantou/1588/00 together with other similar motifs (consensus: A/V-S-N/D/G-R) in older Chinese H9 viruses isolated from ducks between 1976 and 1979 (Li *et al* 2003). The CS sequences in three other European H9 viruses of ISDR, ASDK and ASAR were also chemically similar to this consensus sequence, while the ASNR CS in A/turkey/Netherlands/11015452/11 had been previously observed in A/duck/Hong Kong/Y439/97 (Table 3). These CS sequences differ from the R/K-SSR motifs in the HA genes of the G1 viruses and the one Y280 virus sequenced in the current study, and may be characteristic of the Y439 lineage and related H9 viruses from non-Asian regions (Fig 1 and Table 3). Similar consensus sequences have been described for CS motifs in 27 H9N2 viruses isolated from chickens and ducks in Korean live poultry markets, and one H9N1 Y439 isolate (2006) possessed

the same ASDR sequence (Moon *et al* 2010) observed in four European H9 isolates (Table 3). The majority of characterised Eurasian H9 viruses are H9N2 subtypes from Asian regions, while European H9 viruses investigated in the present study had several different NA subtypes: two, from a chicken and a teal were H9N1, five, from mallards, turkeys and a chicken were H9N2, and one each were H9N8 (duck) and H9N9 (knot) (Fig 1). H9N8 and H9N9 isolates have also been reported from domestic geese in Italy in 2004 and from turkeys in France in 2003 (Alexander 2007).

One factor which relates to the adaptation of AIVs to humans (mammals) relates to the ability of the HA RBS to recognise different sialic acid cellular receptors. Possession of Q226 in the RBS determines a preference to infect avian cells, which are characterised by the presence of cell receptors that possess alpha 2,3-linked sialic acids. L226 shows a preference for cell receptors that posses alpha 2,6-linked sialic acids, which typically occur on mammalian cells although they are also in the chicken respiratory and intestinal tracts, which possess both types of linkages (Matrosovich *et al* 2001). In the present study, the majority of the sequenced G1 isolates from South Asia and the Middle-East possess L226, the exceptions being the chemically similar I226 in two isolates from Nepal (2010) and the avian-like Q226 in A/avian/Jordan/7-Y1/04 (Table 3). Q226 was observed in older Chinese H9 viruses isolated from ducks between 1976 and 1979 (Li *et al* 2003). However, subsequent to the spread of H9N2 among terrestrial poultry in China during the 1990s, H9N2 viruses with L226 emerged and have been observed frequently in more recent years, particularly within the Y280 lineage, which now appears to dominate epidemiologically in China (Xu *et al* 2007, Zhang *et al* 2009, Wu *et al* 2010). L226 in South Asian G1 H9 viruses have also been in observed in India (2003-2004, Nagarajan *et al* 2009) and Pakistan (2005-

2008, Iqbal *et al* 2009). Perk *et al* (2009) examined the HA genes from 46 H9N2 Israeli poultry isolates (2000-2005) and observed Q226 in 29 and L226 in 17. L226 has been reported in six of seven G1 lineage poultry isolates in the UAE (2000-2003; Aamir *et al* 2007).

In contrast, the eight sequenced European H9 viruses all possessed Q226, as did the prototype isolates for the Y439-like lineage, and the North American H9 wild bird isolates, which also clustered within this broad grouping (Fig 1 and Table 3). H9N2 outbreaks in Korea have attracted poultry concerns for several years, and chicken isolates from live poultry markets and slaughterhouses during 2005-2009 have all possessed Q226 (Moon *et al* 2010, Park *et al* 2011). Therefore the H9 Y439 lineage and its related viruses in other geographical regions maintain a RBS preference for avian-like receptors.

Examination of HA amino acid positions in the RBS together with the distribution of predicted glycosylation sites, suggests that certain amino acid combinations may be associated with particular circulating H9 lineages or possibly sublineages (Table 3). For example, the close relationship of the HA gene of the European H9N1 virus A/chicken/England/1415-51184/10 to those in four recent North American H9 wild bird isolates is apparent (Fig 1), and it has 93.6% nucleotide sequence similarity to the HA gene of isolate A/laughing gull/Delaware/5/03 (H9N1). With the exception of one amino acid difference in the CS motif, the four North American H9 wild bird isolates' obtained during 2003-2007 have deduced HA amino acid sequences with the same signatures across the RBS and associated residues, plus the same predicted glycosylation pattern as A/chicken/England/1415-51184/10 (Table 3). This

distinguished A/chicken/England/1415-51184/10 from other European H9 viruses, and strengthened the suggestion that its H9 gene was of North American H9 wild bird origin (Parker *et al* 2012).

## Conclusion

Although the H9 AIVs are classified as LP viruses, their association with morbidity and mortality in poultry in many parts of Asia and the Middle-East, together with a potential zoonotic threat, has resulted in considerable epidemiological interest. Validation of the Monne-mod H9 RRT-PCR in the present study showed that it is able to detect viruses that belong to all three Eurasian H9 lineages, including European H9 AIVs, which appear to be part of a broader global grouping that includes the Y439 lineage sensitively. A proven H9 RRT-PCR is a valuable tool not only for outbreak investigation, but as a sensitive and specific test that can rapidly identify H9 positive specimens meriting further molecular epidemiological investigations. Furthermore the current study underlines the importance of continuing validation of diagnostic tools using contemporary viruses especially when they relate to highly mutable taxa that are under constant selection pressures in nature.

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**Conflict of interest**

None of the authors have any personal or financial conflicts of interest relating to the outcomes of this study.

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**Figure captions**

**Fig 1: HA phylogenetic analysis of 70 H9 AIVs.**

There were a total of 1525 nucleotide positions in the final dataset. The 34 H9 viruses sequenced in this study are indicated in bold type and a black triangle.

**Fig 2. Linear regression plots (unbroken lines) of Ct values for M gene RRT-PCR in comparison to the (a) Monne-mod H9 (n=88) and (b) Ben Shabat H9 (n=22) RRT-PCRs in testing clinical specimens.**

**Fig 3. Entropy plots for the primer / probe binding sequences of both the H9 RRT-PCRs.**

Entropy demonstrates nucleotide variability at each position for the sequenced European (8 viruses, open bars) and G1 (26 viruses, filled bars / solid lines) H9 viruses that had been tested by both H9 RRT-PCRs (Figs S1 and S2). Lower-case nucleotides in the Ben Shabat F primer indicate three conserved positions among all eight European H9 viruses (therefore zero entropy), but all eight European H9 viruses are mismatched with this F primer at these positions (Fig S2).

**Table 1**

**Table 1: Ct values of H9 AIV isolates (n=66) tested by M gene and both H9 RRT-PCRs**

| H9s: All H9N2 unless stated | Isolate name | M gene RRT-PCR | Monne-mod H9 RRT-PCR | Ben Shabat H9 RRT-PCR |
|---|---|---|---|---|
| Prototype (North American) H9 AIV | | | | |
| H9N2 | A/turkey/Wisconsin/1/66* | 21.38 | 22.15 | No Ct |
| European H9 AIVs | | | | |
| **H9N2** | **A/chicken/Hungary/11-459/01** | 28.28 | 32.51 | No Ct |
| **H9N8** | **A/duck/Italy/260/04** | 28.22 | 25.62 | No Ct |
| **H9N2** | **A/mallard/England/7798-6499/06** | 26.37 | 23.97 | No Ct |
| **H9N9** | **A/knot/England/497/02** | 29.5 | 28.67 | No Ct |
| H9N2 | A/chicken/Hungary/11-461/01 | 27.13 | 26.54 | No Ct |
| H9Nx | A/turkey/Germany/726/96 | 23.73 | 21.83 | No Ct |
| **H9N2** | **A/turkey/Germany/EK 224/95** | 25.91 | 23.89 | No Ct |
| H9N2 | A/turkey/Germany/EK 235/95 | 25.45 | 23.6 | No Ct |
| H9Nx | A/avian other/Germany/95 | 26.39 | 24.75 | No Ct |
| H9N3 | A/mallard/Ireland/PV46b/93 | 22.29 | 23.46 | No Ct |
| **H9N1** | **A/teal/N.Ireland/14567-10-5257/07** | 28.98 | 24.01 | No Ct |
| H9N2 | A/mallard/Finland/Li13384/10 ~ | 16.86 | 17.66 | No Ct |
| **H9N2** | **A/turkey/Netherlands/11015452/11** | 22.57 | 24.7 | No Ct |
| Middle-East H9 AIVs | | | | |
| **H9N2** | **A/chicken/Saudi Arabia/582/05** | 25.07 | 23.32 | 23.01 |
| H9Nx | A/chicken/Saudi Arabia/Hse4/Tr/04 | 24.61 | 20.71 | 21.9 |
| H9N2 | A/chicken/Iraq/EKI 1/08 | 25.15 | 21.63 | 25.15 |
| **H9N2** | **A/chicken/Iraq/EKI 14/08** | 22.97 | 21.22 | 25.28 |
| H9N2 | A/chicken/Saudi Arabia/1BL15/06 | 26.47 | 25.33 | 26 |
| **H9N2** | **A/chicken/Saudi Arabia/2BL09/06** | 21.54 | 19.95 | 20.85 |
| **H9N2** | **A/Libya/RV35D/06** | 22.49 | 21.59 | 24.15 |
| H9N2 | A/Libya/RV35E/06 | 24.99 | 23.99 | 26.75 |
| H9N2 | A/chicken/Jordan/2/04 | 33.53 | 27.3 | 26.92 |
| **H9N2** | **A/avian/Jordan/7-Y1/04** | 31.26 | 24.06 | 24.27 |
| **H9N2** | **A/chicken/Kuwait/9/04** | 26.44 | 24.47 | No Ct |
| **H9N2** | **A/chicken/Israel/421201/ 04** | 30.29 | 23.75 | 25.25 |
| H9N2 | A/chicken/Israel/421203/ 04 | 29.9 | 23.51 | 24.68 |
| **H9N2** | **A/chicken/Lebanon/1080/04** | 28.36 | 23.47 | 24.77 |
| H9N2 | A/chicken/Saudi Arabia/A-36360/10 | 22.82 | 24.51 | 26.47 |
| H9N2 | A/chicken/Saudi Arabia/B-36361/10 | 25.59 | 26.82 | No Ct |
| **H9N2** | **A/chicken/Saudi Arabia/C-36362/10** | 22.52 | 23.89 | 28.62 |
| **H9N2** | **A/chicken/Saudi Arabia/D-36363/10** | 23.92 | 25.29 | No Ct |
| **H9N2** | **A/chicken/Saudi Arabia/E-36364/06** | 22.9 | 23.8 | 25.52 |
| **H9N2** | **A/chicken/Saudi Arabia/F-36365/06** | 20.87 | 22.77 | 22.53 |
| H9N2 | A/chicken/Saudi Arabia/G-36366/06 | 23.73 | 24.33 | 24.15 |
| H9N2 | A/chicken/Saudi Arabia/H-36367/06 | 26.81 | 22.98 | 22.04 |
| H9Nx | A/chicken/Saudi Arabia/36/99 | 23.24 | 23.69 | 24.51 |
| H9Nx | A/chicken/Saudi Arabia/01/99 | 24.59 | 23.88 | 23.73 |
| H9Nx | A/chicken/Saudi Arabia/42/99 | 24.4 | 24.52 | 25.83 |
| H9Nx | A/chicken/Iran/SL-1096-05/00 | 26.63 | 24.92 | 29.55 |
| H9Nx | A/chicken/Iran/SL-1096-12/00 | 25.57 | 23.98 | 29.31 |
| **H9Nx** | **A/chicken/Saudi Arabia/H29TR/11** | 22.92 | 25.84 | No ct |
| **H9Nx** | **A/chicken/UAE/F1P7/11** | 26.22 | 25.35 | No ct |
| South Asian H9 AIVs | | | | |
| H9N2 | A/chicken/Bangladesh/FDIL(S)-102/07 | 23.02 | 21.53 | 22.02 |
| **H9N2** | **A/chicken/Bangladesh/627/07** | 23.71 | 20.19 | 23.78 |
| **N9Nx** | **A/chicken/Bangladesh/FDIL(M)-112/07** | 23.03 | 21.26 | 21.94 |
| **H9N2** | **A/chicken/Pakistan/NARC-2434/06** | 23.6 | 20.95 | 22.73 |
| **H9N2** | **A/chicken/Pakistan/G-Karachi/03** | 25.75 | 25.5 | 25.32 |
| **H9Nx** | **A/chicken/Pakistan/47/03** | 23.33 | 24.95 | 23.99 |
| H9N2 | A/chicken/Pakistan/086/99 | 23.44 | 23.81 | 23.48 |
| **H9N2** | **A/chicken/India/3/03** | 22.58 | 23.14 | 22.05 |
| H9N2 | A/chicken/India/4/03 | 24.3 | 22.44 | 22.29 |
| **H9Nx** | **A/chicken/Nepal/2490/09** | 22.46 | 22.4 | 23.29 |
| H9Nx | A/chicken/Nepal/2491/09 | 22.63 | 22.5 | 22.78 |
| H9Nx | A/chicken/Nepal/2492/09 | 31.78 | 30.1 | 35.08 |
| **H9Nx** | **A/chicken/Nepal/2493/09** | 21.12 | 20.8 | 22.14 |
| H9Nx | A/chicken/Nepal/2494/09 | 22.99 | 22.3 | 22.59 |
| **H9Nx** | **A/chicken/Nepal/5cl/10** | 33.92 | 31.32 | 35.23 |
| **H9Nx** | **A/chicken/Nepal/6cl/10** | 29.24 | 26.87 | No Ct |
| **H9N2** | **A/chicken/Nepal/5430/11** | 24.91 | 24.03 | 29.19 |
| Chinese H9 AIVs | | | | |
| **H9N2** | **A/duck/Hunan/1/06** | 24.38 | 25.92 | No Ct |
| H9N2 | A/duck/Hunan/2/06 | 22.48 | 25.61 | No Ct |
| H9Nx | A/chicken/Shandong/1/07 | 23.34 | 22.63 | No Ct |
| H9N2 | A/quail/Hong Kong/G1/97* | 28.98 | 29.74 | 30.08 |
| H9Nx | A/chicken/China/428/99 | 24.4 | 23.92 | 26.88 |
| H9Nx | A/chicken/China/437/99 | 23.07 | 22.57 | 26.64 |
| H9Nx | A/chicken/China/440/99 | 27.41 | 27.22 | 29.06 |

