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Identification of Molecular Markers Associated with Alteration of Receptor-Binding Specificity in a Novel Genotype of Highly Pathogenic Avian Influenza A(H5N1) Viruses Detected in Cambodia in 2013

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Human infections with influenza A(H5N1) virus in Cambodia increased sharply during 2013. Molecular characterization of viruses detected in clinical specimens from human cases revealed the presence of mutations associated with the alteration of receptor-binding specificity (K189R, Q222L) and respiratory droplet transmission in ferrets (N220K with Q222L). Discovery of quasispecies at position 222 (Q/L), in addition to the absence of the mutations in poultry/environmental samples, suggested that the mutations occurred during human infection and did not transmit further.

Early detection of avian influenza A virus infection in humans and characterization of the viruses remain a global public health goal to better anticipate pandemic threats. Although there is little evidence of person-to-person transmission of these viruses to date, mutations in the hemagglutinin (HA) and internal protein genes associated with human adaptation have been reported in some groups of highly pathogenic avian influenza (HPAI) A(H5N1) viruses, as well as in A(H7N9) viruses in China (1–3). A glutamine-to-leucine substitution in the HA receptor-binding domain 220-loop (Q222L by H5 numbering) is recognized as one of the critical substitutions, altering the binding preference of the virus for host cells displaying sialic acid in α2,3 linkage to galactose on carbohydrate side chains (avian receptor) to sialic acid in α2,6 linkage to galactose (human receptor) (4, 5). Two recent studies identified additional HA (6, 7) and internal gene (7) mutations in H5N1 viruses with efficient respiratory droplet transmission in ferrets. While the exact contribution of each individual mutation described in either study has not been fully elucidated, the combination of as few as four HA mutations compared to parental strains (N154D, N220K, Q222L, T315I [6] and H103Y, T156A, Q222L, G224S [7]) was sufficient to facilitate aerosol droplet transmission. To date, only one of these four mutations, which results in loss of a glycosylation site at the 150 loop (either N154D or T156A), has been routinely identified in naturally occurring viruses detected in birds and humans. The other mutations remain extremely rare or undetected among circulating strains of HPAI H5N1 viruses (2).

During 2013, the number of reported H5N1-positive human cases detected in Cambodia ($n = 26$) exceeded the cumulative number identified from 2005 to 2012 ($n = 21$) (8) (Table 1). Twenty-six clinical specimens, one from each of the human cases positive for H5 HA by real-time reverse transcription (RT)-PCR, were inoculated into MDCK cells and/or embryonated chicken eggs, yielding 15 virus isolates (Table 1). The HA genes from 20 cases were sequenced directly from clinical specimens and/or virus isolates, and full-genome sequences were generated from the cases producing virus isolates. In addition to sequences obtained from human cases, sequences from 72 viruses obtained from poultry, environmental sources, and civet cats were determined.

Nucleotide sequence analysis of the HA genes revealed that all viruses belong to the clade 1.1.2 phylogenetic group (9, 10). While all 2013 data from neighboring countries may not be available, the Cambodian viruses clustered in a discrete group made up of samples collected only in Cambodia during 2013 (Fig. 1). Amino acid sequence comparisons to clade 1 progenitor strains (e.g., A/Vietnam/1203/2004) revealed that all viruses in the 2013 group shared mutations at four positions (HA S123P, S133A, S155N, and K266R). Three of these four HA substitutions were previously recognized as contributing to increased binding of H5 viruses to mammalian host cell sialic acid receptors in α2,6 linkage either alone (S133A, S155N) or in combination with other mutations (S123P) (11–13). While the S155N mutation resides within the 150-loop glycosylation motif, the mutation of serine to asparagine does not change the predicted N-linked glycosylation motif

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TABLE 1 Characteristics of 2,013 human cases of HPAI A(H5N1) virus infection detected in Cambodia