**Bold-type** isolate name indicates HA gene sequenced in current study, * indicates previously sequenced HA

~ Sequence of A/mallard/Finland/Li13384/10 is forthcoming (Lindh *et al* , manuscript in preparation)

**Table 2.** Bayesian estimates of the sensitivity of three AI RRT-PCRs for detection of H9 avian influenza in clinical specimens

| AI RRT-PCR | Estimate from Bayesian model* | | |
|---|---|---|---|
| | Median | 2.5 percentile | 97.5 percentile |
| M gene | 0.82 | 0.71 | 0.91 |
| H9 Monne-mod | 0.97 | 0.82 | 0.99 |
| H9 Ben Shabat | 0.31 | 0.20 | 0.44 |

* Determined from the results of testing 139 clinical specimens (Table S2), where indeterminate Ct values (36.01-39.99) were classified as negative.

# Table 3

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**Table 3. Amino-acids of interest in the haemagglutinin peptide sequence of H9 viruses**

| Lineage | H9 isolate / virus name | CS motif | Receptor binding site (RBS) | | | | | | | | | Glycosylation sites | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | H9 pre-HA0 numbering | 335-338 | 191 | 198 | 202 | 232 | 233 | 234 | 235 | 149 | 150 | 29 | 105 | 141 | 218 | 282 | 298 | 305 | 313 | 492 | 551 |
| | H3 numbering: | 326-329 | 183 | 190 | 194 | 224 | 225 | 226 | 227 | 137 | 138 | 21 | 95 | 129 | 210 | 273 | 289 | 296 | 304 | 483 | 541 |
| **North American poultry** | **A/turkey/Wisconsin/1/66** | VSSR | H | E | L | N | G | Q | Q | R | A | NST | - | NVT | NRT | - | NTT | NIS | - | NGT | NGS |
| **Y439 or Korean-like** | **A/duck/Hong Kong/Y439/97** | ASNR | H | E | L | N | D | Q | Q | R | A | NST | - | NVT | NRT | - | NTT | NVS | - | NGT | ~ |
| | **A/chicken/Korea/S1/03** | ASGR | H | E | L | N | G | Q | Q | K | A | NST | - | NVT | NRT | - | NTT | NVS | - | NGT | NGS |
| | A/chicken/Hungary/11-459/01 | ISDR | H | E | L | N | G | Q | Q | K | A | NST | - | NVT | NRT | - | NTT | NVS | - | NGT | NGS |
| | A/duck/Italy/260/04 | ASDR | H | E | L | N | G | Q | Q | K | A | NST | - | NVT | NRT | NSS | NTT | NVS | - | NGT | NGS |
| | A/mallard/England/7798-6499/06 | ASDR | H | E | L | N | G | Q | Q | K | A | NST | - | NVT | NRT | - | NTT | NVS | - | NGT | NGS |
| | A/knot/England/497/02 | ASDR | H | E | L | N | G | Q | Q | K | A | NST | - | NVT | NRT | - | NTT | NVS | - | NGT | NGS |
| | A/teal/Northern Ireland/14567-10-5257/07 | ASDR | H | E | L | N | G | Q | Q | K | A | NST | - | NVT | NRT | - | NTT | NVS | - | NGT | NGS |
| | A/chicken/England/1415-51184/10 | ASDK | H | E | L | K | G | Q | Q | A | A | NST | NGT | NVT | NRT | - | NTT | NVS | - | NGT | NGS |
| | A/turkey/Germany/EK224/95 | ASAR | H | E | L | N | G | Q | Q | S | A | NST | - | NVT | NRT | - | NTT | NVS | - | NGT | NGS |
| | A/turkey/Netherlands/11015452/11 | ASNR | H | E | L | N | G | Q | Q | R | A | NST | - | NVT | NRT | - | NTT | NVS | - | NGT | NGS |
| Other previously-sequenced H9 viruses related to Y439 | A/duck/Vietnam/OIE-2313/09 | VSGR | H | E | L | N | G | Q | Q | A | A | NST | - | NVT | NRT | - | NTT | NVS | - | NGT | NGS |
| | A/chicken/Korea/HC0410/09 | TSGR | H | E | L | N | G | Q | Q | K | A | NST | - | NVT | NRT | - | NTT | NVS | - | NGT | NGS |
| | A/shorebird/Delaware/554/07 | ASDR | H | E | L | K | G | Q | Q | A | A | NST | NGT | NVT | NRT | - | NTT | NVS | - | NGT | NGS |
| | A/laughing gull/Delaware/12/06 | ASDR | H | E | L | K | G | Q | Q | A | A | NST | NGT | NVT | NRT | - | NTT | NVS | - | NGT | NGS |
| | A/laughing gull/Delaware/5/03 | ASDR | H | E | L | K | G | Q | Q | A | A | NST | NGT | NVT | NRT | - | NTT | NVS | - | NGT | NGS |
| | A/common murre/Oregon/19497-004/05 | ASDR | H | E | L | K | G | Q | Q | A | A | NST | NGT | NVT | NRT | - | NTT | NVS | - | NGT | NGS |
| | A/teal/Primorie/3628/02 | ASGR | H | E | L | N | G | Q | Q | K | A | NST | - | NVT | NRT | - | NTT | NVS | - | NGT | NGS |
| **chicken/Beijing/94 or Y280-like** | **A/duck/Hong Kong/Y280/97** | RSSR | N | T | L | N | G | L | Q | K | A | NST | - | NVS | NRT | - | NTT | NVS | - | NGT | ~ |
| | **A/chicken/Beijing/1/94** | RSSR | N | V | L | N | G | Q | Q | K | A | NST | - | NVT | NRT | - | NTT | NVS | - | NGT | NGS |
| | A/duck/Hunan/1/06 | RSSR | N | A | L | N | G | L | Q | K | A | NST | - | NVS | NRT | - | NTT | NVS | NCS | NGT | NGS |
| **G1-like** | **A/quail/Hong Kong/G1/97** | RSSR | H | E | L | N | D | L | Q | R | A | NST | NGT | NVT | NRT | - | NST | NIS | - | NGT | NGS |
| Middle-East | A/chicken/Saudi Arabia/582/05 | RSSR | H | T | L | N | G | L | I | K | S | NST | NGT | NVT | - | - | NST | NIS | - | NGT | NGS |
| | A/chicken/Iraq/EKI 14/08 | RSSR | H | A | L | N | G | L | I | K | S | NST | NGT | NVT | - | - | NST | NIS | - | NGT | NGS |
| | A/chicken/Saudi Arabia/2BL09/06 | RSSR | H | A | L | N | G | L | I | K | S | NST | NGT | NVT | - | - | NST | NIS | - | NGT | NGS |
| | A/avian/Libya/RV35D/06 | RSSR | H | T | L | N | G | L | I | K | S | NST | NGT | NVT | - | - | NST | NIS | - | NGT | NGS |
| | A/avian/Jordan/7-Y1/04 | RSSR | H | A | L | N | G | Q | Q | K | A | NST | NGT | NVT | - | - | NST | NIS | - | NGT | NGS |
| | A/chicken/Kuwait/9/04 | KSSR | H | A | L | N | G | L | I | K | S | NST | NGT | NVT | - | - | NST | NIS | - | NGT | NGS |
| | A/chicken/Israel/421201/ 04 | RSSR | H | A | L | N | G | L | Q | K | A | NST | NGT | NVT | - | - | NST | NIS | - | NGT | NGS |
| | A/chicken/Lebanon/1080/04 | RSSR | H | A | L | N | G | L | Q | K | A | NST | NGT | NVT | - | - | NST | NIS | - | NGT | NGS |
| | A/chicken/Saudi Arabia/C-36362/10 | RSSR | H | A | L | N | G | L | I | K | S | NST | NGT | NVT | - | - | NST | NIS | - | NGT | NGS |
| | A/chicken/Saudi Arabia/D-36363/10 | RSSR | H | A | L | N | G | L | I | K | S | NST | NGT | NVT | - | - | NST | NIS | - | NGT | NGS |
| | A/chicken/Saudi Arabia/E-36364/06 | RSSR | H | V | L | N | G | L | I | K | S | NST | NGT | NVT | - | - | NST | NIS | - | NGT | NGS |
| | A/chicken/Saudi Arabia/F-36365/06 | RSSR | H | A | L | N | G | L | I | K | S | NST | NGT | NVT | - | - | NST | NIS | - | NGT | NGS |
| | A/chicken/Saudi Arabia/H29TR/11 | RSSR | H | V | L | N | G | L | I | K | S | NST | NGT | NVT | - | - | NST | NIS | - | NGT | NGS |
| | A/chicken/UAE/F1P7/11 | RSSR | H | A | L | N | G | L | L | K | S | NST | NGT | NVT | - | - | NST | NIS | - | NGT | NGS |
| South Asia | A/chicken/Bangladesh/627/07 | KSSR | H | A | L | N | G | L | I | K | S | NST | NGT | NVT | - | - | NST | NIS | - | NGT | NGS |
| | A/chicken/Bangladesh/FDIL(M)-112/07 | RSSR | H | A | L | N | G | L | I | K | S | NST | NGT | NVT | - | - | NST | NIS | - | NGT | NGS |
| | A/chicken/Pakistan/NARC-2434/06 | RSSR | H | A | L | N | G | L | I | K | S | NST | NGT | NVT | - | - | NST | NIS | - | NGT | NGS |
| | A/chicken/Pakistan/G-Karachi/03 | RSSR | H | A | L | N | G | L | I | K | S | NST | NGT | NVT | - | - | NST | NIS | - | NGT | NGS |
| | A/chicken/Pakistan/47/03 | RSSR | H | A | L | N | G | L | I | K | S | NST | NGT | NVT | - | - | NST | NIS | - | NGT | NGS |
| | A/chicken/India/3/03 | RSSR | H | A | L | N | G | L | I | K | S | NST | NGT | NVT | - | - | NST | NIS | - | NGT | NGS |
| | A/chicken/Nepal/2490/09 | RSSR | H | A | L | N | G | L | I | K | S | NST | NGT | NVT | - | - | NST | NIS | - | NGT | NGS |
| | A/chicken/Nepal/2493/09 | RSSR | H | A | L | N | G | L | I | K | S | NST | NGT | NVT | - | - | NST | NIS | - | NGT | NGS |
| | A/chicken/Nepal/5cl/10 | KSSR | N | V | L | N | G | I | I | K | S | NST | NGT | NVT | - | - | NST | NIS | - | NGT | NGS |
| | A/chicken/Nepal/6cl/10 | KSSR | N | V | L | N | G | I | I | K | S | NST | NGT | NVT | - | - | NST | NIS | - | NGT | NGS |
| | A/chicken/Nepal/5430/11 | KSSR | N | A | L | N | G | L | I | K | S | NST | NGT | NVT | - | - | NST | NIS | - | NGT | NGS |

All H9 viruses were sequenced in this study except where stated, with previously-sequenced prototype viruses of the H9 lineages indicated in **bold,** ~ aminoacid at this position unknown due to insufficient length of deposited sequence.

**Figure 1**

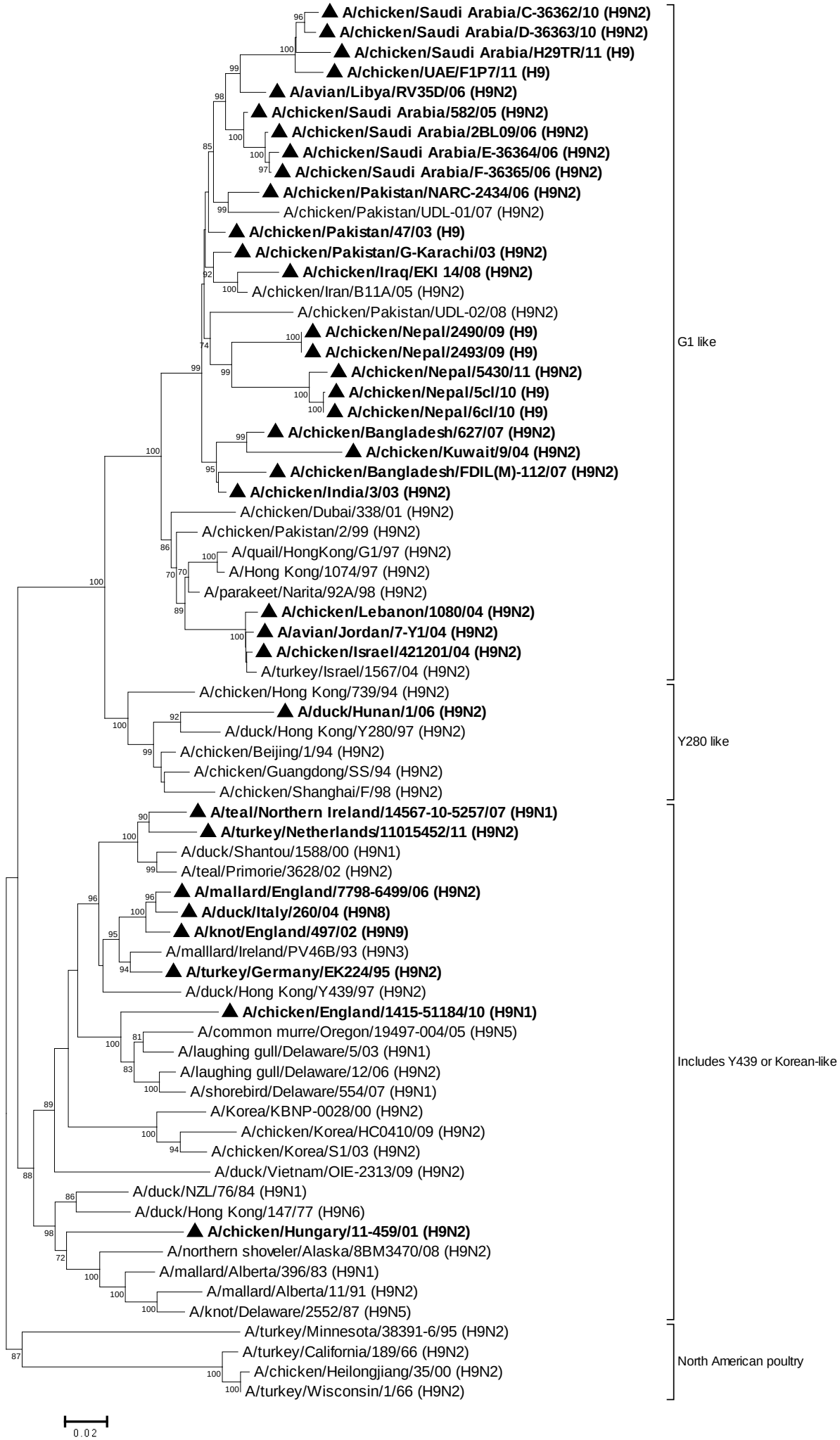
A/chicken/Saudi Arabia/C-36362/10 (H9N2)
A/chicken/Saudi Arabia/D-36363/10 (H9N2)
A/chicken/Saudi Arabia/H29TR/11 (H9)
A/chicken/UAE/F1P7/11 (H9)
A/avian/Libya/RV35D/06 (H9N2)
A/chicken/Saudi Arabia/582/05 (H9N2)
A/chicken/Saudi Arabia/2BL09/06 (H9N2)
A/chicken/Saudi Arabia/E-36364/06 (H9N2)
A/chicken/Saudi Arabia/F-36365/06 (H9N2)
A/chicken/Pakistan/NARC-2434/06 (H9N2)
A/chicken/Pakistan/UDL-01/07 (H9N2)
A/chicken/Pakistan/47/03 (H9)
A/chicken/Pakistan/G-Karachi/03 (H9N2)
A/chicken/Iraq/EKI 14/08 (H9N2)
A/chicken/Iran/B11A/05 (H9N2)
A/chicken/Pakistan/UDL-02/08 (H9N2)
A/chicken/Nepal/2490/09 (H9)
A/chicken/Nepal/2493/09 (H9)
A/chicken/Nepal/5430/11 (H9N2)
A/chicken/Nepal/5cl/10 (H9)
A/chicken/Nepal/6cl/10 (H9)
A/chicken/Bangladesh/627/07 (H9N2)
A/chicken/Kuwait/9/04 (H9N2)
A/chicken/Bangladesh/FDIL(M)-112/07 (H9N2)
A/chicken/India/3/03 (H9N2)
A/chicken/Dubai/338/01 (H9N2)
A/chicken/Pakistan/2/99 (H9N2)
A/quail/HongKong/G1/97 (H9N2)
A/Hong Kong/1074/97 (H9N2)
A/parakeet/Narita/92A/98 (H9N2)
A/chicken/Lebanon/1080/04 (H9N2)
A/avian/Jordan/7-Y1/04 (H9N2)
A/chicken/Israel/421201/04 (H9N2)
A/turkey/Israel/1567/04 (H9N2)
G1 like
A/chicken/Hong Kong/739/94 (H9N2)
A/duck/Hunan/1/06 (H9N2)
A/duck/Hong Kong/Y280/97 (H9N2)
A/chicken/Beijing/1/94 (H9N2)
A/chicken/Guangdong/SS/94 (H9N2)
A/chicken/Shanghai/F/98 (H9N2)
Y280 like
A/teal/Northern Ireland/14567-10-5257/07 (H9N1)
A/turkey/Netherlands/11015452/11 (H9N2)
A/duck/Shantou/1588/00 (H9N1)
A/teal/Primorie/3628/02 (H9N2)
A/mallard/England/7798-6499/06 (H9N2)
A/duck/Italy/260/04 (H9N8)
A/knot/England/497/02 (H9N9)
A/malllard/Ireland/PV46B/93 (H9N3)
A/turkey/Germany/EK224/95 (H9N2)
A/duck/Hong Kong/Y439/97 (H9N2)
A/chicken/England/1415-51184/10 (H9N1)
A/common murre/Oregon/19497-004/05 (H9N5)
A/laughing gull/Delaware/5/03 (H9N1)
A/laughing gull/Delaware/12/06 (H9N2)
A/shorebird/Delaware/554/07 (H9N1)
A/Korea/KBNP-0028/00 (H9N2)
A/chicken/Korea/HC0410/09 (H9N2)
A/chicken/Korea/S1/03 (H9N2)
A/duck/Vietnam/OIE-2313/09 (H9N2)
A/duck/NZL/76/84 (H9N1)
A/duck/Hong Kong/147/77 (H9N6)
A/chicken/Hungary/11-459/01 (H9N2)
A/northern shoveler/Alaska/8BM3470/08 (H9N2)
A/mallard/Alberta/396/83 (H9N1)
A/mallard/Alberta/11/91 (H9N2)
A/knot/Delaware/2552/87 (H9N5)
Includes Y439 or Korean-like
A/turkey/Minnesota/38391-6/95 (H9N2)
A/turkey/California/189/66 (H9N2)
A/chicken/Heilongjiang/35/00 (H9N2)
A/turkey/Wisconsin/1/66 (H9N2)
North American poultry
0.02

**Fig 2. HA phylogenetic analysis of seventy H9 AIVs**

There were a total of 1525 nucleotide positions in the final dataset. The 34 H9 viruses sequenced in this study are indicated in bold type and a black triangle.

**Figure 2**

Fig 2. Linear regression plots (unbroken lines) of Ct values for M gene RRT-PCR in comparison to the (a) Monne-mod H9 (n=88) and (b) Ben Shabat H9 (n=22) RRT-PCRs in testing clinical specimens

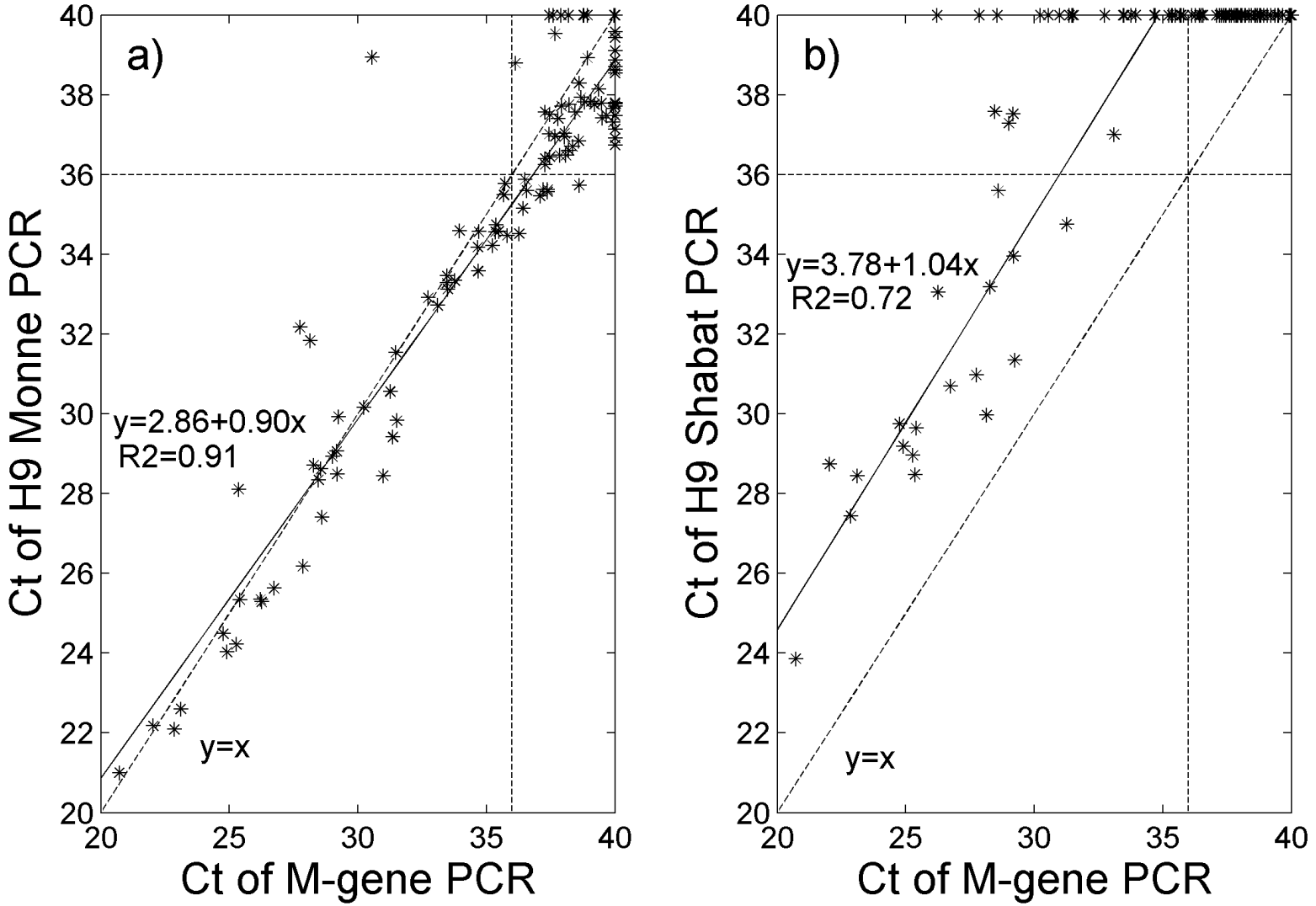

Figure 3

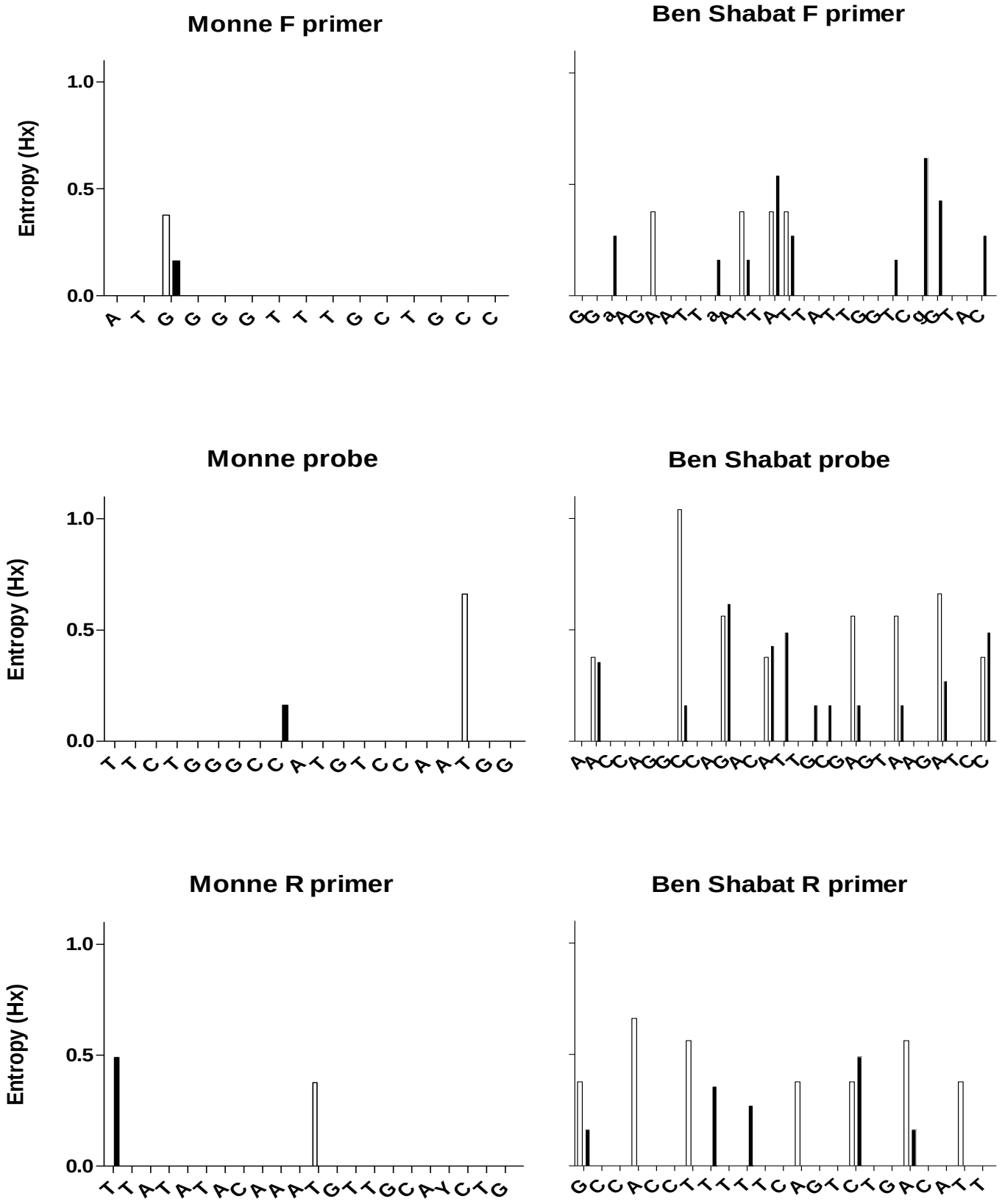


**Fig 3: Entropy plots for the primer / probe binding sequences of both the H9 RRT-PCRs.**
Entropy demonstrates nucleotide variability at each position for the sequenced European (8 viruses, open bars) and G1 (26 viruses, filled bars / solid lines) H9 viruses that had been tested by both H9 RRT-PCRs (Figs S1 and S2). Lower-case nucleotides in the Ben Shabat F primer indicate three conserved positions among all eight European H9 viruses (therefore zero entropy), but all eight European H9 viruses are mismatched with this F primer at these positions (Fig S2).

## Optional e-only supplementary files

**Fig S1: Primer / probe (Monne-mod H9 RRT-PCR) binding sequence mismatches in Eurasian H9 AIVs**
Names of prototype isolates for the H9 lineages indicated in **bold**
Mismatches in the primer/probe binding sequences indicated by **bold** nucleotides

| Lineage | H9 isolate | H9for (5'-3')<br>ATGGGGTTTGCTGCC | H9pro (5'-3')<br>TTCTGGGCCATGTCCAATGG | H9rev (5'-3')<br>TTATATACAAATGTTGCAYCTG |
|---|---|---|---|---|
| **Original historic H9 isolate** | **A/turkey/Wisconsin/1/66** | ATGGGGTTTGCTGCC | TTCTGGGCCATGTCCAATGG | TTATATACAAATGTTGCATCTG |
| **Y439/Korean-like** | **A/duck/Hong Kong/Y439/97 #** | no sequence | no sequence | no sequence |
| | **A/chicken/Korea/S1/03 #** | AT**A**GGGTTTGCTGCC | TTCTGGGCCATGTCCAA**C**GG | TTATATACAAATGTTGCATCTG |
| Europe | A/chicken/Hungary/11-459/01 | ATGGGGTTTGCTGCC | TTCTGGGCCATGTCCAATGG | TTATATACAAATGTTGCATCTG |
| | A/duck/Italy/260/04 | ATGGGGTTTGCTGCC | TTCTGGGCCATGTCCAA**C**GG | TTATATACAAATGTTGCATCTG |
| | A/mallard/England/7798-6499/06 | ATGGGGTTTGCTGCC | TTCTGGGCCATGTCCAA**C**GG | TTATATACAAATGTTGCATCTG |
| | A/knot/England/497/02 | ATGGGGTTTGCTGCC | TTCTGGGCCATGTCCAA**C**GG | TTATATACAAATGTTGCATCTG |
| | A/teal/N Ireland/14567-10-5257/07 | ATGGGGTTTGCTGCC | TTCTGGGCCATGTCCAATGG | TTATATACAAATGTTGCATCTG |
| | A/chicken/England/1415-51184/10 * | AT**A**GGGTTTGCTGCC | TTCTGGGCCATGTCCAATGG | TTATATACAAA**C**GTTGCATCTG |
| | A/turkey/Germany/EK224/95 | ATGGGGTTTGCTGCC | TTCTGGGCCATGTCCAATGG | TTATATACAAATGTTGCATCTG |
| | A/mallard/Finland/Li13384/10 | ATGGGGTTTGCTGCC | TTCTGGGCCATGTCCAATGG | TTATATACAAATGTTGCATCTG |
| | A/turkey/Netherlands/11015452/11 | ATGGGGTTTGCTGCC | TTCTGGGCCATGTCCAATGG | TTATATACAAATGTTGCATCTG |
| **ck/Beijing/94 or Y280 like** | **A/duck/Hong Kong/Y280/97 #** | no sequence | no sequence | no sequence |
| | **A/chicken/Beijing/1/94 #** | ATGGGGTTTGCTGCC | TTCTGGGCCATGTCCAATGG | no sequence |
| | A/duck/Hunan/1/06 | ATGGGGTTTGCTGCC | TTCTGGGCCATGTCCAATGG | TTATATACAAATGTTGCAT**T**TG |
| **G1 like** | **A/quail/Hong Kong/G1/97** | ATGGGGTTTGCTGCC | TTCTGGGCCATGTCCAATGG | TTATATACAAATGTTGCACCTG |
| Middle-East | A/chicken/S Arabia/582/05 | ATGGGGTTTGCTGCC | TTCTGGGCCATGTCCAATGG | TTATATACAAATGTTGCACCTG |
| | A/chicken/Iraq/EKI 14/08 | ATGGGGTTTGCTGCC | TTCTGGGCCATGTCCAATGG | TTATATACAAATGTTGCACCTG |
| | A/chicken/S Arabia/2BL09/06 | ATGGGGTTTGCTGCC | TTCTGGGCCATGTCCAATGG | TTATATACAAATGTTGCACCTG |
| | A/avian/Libya/RV35D/06 | ATGGGGTTTGCTGCC | TTCTGGGCCATGTCCAATGG | TTATATACAAATGTTGCACCTG |
| | A/avian/Jordan/7-Y1/04 | ATGGGGTTTGCTGCC | TTCTGGGCCATGTCCAATGG | TTATATACAAATGTTGCACCTG |
| | A/chicken/Kuwait/9/04 | ATGGGGTTTGCTGCC | TTCTGGGCCATGTCCAATGG | TTATATACAAATGTTGCACCTG |
| | A/chicken/Israel/421201/ 04 | ATGGGGTTTGCTGCC | TTCTGGGCCATGTCCAATGG | TTATATACAAATGTTGCACCTG |
| | A/chicken/Lebanon/1080/04 | ATGGGGTTTGCTGCC | TTCTGGGCCATGTCCAATGG | TTATATACAAATGTTGCACCTG |
| | A/chicken/S Arabia/C-36362/10 | ATGGGGTTTGCTGCC | TTCTGGGCCATGTCCAATGG | TTATATACAAATGTTGCACCTG |
| | A/chicken/S Arabia/D-36363/10 | AT**A**GGGTTTGCTGCC | TTCTGGGCCATGTCCAATGG | TTATATACAAATGTTGCACCTG |
| | A/chicken/S Arabia/E-36364/06 | ATGGGGTTTGCTGCC | TTCTGGGCCATGTCCAATGG | TTATATACAAATGTTGCACCTG |
| | A/chicken/S Arabia/F-36365/06 | ATGGGGTTTGCTGCC | TTCTGGGCCATGTCCAATGG | TTATATACAAATGTTGCACCTG |
| | A/chicken/Saudi Arabia/H29TR/11 | ATGGGGTTTGCTGCC | TTCTGGGCCATGTCCAATGG | TTATATACAAATGTTGCACCTG |
| | A/chicken/UAE/F1P7/11 | ATGGGGTTTGCTGCC | TTCTGGGC**T**ATGTCCAATGG | TTATATACAAATGTTGCACCTG |
| South Asia | A/chicken/Bangladesh/627/07 | ATGGGGTTTGCTGCC | TTCTGGGCCATGTCCAATGG | TTATATACAAATGTTGCACCTG |
| | A/chicken/Bangladesh/FDIL(M)-112/07 | ATGGGGTTTGCTGCC | TTCTGGGCCATGTCCAATGG | TTATATACAAATGTTGCACCTG |
| | A/chicken/Pakistan/NARC-2434/06 | ATGGGGTTTGCTGCC | TTCTGGGCCATGTCCAATGG | TTATATACAAATGTTGCACCTG |
| | A/chicken/Pakistan/G-Karachi/03 | ATGGGGTTTGCTGCC | TTCTGGGCCATGTCCAATGG | TTATATACAAATGTTGCACCTG |
| | A/chicken/Pakistan/47/03 | ATGGGGTTTGCTGCC | TTCTGGGCCATGTCCAATGG | TTATATACAAATGTTGCACCTG |
| | A/chicken/India/3/03 | ATGGGGTTTGCTGCC | TTCTGGGCCATGTCCAATGG | TTATATACAAATGTTGCACCTG |
| | A/chicken/Nepal/2490/09 | ATGGGGTTTGCTGCC | TTCTGGGCCATGTCCAATGG | **C**TATATACAAATGTTGCACCTG |
| | A/chicken/Nepal/2493/09 | ATGGGGTTTGCTGCC | TTCTGGGCCATGTCCAATGG | **C**TATATACAAATGTTGCACCTG |
| | A/chicken/Nepal/5cl/10 | ATGGGGTTTGCTGCC | TTCTGGGCCATGTCCAATGG | **C**TATATACAAATGTTGCACCTG |
| | A/chicken/Nepal/6cl/10 | ATGGGGTTTGCTGCC | TTCTGGGCCATGTCCAATGG | **C**TATATACAAATGTTGCACCTG |
| | A/chicken/Nepal/5430/11 | ATGGGGTTTGCTGCC | TTCTGGGCCATGTCCAATGG | **C**TATATACAAATGTTGCACCTG |

# Prototype isolate not available for this study, * sequenced from cloacal swab.

## Optional e-only supplementary files

**Fig S2: Primer / probe (Ben Shabat H9 RRT-PCR) binding sequecnce mismatches in Eurasian H9 AIVs**
Names of prototype isolates for the H9 lineages indicated in **bold**
Mismatches in the primer/probe binding sequences indicated by **bold** nucleotides

| Lineage | H9 isolate | H9F (5'-3')<br>GGAAGAATTAATTATTATTGGTCGGTAC | H9PRO (5'-3')<br>AACCAGGCCAGACATTGCGAGTAAGATCC | H9R (5'-3')<br>GCCACCTTTTTCAGTCTGACATT |
|---|---|---|---|---|
| **Original historic H9 isolate** | **A/turkey/Wisconsin/1/66** | GGAAGAAT**CG**ATT**C**TTATTGGTC**T**GTAC | AACC**T**GG**G**CAGAC**T**TTG**A**GA**A**TAAG**G**TCC | **T**CC**T**CCTTTTTCAGTCTGACATT |
| **Y439/Korean like** | **A/duck/Hong Kong/Y439/97** | GG**G**AGAATT**G**ATTATTATTGGTC**A**GTAC | A**G**CCAGG**T**CA**A**ACG**T**TGCG**G**GTG**A**G**G**TC**A** | **A**CCACCTTT**C**TCAGT**T**TGACA**C**T |
| | **A/chicken/Korea/S1/03** | GG**G**AG**G**ATT**G**ATTATTATTGGTC**A**GTAC | A**G**CCAGGCCAGACATTGCGA**A**TAAGATCC | **A**CCACCTTT**C**TCAGT**T**TG**G**CA**C**T |
| Europe | A/chicken/Hungary/11-459/01 | GG**G**AGAATT**G**A**C**TATTATTGGTC**A**GTAC | AACCAGG**T**CAGACATTGCGAGT**G**AGATC**A** | GCCACCTTT**C**TC**G**GTCTGACACT |
| | A/duck/Italy/260/04 | GG**G**AGAATT**G**ATTATTATTGGTC**A**GTAC | A**G**CCAGGCCA**A**ACATTGCG**G**GT**G**AG**G**TCC | **A**CC**G**CCTTT**C**TCAGT**T**TGACATT |
| | A/mallard/England/7798-6499/06 | GG**G**AGAATT**G**ATTATTATTGGTC**A**GTAC | A**G**CCAGGCCA**A**ACATTGCG**G**GT**G**AG**G**TCC | **A**CC**G**CCTTT**C**TCAGT**T**TGACATT |
| | A/knot/England/497/02 | GG**G**AG**G**ATT**G**ATTATTATTGGTC**A**GTAC | A**G**CCAGG**T**CA**A**ACATTGCG**G**GT**G**AG**G**TCC | **A**CC**G**CCTTT**C**TCAGT**T**TGACATT |
| | A/teal/N Ireland/14567-10-5257/07 | GG**G**AGAATT**G**ATT**C**TTATTGGTC**A**GTAC | A**G**CCAGG**G**CA**A**ACATTGCG**G**GT**G**AGATCC | **A**CC**G**CC**C**TT**C**TCAGT**T**TG**G**CATT |
| | A/chicken/England/1415-51184/10 * | GG**G**AGAATT**G**ATTATTATTGGTC**A**GTAC | A**G**CCAGG**T**CAGACATTGCGAGTAAGATCC | **A**CCACCTTT**C**TCAGT**T**TGACATT |
| | A/turkey/Germany/EK224/95 | GG**G**AGAATT**G**ATTATTATTGGTC**A**GTAC | A**G**CCAGG**T**CA**A**ACG**T**TGCG**G**GT**G**AGATCC | **A**CCACCTTT**C**TCAGT**T**TGACATT |
| | A/mallard/Finland/Li13384/10 | GG**G**AGAATT**G**ATTA**C**TATTGGTC**A**GTAC | A**G**CCAGG**G**CA**A**ACATTGCG**G**GT**G**AGATCC | **A**CC**G**CC**C**TTTTCAGT**T**TG**G**CAT**C** |
| | A/turkey/Netherlands/11015452/11 | GG**G**AGAATT**G**ATTA**C**TATTGGTC**A**GTAC | A**G**CCAGG**G**CA**A**ACATTGCG**G**GTAAGATCC | **A**CC**G**CC**C**TT**C**TCAGT**T**TG**G**CATT |
| **ck/Beijing/94 or Y280 like** | **A/duck/Hong Kong/Y280/97** | GGAAGAATT**G**ATTATTATTGGTC**G**GTA**T** | AACCAGG**T**CAGACATTGCGAGTAAGATCC | GCCACC**T**C**T**TTC**T**GTCTGACATT |
| | **A/chicken/Beijing/1/94** | GGAAGAATT**G**ATTATTATTGGTCGGTA**T** | AACCAGG**T**CAGACATTGCGAGTAAGATCC | GCCACC**T**C**T**TTCAGTCTGACATT |
| | A/duck/Hunan/1/06 | GGAAGAATT**GA**C**T**ATTATTGGTCGGTA**T** | A**G**CCAGG**T**CA**A**ACA**C**TGCGA**A**TAAGATCC | GCCACCTTTTTC**T**GTCTGACATT |
| **G1 like** | **A/quail/Hong Kong/G1/97** | GGAAGAATT**G**ATTATTATTGGTCGGTAC | AACCAGGCCAGACATTGCGAGTAAGATCC | GCCACCTTTTTCAGTCTGACATT |
| Middle-East | A/chicken/S Arabia/582/05 | GGAAGAATTAATT**C**TTATTGGTCGGTAC | AACCAGGCCAGACATTGCGAGTAAGATCC | GCCACCTTTTTCAGTCTGACATT |
| | A/chicken/Iraq/EKI 14/08 | GG**C**AGAATTAATTATTATTGGTCGGTAC | AACCAGGCCAGACATTGCG**G**GTAAG**G**TC**A** | GCCACCTTT**C**TCAGTCTGACATT |
| | A/chicken/S Arabia/2BL09/06 | GGAAGAATTAATT**C**TTATTGGTCGGTAC | AACCAGGCCAGACATTGCGAGTAAGATCC | GCCACCTTTTTCAGTCTGACATT |
| | A/avian/Libya/RV35D/06 | GGAAGAATTAATTATTATTGGTCGGTAC | AACCAGGCCA**A**AC**G**TTGCGAGTAAGATCC | GCCACCTTTTTCAGTCTGACATT |
| | A/avian/Jordan/7-Y1/04 | GGAAGAATTAATTATTATTGGTCGGTAC | AACCAGGCCAGACATTGCGAGTAAGATCC | GCCACCTTTTTCAGTCTGACATT |
| | A/chicken/Kuwait/9/04 | GGAAGAATTAATTATTATTGGTC**A**GTAC | AACCAGGCCAGACATTGCGAGTAAGATCC | **A**CCACCTTTTTCAGTCTGACATT |
| | A/chicken/Israel/421201/ 04 | GGAAGAATTAATTATTATTGGTCGGTAC | AACCAGGCCAGACATTGCGAGTAAGATCC | GCCACCTTTTTCAGTCTGACATT |
| | A/chicken/Lebanon/1080/04 | GGAAGAATTAATTATTATTGGTCGGTAC | AACCAGGCCAGACATTGCGAGTAAGATCC | GCCACCTTTTTCAGTCTGACATT |
| | A/chicken/S Arabia/C-36362/10 | GGAAGAATTAATT**C**TTATTGGTCGGTAC | AACCAGG**T**CA**A**ACATTGCGAGTAAGATC**A** | GCCACCTTTTTCAGTCTGACATT |
| | A/chicken/S Arabia/D-36363/10 | GGAAGAATTAATT**C**TTATTGGTCGGTAC | AACCAGGCCA**A**AC**G**TT**A**CGAGTAAGATC**A** | GCCACCTTTTTCAGTCTGACATT |
| | A/chicken/S Arabia/E-36364/06 | GGAAGAATTAATT**C**TTATTGGTCGGTAC | AACCAGGCCAGACATTGCGAGTAAGATCC | GCCACCTTTTTCAGTCTGACATT |
| | A/chicken/S Arabia/F-36365/06 | GGAAGAATTAATT**C**TTATTGGTCGGTAC | AACCAGGCCAGACATTGCGAGTAAGATCC | GCCACCTTTTTCAGTCTGACATT |
| | A/chicken/Saudi Arabia/H29TR/11 | GGAAGAATTAATTA**C**TATTGGTCGGTAC | AACCAGGCCA**A**AC**G**TTG**A**GAGTAAGATC**A** | GCCACCTTTTTCAGTCTGACATT |
| | A/chicken/UAE/F1P7/11 | GGAAGAATTAATTA**C**TATTGGTCG**A**TAC | AACCAGGCCA**A**AC**G**TTGCGAGT**G**AGATC**A** | GCCACCTTTTTCAGTCTGACATT |
| South Asia | A/chicken/Bangladesh/627/07 | GGAAGAATTAATTATTATTGGTC**A**GTAC | AACCAGGCCAGACATTGCGAGTAAG**G**TCC | GCCACCTTTTTCAGTCTGACATT |
| | A/chicken/Bangladesh/FDIL(M)-112/07 | GGAAGAATTAATTATTATTGGTC**A**GTAC | AACCAGGCCAGACATTGCGAGTAAGATCC | GCCACCTTTTTCAGTCTGACATT |
| | A/chicken/Pakistan/NARC-2434/06 | GGAAGAATTAA**C**TATTATTGGTCGGTAC | A**G**CCAGGCCAGACATTGCGAGTAAGATCC | GCCACCTTTTTCAGTCTG**G**CATT |
| | A/chicken/Pakistan/G-Karachi/03 | GG**C**AGAATTAATTATTATTGGTCGGTAC | AACCAGGCCAGACATTGCGAGTAAGATCC | GCCACCTTT**C**TCAGTCTGACATT |
| | A/chicken/Pakistan/47/03 | GGAAGAATTAATTATTATTGG**G**CGGTAC | AACCAGGCCAGACATTGCGAGTAAGATCC | GCCACCTTTTTCAGTCTGACATT |
| | A/chicken/India/3/03 | GGAAGAATTAATTATTATTGGTCGGTAC | AACCAGGCCAGACATTGCGAGTAAGATCC | GCCACCTTTTTCAGTCTGACATT |
| | A/chicken/Nepal/2490/09 | GGAAGAATTAATTATTATTGGTCGGTA**T** | A**G**CCAGGCCAGACA**C**TGCGAGTAAGATCC | GCCACCTTTTTCAGT**T**TGACATT |
| | A/chicken/Nepal/2493/09 | GGAAGAATTAATTATTATTGGTCGGTA**T** | A**G**CCAGGCCAGACA**C**TGCGAGTAAGATCC | GCCACCTTTTTCAGT**T**TGACATT |
| | A/chicken/Nepal/5cl/10 | GGAAGAATTAATTATTATTGGTC**TA**TAC | AACCAGGCCA**A**ACA**C**TGCGAGTAAGATCC | GCCACC**T**C**T**TTCAGT**T**TGACATT |
| | A/chicken/Nepal/6cl/10 | GGAAGAATTAATTATTATTGGTC**TA**TAC | AACCAGGCCA**A**ACA**C**TGCGAGTAAGATCC | GCCACC**T**C**T**TTCAGT**T**TGACATT |
| | A/chicken/Nepal/5430/11 | GGAAGAATTAATTATTATTGGTCG**A**TAC | AACCAGGCCA**A**ACA**C**TGCGAGTAAGATCC | GCCACC**T**C**T**TTCAGT**T**TGACATT |

# Prototype isolate not available for this study, * sequenced from cloacal swab.

## Optional e-only supplementary files

**Table S1: Ct values of non-H9 AIV isolates (n=74) tested by M gene and both H9 RRT-PCRs**

| Subtype and pathogenicity for H5 and H7 isolates | Isolate name | M gene RRT-PCR | Monne-mod H9 RRT-PCR | Ben Shabat H9 RRT-PCR |
|---|---|---|---|---|
| H1N1 | A/duck/Alberta/35/76 | 26.72 | No Ct | No Ct |
| H2N2 | A/goose/England/21347/07 | 24.21 | No Ct | No Ct |
| H2N3 | A/duck/Germany/1215/73 | 32.22 | No Ct | No Ct |
| H2N3 | A/mallard/England/7277/06 | 19.84 | No Ct | No Ct |
| H3N2 | A/duck/Malaysia/F11107/02 | 34.73 | No Ct | No Ct |
| H3N8 | A/swan/England/08 | 19.73 | No Ct | No Ct |
| H4N6 | A/duck/Czechoslovakia/56 | 28.73 | No Ct | No Ct |
| H5N1 HP | A/chicken/Scotland/59 | 21.24 | No Ct | No Ct |
| H5N3 HP | A/tern/South Africa/61 | 21.97 | No Ct | No Ct |
| H5N2 LP | A/chicken/Mexico/94 | 20.09 | No Ct | No Ct |
| H5N2 LP | A/ostrich/Denmark/72420/96 | 21.44 | No Ct | No Ct |
| H5N2 HP | A/chicken/Italy/97 | 26.66 | No Ct | No Ct |
| H5N9 LP | A/chicken/Italy/97 | 30.33 | No Ct | No Ct |
| H5N9 LP | A/chicken/Italy/22A/98 | 31.97 | No Ct | No Ct |
| H5N2 LP | A/chicken/Belgium/99 | 21.12 | No Ct | No Ct |
| H5N2 LP | A/chicken/France/02 | 30.15 | No Ct | No Ct |
| H5N3 LP | A/duck/France/02 | 28.71 | No Ct | No Ct |
| H5N2 LP | A/duck/Singapore/F118/04 | 32.25 | No Ct | No Ct |
| H5N2 LP | A/wild birds/Denmark/04 | 31.94 | No Ct | No Ct |
| H5N1 HP | A/chicken/Indonesia/04 | 24.09 | No Ct | No Ct |
| H5N1 HP | A/chicken/Thailand//172/04 | 31.37 | No Ct | No Ct |
| H5N1 HP | A/Vietnam/1194/04 | 19.74 | No Ct | No Ct |
| H5N3 LP | A/turkey/Italy/05 | 30.88 | No Ct | No Ct |
| H5N1 HP | A/chicken/Indonesia/5/05 | 19.63 | No Ct | No Ct |
| H5N1 HP | A/turkey/Turkey/1/05 | 31.91 | No Ct | No Ct |
| H5N1 HP | A/goose/Hungary/2823/07 | 26.78 | No Ct | No Ct |
| H5N1 HP | A/chicken/Thailand-Nongkhai/NIAH400802/07 | 31.29 | No Ct | No Ct |
| H5N1 HP | A/chicken/Thailand-Phichit-NIAH/600674/08 | 28.46 | No Ct | No Ct |
| H5N1 HP | A/swan/England/70/08 | 24.27 | No Ct | No Ct |
| H6N1 | A/turkey/Ontario/98 | 22.4 | No Ct | No Ct |
| H6N1 | A/turkey/England/09 | 20.65 | No Ct | No Ct |
| H6N2 | A/teal/England/7440/06 | 32.79 | No Ct | No Ct |
| H6N8 | A/duck/Denmark/883/02 | 30.77 | No Ct | No Ct |
| H7N1 LP | A/African starling/Q-England/983/79 | 27.03 | No Ct | No Ct |
| H7N7 HP | A/chicken/Australia/Bendigo/85 | 25.15 | No Ct | No Ct |
| H7N2 LP | A/psittacine/Italy/1/91 | 21.1 | No Ct | No Ct |
| H7N1 LP | A/ostrich/South Africa/1609/91 | 24.08 | No Ct | No Ct |
| H7N1 LP | A/ostrich/South Africa/(Oudtshorn)/5352/92 | 22.75 | No Ct | No Ct |
| H7N1 LP | A/conure/England/766/94 | 29.31 | No Ct | No Ct |
| H7N1 LP | A/parrot/England/1174/94 | 26.57 | No Ct | No Ct |
| H7N1 LP | A/fairy bluebird/Singapore/F92/94 | 21.87 | No Ct | No Ct |
| H7N2 LP | A/turkey/Poland/85/95 | 19.63 | No Ct | No Ct |
| H7N1 LP | A/common iora/Singapore/F89/95 | 24.06 | No Ct | No Ct |
| H7N3 HP | A/chicken/Pakistan/CR2/95 | 30.55 | No Ct | No Ct |
| H7N7 LP | A/England/268/96 | 31.19 | No Ct | No Ct |
| H7N1 LP | A/ostrich/Zimbabwe/222/96 | 25.89 | No Ct | No Ct |
| H7N7 LP | A/turkey/Ireland/317/98 | 24.73 | No Ct | No Ct |
| H7N1 LP | A/duck/Taiwan/25.2-54-8/98 | 25.9 | No Ct | No Ct |
| H7N1 LP | A/teal/Taiwan/WB2-37-2TPFE2/98 | 25.19 | No Ct | No Ct |
| H7N3 HP | A/Peregrine falcon/UAE/188/99 | 25.29 | No Ct | No Ct |
| H7N1 LP | A/turkey/Italy/977/99 | 23.22 | No Ct | No Ct |
| H7N1 LP | A/turkey/Italy/4829/99 | 24.6 | No Ct | No Ct |
| H7N1 LP | A/chicken/Italy/1081/99 | 22.5 | No Ct | No Ct |
| H7N1 HP | A/turkey/Italy/4640/99 | 24.65 | No Ct | No Ct |
| H7N1 HP | A/chicken/Italy/4746/99 | 21.74 | No Ct | No Ct |
| H7N1 LP | A/turkey/Italy/117/00 | 22.85 | No Ct | No Ct |
| H7N1 HP | A/turkey/Italy/3/00 | 25.28 | No Ct | No Ct |
| H7N1 HP | A/chicken/Italy/4/00 | 24.71 | No Ct | No Ct |
| H7N1 HP | A/falcon/Italy/2985/00 | 26.28 | No Ct | No Ct |
| H7N7 HP | A/chicken/Netherlands/3227-8/03 | 23.91 | No Ct | No Ct |
| H7N3 LP | A/chicken/England/06 | 19.1 | No Ct | No Ct |
| H7N7 HP | A/chicken/Eng/011406/08 | 19.11 | No Ct | No Ct |
| H8N4 | A/turkey/Ontario/6118/68 | 28.56 | No Ct | No Ct |
| H8N4 | A/teal/England/7486/04 | 20.78 | No Ct | No Ct |
| H10N7 | A/chicken/England/279/01 | 25.36 | No Ct | No Ct |
| H10N7 | A/mallard/England7495/06 | 33.39 | No Ct | No Ct |
| H11N6 | A/duck/England/56 | 29.1 | No Ct | No Ct |
| H11N3 | A/duck broiler/Singapore/F107/05/02 | 32.31 | No Ct | No Ct |
| H12N5 | A/duck/Alberta/60/76 | 26.14 | No Ct | No Ct |
| H13N6 | A/gull/Maryland/704/77 | 30.61 | No Ct | No Ct |
| H14N6 | A/mallard/Gurjev/244/82 | 34.35 | No Ct | No Ct |
| H15N6 | A/shearwater/Western Australia/79 | 27.91 | No Ct | No Ct |
| H16N3 | A/gull/Denmark/68110/02 | 30.3 | No Ct | No Ct |
| H16N3 | A/gull/Sweden/03 | 33.97 | No Ct | No Ct |

# Optional e-only supplementary files

**Table S2: Testing of clinical specimens (n=139) from H9 poultry submissions (2010 and 2011)**

Ct values from testing of these clinical specimens were used for the linear regression and Bayesian analyses shown in Fig 2 and Table 2 respectively.

Indeterminate Ct values are shown in *italic type.*

| Submission details for H9 outbreak (AHVLA reference - Year) | Clinical specimen (swab unless stated) | Ct values for AI RRT-PCRs | | | | VI result |
|---|---|---|---|---|---|---|
| | | M gene | Monne-mod H9 | Ben Shabat H9 | N1 | |
| Nepal, chickens (AV1095-10) | Tracheal | 20.72 | 21 | 23.86 | NA | + |
| | Tracheal | 29.24 | 29.93 | 31.35 | NA | - |
| | Tracheal | 40 | 40 | 40 | NA | - |
| | Tracheal | 40 | 40 | 40 | NA | - |
| | Tracheal | *38.77* | 40 | 40 | NA | - |
| | Tracheal | 33.95 | 34.59 | 40 | NA | - |
| Nepal, chickens (AV1174-10) | Tracheal | 28.15 | 31.84 | 29.97 | NA | + |
| | Tracheal | 27.74 | 32.17 | 30.97 | NA | + |
| | Tracheal | 30.54 | *38.94* | 40 | NA | - |
| | Tracheal | 25.36 | 28.11 | 28.48 | NA | + |
| Nepal, chickens (AV218-11) | Tracheal | 40 | 40 | 40 | NA | - |
| | Tracheal | 40 | 40 | 40 | NA | - |
| | Tracheal | 25.28 | 24.22 | 28.96 | NA | + |
| | Tracheal | 24.91 | 24.03 | 29.19 | NA | + |
| | Tracheal | 26.25 | 25.29 | 33.05 | NA | + |
| | Tracheal | 26.73 | 25.63 | 30.69 | NA | + |
| UAE, chickens (AV281-11) | Flock 1 trachea pool | 27.86 | 26.18 | 40 | NA | + |
| | Flock 1 caecal tonsil pool | *37.38* | 35.62 | 40 | NA | + |
| | Flock 1 mixed viscera pool | *37.09* | 35.46 | 40 | NA | + |
| | Flock 1 mixed viscera pool | 26.22 | 25.35 | 40 | NA | + |
| | Flock 2 mixed viscera pool | 30.98 | 28.45 | 40 | NA | + |
| | Flock 2 trachea / lung pool | 31.52 | 29.83 | 40 | NA | + |
| | Flock 2 caecal tonsil pool | 31.36 | 29.42 | 40 | NA | + |
| UK, chickens (AV1414-10 and AV1415-10) | Tracheal | 40 | 40 | 40 | ND | - * |
| | Tracheal | 40 | *39.43* | 40 | ND | - * |
| | Tracheal | *37.91* | 40 | 40 | ND | - * |
| | Tracheal | *40* | *38.54* | 40 | ND | - * |
| | Cloacal | 35.72 | 35.78 | 40 | *37.24* | - * |
| | Cloacal | *37.21* | 35.62 | 40 | ND | - * |
| | Cloacal | *38.19* | 40 | 40 | ND | - * |
| | Cloacal | 40 | *37.71* | 40 | ND | - * |
| | Cloacal | 40 | 40 | 40 | ND | - * |
| | Cloacal | *38.04* | *36.94* | 40 | ND | - * |
| | Cloacal | 40 | 40 | 40 | ND | - * |
| | Tracheal | *38.22* | *37.75* | 40 | ND | - * |
| | Tracheal | 40 | *37.14* | 40 | ND | - * |
| | Tracheal | 40 | 40 | 40 | ND | - * |
| | Tracheal | *38.2* | *36.6* | 40 | ND | - * |
| | Cloacal | *38.82* | *37.82* | 40 | ND | - * |
| | Cloacal | 40 | *38.61* | 40 | ND | - * |
| | Cloacal | 40 | *37.48* | 40 | ND | - * |
| | Cloacal | 40 | 40 | 40 | ND | - * |
| | Cloacal | *37.28* | *36.4* | 40 | ND | - * |
| | Cloacal | *36.57* | 35.6 | 40 | ND | - * |
| | Cloacal | 40 | 40 | 40 | ND | - * |
| | Tracheal | *39.37* | *38.14* | 40 | ND | - * |
| | Tracheal | 35.38 | 34.74 | 40 | *36.38* | - * |
| | Tracheal | 40 | 40 | 40 | ND | - * |
| | Tracheal | *37.47* | *36.44* | 40 | ND | - * |
| | Cloacal | *38.93* | *38.92* | 40 | ND | - * |
| | Cloacal | 31.48 | 31.54 | 40 | 32.67 | - * |
| | Cloacal | *36.13* | *38.79* | 40 | ND | - * |
| | Cloacal | 35.82 | 34.47 | 40 | No Ct | - * |
| | Cloacal | 40 | 40 | 40 | ND | - * |
| | Cloacal | *39.5* | *37.41* | 40 | ND | - * |
| | Cloacal | 35.36 | 34.56 | 40 | ND | - * |
| | Tracheal | 40 | 40 | 40 | ND | - * |
| | Tracheal | 40 | 40 | 40 | ND | - * |
| | Tracheal | *38.46* | *37.56* | 40 | ND | - * |
| | Tracheal | *37.92* | *37.71* | 40 | ND | - * |
| | Cloacal | 34.7 | 34.57 | 40 | 35.67 | - * |
| | Cloacal | *38.59* | *36.84* | 40 | ND | - * |
| | Cloacal | *36.28* | 34.52 | 40 | ND | - * |
| | Cloacal | *37.68* | *36.96* | 40 | ND | - * |
| | Cloacal | *38.35* | *36.7* | 40 | ND | - * |
| | Cloacal | 40 | *38.7* | 40 | ND | - * |
| | Cloacal | 29.18 | 29.06 | *37.52* | 29.06 | - * |
| | Tracheal | 40 | 40 | 40 | ND | - * |
| | Tracheal | *38.93* | 40 | 40 | ND | - * |
| | Tracheal | 40 | 37.8 | 40 | ND | - * |

| Submission details for H9 outbreak (AHVLA reference - Year) | Clinical specimen (swab unless stated) | Ct values for AI RRT-PCRs | | | | VI result |
|---|---|---|---|---|---|---|
| | | M gene | Monne-mod H9 | Ben Shabat H9 | N1 | |
| UK, chickens continued (AV1414-10 and AV1415-10) | Tracheal | *37.28* | *36.25* | 40 | ND | - * |
| | Cloacal | *39.48* | *37.79* | 40 | ND | - * |
| | Cloacal | 32.74 | 32.91 | 40 | 35.5 | - * |
| | Cloacal | *37.6* | 40 | 40 | ND | - * |
| | Cloacal | 35.48 | 34.58 | 40 | ND | - * |
| | Cloacal | *37.47* | *37.5* | 40 | ND | - * |
| | Cloacal | 40 | 40 | 40 | ND | - * |
| | Cloacal | *38.8* | 40 | 40 | ND | - * |
| | Tracheal | 40 | *38.87* | 40 | ND | - * |
| | Tracheal | *39.95* | *37.65* | 40 | ND | - * |
| | Tracheal | 40 | *37.13* | 40 | ND | - * |
| | Tracheal | *37.28* | *37.57* | 40 | ND | - * |
| | Cloacal | 28.56 | 28.62 | 40 | ND | - * |
| | Cloacal | *39.08* | *37.83* | 40 | ND | - * |
| | Cloacal | 40 | *39.11* | 40 | ND | - * |
| | Cloacal | 40 | 40 | 40 | ND | - * |
| | Cloacal | *39.67* | *37.48* | 40 | ND | - * |
| | Cloacal | 30.23 | 30.17 | 40 | ND | - * |
| | Cloacal | 40 | *36.92* | 40 | ND | - * |
| | Tracheal | 40 | 40 | 40 | ND | - * |
| | Tracheal | 40 | 40 | 40 | ND | - * |
| | Tracheal | 40 | *39.58* | 40 | ND | - * |
| | Tracheal | *38.09* | *36.48* | 40 | ND | - * |
| | Cloacal | *38.61* | 35.73 | 40 | ND | - * |
| | Cloacal | *37.86* | *36.48* | 40 | ND | - * |
| | Cloacal | 34.67 | 34.18 | 40 | ND | - * |
| | Cloacal | *37.38* | 35.57 | 40 | ND | - * |
| | Cloacal | 40 | 40 | 40 | ND | - * |
| | Cloacal | 33.47 | 33.46 | 40 | ND | - * |
| | Cloacal | *38.68* | *37.93* | 40 | ND | - * |
| | Tracheal | 40 | 40 | 40 | ND | - * |
| | Tracheal | *37.79* | *37.4* | 40 | ND | - * |
| | Tracheal | *39.93* | *37.31* | 40 | ND | - * |
| | Tracheal | 35.68 | 35.49 | 40 | *37.85* | - * |
| | Cloacal | 33.77 | 33.34 | 40 | ND | - * |
| | Cloacal | *37.44* | *37.01* | 40 | ND | - * |
| | Cloacal | 40 | 40 | 40 | ND | - * |
| | Cloacal | 40 | *37.77* | 40 | ND | - * |
| | Cloacal | *38.04* | *37.03* | 40 | ND | - * |
| | Cloacal ~ | 29.01 | 28.93 | 37.29 | ND | - * |
| | Cloacal | *39.2* | *37.76* | 40 | ND | - * |
| Saudi Arabia, chickens (AV54-10), submitted on FTA cards | Kidney | 28.28 | 28.71 | 33.18 | NA | NA |
| | Trachea scrapings | 24.76 | 24.49 | 29.75 | NA | NA |
| | Lung | 23.11 | 22.6 | 28.45 | NA | NA |
| | Caecal tonsil | 28.6 | 27.41 | 35.59 | NA | NA |
| | Kidney | *37.45* | 40 | 40 | NA | NA |
| | Trachea scrapings | 22.04 | 22.18 | 28.74 | NA | NA |
| | Lung | 29.19 | 28.49 | 33.96 | NA | NA |
| | Caecal tonsil | 35.23 | 34.22 | 40 | NA | NA |
| | Kidney | 33.46 | 33.28 | 40 | NA | NA |
| | Trachea scrapings | *37.67* | *39.53* | 40 | NA | NA |
| | Lung | 40 | *36.74* | 40 | NA | NA |
| | Caecal tonsil | *38.6* | *38.29* | 40 | NA | NA |
| | Kidney | 33.1 | 32.72 | 37 | NA | NA |
| | Trachea scrapings | 40 | 40 | 40 | NA | NA |
| | Lung | *36.5* | 35.87 | 40 | NA | NA |
| | Caecal tonsil | *39.97* | 40 | 40 | NA | NA |
| | Kidney | 31.26 | 30.56 | 34.75 | NA | NA |
| | Trachea scrapings | 25.4 | 25.34 | 29.64 | NA | NA |
| | Lung | 22.86 | 22.09 | 27.44 | NA | NA |
| | Caecal tonsil | 28.47 | 28.34 | *37.58* | NA | NA |
| | Kidney | 40 | 40 | 40 | NA | NA |
| | Trachea scrapings | 40 | 40 | 40 | NA | NA |
| | Lung | 40 | 40 | 40 | NA | NA |
| | Caecal tonsil | 40 | 40 | 40 | NA | NA |
| | Kidney | 33.5 | 33.13 | 40 | NA | NA |
| | Trachea scrapings | 34.68 | 33.58 | 40 | NA | NA |
| | Lung | *36.42* | 35.15 | 40 | NA | NA |
| | Caecal tonsil | 40 | 40 | 40 | NA | NA |

* VI testing done as pools of five swabs at time of original submission, ~ RNA extracted from cloacal swab used for HA sequencing, NA=not applicable, ND=not done.

**Table S3: Details of cDNA synthesis and conventional RT-PCR primers for amplifying and sequencing Eurasian H9 AIVs.**

2.5ul extracted RNA was added to a mix that included 1ul of the universal influenza A cDNA primer (5'-AGCAAAAGCAGG-3', 50uM stock; Iqbal *et al* 2009) and the annealing buffer supplied in the SuperScript™ III First-Strand Synthesis SuperMix kit (Invitrogen, Paisley, UK), made up to 8ul with RNase-free water. This was denatured (65°C for 5mins) and snap-cooled on ice, and other kit reagents then added for cDNA synthesis to proceed in a 20ul final volume at 50C for 50 mins as per the manufacturer's instructions. This was inactivated at 95°C for 3 mins and chilled. cDNA was diluted x5 fold in molecular-grade water and 5ul amplified using the Platinum® *Taq* DNA Polymerase kit (LifeTechnologies, Paisley, UK) with either universal HA primers (Hoffmann *et al* 2001) at a final concentration of 0.2µM, or the listed primers (below) which were designed to amplify overlapping regions of the HA gene from Eurasian H9 AIVs of different geographic origins. The total reaction volume was 50ul and cycling was conducted on a conventional PCR thermocycler: 94°C for 1 min, followed by x40 cycles: 94°C for 45 seconds, 50°C for 45 secs and 72°C for 2 minutes; with a final 72°C extension for 3 minutes. If the initial amplification was with the universal HA primers, a nested PCR would follow where 0.5ul from the first round was amplified with the Platinum® *Taq* DNA Polymerase and the specific H9 primers (0.2 µM final concentration) for European and Middle Eastern / South Asian isolates (below) using the same thermocycling conditions. Amplicons were electrophoresed in 2.0% w/v agarose gel and bands purified using the QIAquick Gel Extraction Kit (Qiagen)

Conventional RT-PCR primers for European H9 AIVs:

| **HA portion** | **Primers*** | **Primer sequence (5'-3')** |
|---|---|---|
| 5’ end | F233 Eur | AAAGCAGGGGAA**W**TTCA**Y**AACCA |
| | R233 Eur1 | GAGAGGGTGCCCCAGGTTTG |
| HA1 | F3 H9 Eur | CA**Y**AACCAGCTAAAATGGAAATA |
| | R767 H9Eur | CAATGTTTG**S**CCTGGCTTTAG |
| HA1/2 | F622 H9 Eur | AACAACAAGCGTGAC**M**ACAGAAGA**Y**ATC |
| | R622 H9 Eur | GAGTCTGGCTTC**R**ATCTCACTGAATT |
| HA2 | F582 H9 Eur | GCTGCAGACAGGGAATC**R**ACTC |
| | R1 H9 Eur | GGGTGTTTTT**R**CTAATTATATACAA |
| 3’ end | F254 Eur | ATTAGAAATGGAAC**Y**TATGACAGG |
| | R254 Eur | TCGTCTCGTATTAGTAGAAACAAG |

Conventional RT-PCR primers for Middle-East and South Asian H9 AIVs. These primers also served to amplify and sequence the Chinese H9 AIV in this study:

| **HA portion** | **Primers*** | **Primer sequence (5'-3')** |
|---|---|---|
| 5’ end | F308 MEIS | GAGCAAAAGCAGGGGAATTTCTTA |
| | R308 MEIS | TCCTCCCAACAGCAG**R**TCACA |
| HA1 | F754 MEIS | TGGAAACAATATCACTGATAACTATACT |
| | R754 MEIS | CGCAATGTCTGGCCTGGTTTTA |
| HA1/2 | F723 MEIS | ATCCACCCAC**Y**GATACTGCACAGAC |
| | R723 MEIS | TATATGCCCATACGTCTTGTATTTGGTC |
| HA2 | F531 MEIS | ATAAAATAACATCCAAGGTGAATAATAT |
| | R531 MEIS | ATATACAAATGTTGCACCTG |
| 3’ end | F299 MEIS | GAAGATGGGAA**R**GGCTGTTT |
| | R299 MEIS | AGAAACAAGGGTGTTTTTGCT |

* Forward and reverse primers indicated by F and R prefixes respectively.

# Phylogenetic and molecular characteristics of Eurasian H9 avian influenza viruses and their detection by two different H9-specific RealTime reverse transcriptase polymerase chain reaction tests

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