WHO case no.[a]	Strain name	GenBank/GISAID accession number(s)	Isolate passage history and/or sequence availability[b]	Location[d]	Age[e]	Sex	Illness onset date	Specimen collection date	Antiviral treatment start date[f]	Outcome	Detection method[g]
CD22 (1)	A/Cambodia/X0123311/2013	PB2, KF001369; PB1, KF001370; PA, KF001371; HA, KF001372; NP, KF001373; NA, KF001374; M, KF001375; NS, KF001376	C1 and C1/E1	Phnom Penh	8 mo	M	8-Jan-13	9-Jan-13	NA	Recovered	ILI surveillance
CD23 (2)	A/Cambodia/X0121311/2013	No data	VNR (no data)	Takeo	17	F	11-Jan-13	21-Jan-13	NA	Fatal	Event based
CD24 (3)	A/Cambodia/X0123312/2013	PB2, KF001377; PB1, KF001378; PA, KF001379; HA, KF001380; NP, KF001381; NA, KF001382; M, KF001383; NS, KF001384	C1 and C1/E1	Kampong Speu	35	M	13-Jan-13	21-Jan-13	NA	Fatal	SARI surveillance
CD25 (4)	A/Cambodia/X0125302/2013	PB2, KF001385; PB1, KF001386; PA, KF001387; HA, KF001388; NP, KF001389; NA, KF001390; M, KF001391; NS, KF001392	C1 and C1/E1	Kampong Speu	17 mo	F	13-Jan-13	25-Jan-13	26-Jan-13	Fatal	Event based
CD26 (5)	A/Cambodia/X0128304/2013	No data	VNR (no data)	Kampot	9	F	15-Jan-13	27-Jan-13	27-Jan-13	Fatal	Event based
CD27 (6)	A/Cambodia/X0207301/2013	PB2, KF001393; PB1, KF001394; PA, KF001395; HA, KF001396; NP, KF001397; NA, KF001398; M, KF001399; NS, KF001400	C1	Takeo	5	F	25-Jan-13	6-Feb-13	6-Feb-13	Fatal	Event based
CD28 (7)	A/Cambodia/X0212301/2013	PB2, KF001401; PB1, KF001402; PA, KF001403; HA, KF001404; NP, KF001405; NA, KF001406; M, KF001407; NS, KF001408	C1	Kampot	3	F	3-Feb-13	11-Feb-13	11-Feb-13	Fatal	Event based
CD29 (8)	A/Cambodia/X0219301/2013	PB2, KF001409; PB1, KF001410; PA, KF001411; HA, KF001412; NP, KF001413; NA, KF001414; M, KF001415; NS, KF001416	C1 and C1/E1	Kampot	2	M	6-Feb-13	18-Feb-13	NA	Fatal	Event based
CD30 (9)	A/Cambodia/X0215301/2013	HA, KF001417; NA, KF001418	VNR (HA, NA sequence)[c]	Kampong Cham	35	M	13-Feb-13	13-Feb-13	13-Feb-13	Fatal	Event based
CD31 (10)	A/Cambodia/X0401302/2013	No data	VNR (no data)	Kampot	5	M	27-Mar-13	1-Apr-13	1-Apr-13	Recovered	Event based
CD32 (11)	A/Cambodia/X0502302/2013	HA, KF369257	VNR (HA sequence)	Kampong Speu	5	F	2-May-13	2-May-13	NA	Recovered	Fever surveillance
CD33 (12)	A/Cambodia/X0621333/2013	HA, EPI538833	VNR (HA sequence)	Phnom Penh	58	M	Unclear	8-Jan-13	NA	Recovered	SARI surveillance
CD34 (13)	A/Cambodia/X0628313/2013	PB2, KF918453; PB1, KF918454; PA, KF918455; HA, KF918456; NP, KF918457; NA, KF918458; M, KF918459; NS, KF918460	C1 and C1/E1	Kampot	6	F	24-Jun-13	28-Jun-13	28-Jun-13	Fatal	Event based
CD35 (14)	A/Cambodia/X0709301/2013	No data	VNR (no data)	Prey Veng	3	M	4-Jul-13	9-Jul-13	9-Jul-13	Recovered	Event based
CD36 (15)	A/Cambodia/X0808305/2013	PB2, KF918461; PB1, KF918462; PA, KF918463; HA, KF918464; NP, KF918465; NA, KF918466; M, KF918467; NS, KF918468	C1	Battambang	7	M	26-Jul-13	4-Aug-13	9-Aug-13	Fatal	Event based
CD37 (16)	A/Cambodia/X0810301/2013	PB2, EPI537652; PB1, EPI537653, PA, KF918469; HA, KF918470; NP, KF918471; NA, KF918472; M, KF918473; NS, KF918474	E1 and E2	Kandal	5	F	1-Aug-13	9-Aug-13	10-Aug-13	Recovered	Event based
CD38 (17)	A/Cambodia/X0817302/2013	PB2, KF918487; PB1, KF918488; PA, KF918489; HA, KF918490; NP, KF918491; NA, KF918492; M, KF918493; NS, KF918494	C1 and C1/E1	Kandal	6	M	21-Jul-13	23-Jul-13	NA	Recovered	Fever surveillance
CD39 (18)	A/Cambodia/X0828324/2013	PB2, KF918495; PB1, KF918496; PA, KF918497; HA, KF918498; NP, KF918499; NA, KF918500; M, KF918501; NS, KF918502	C1 and C1/E1	Phnom Penh	15 mo	M	16-Aug-13	27-Aug-13	27-Aug-13	Recovered	Event based

CD40 (19)	A/Cambodia/X0913301/2013	PB2, KF918503; PB1, KF918504; PA, KF918505; HA, KF918506; NP, KF918507; NA, KF918508; M, KF918509; NS, KF918510	C1	Takeo	5	F	7-Sept-13	13-Sept-13	13-Sept-13	Recovered	Event based
CD41 (20)	A/Cambodia/X0916322/2013	PB2, KF918511; PB1, KF918512; PA, KF918513; HA, KF918514; NP, KF918515; NA, KF918516; M, KF918517; NS, KF918518	C1 and C1/E1	Kampot	2	F	11-Sept-13	16-Sept-13	16-Sept-13	Fatal	Event based
CD42 (21)	A/Cambodia/X1014305/2013	No data	VNR (no data)	Kampong Thom	8	F	8-Oct-13	11-Oct-13	14-Oct-13	Recovered	Event based
CD43 (22)	A/Cambodia/X1024307/2013	PB2, KF918527; PB1, KF918528; PA, KF918529; HA, KF918530; NP, KF918531; NA, KF918532; M, KF918533; NS, KF918534	C1	Battambang	6	F	14-Oct-13	22-Oct-13	24-Oct-13	Recovered	Event based
CD44 (23)	A/Cambodia/X1030304/2013	PB2, EPI537641; PB1, EPI537642; PA, EPI537643; HA, EPI497961; NP, EPI537644; NA, EPI497962; M, EPI537645; NS, EPI537646	C1	Pursat	3	F	17-Oct-13	25-Oct-13	NA	Fatal	Event based
CD45 (24)	A/Cambodia/X1107305/2013	HA, EPI537657	VNR (HA sequence)	Kampot	10	M	30-Oct-13	7-Nov-13	7-Nov-13	Fatal	Event based
CD46 (25)	A/Cambodia/X1108306/2013	HA, EPI537675	VNR (HA sequence)	Phnom Penh	3	M	5-Nov-13	8-Nov-13	8-Nov-13	Recovered	Event based
CD47 (26)	A/Cambodia/X1108305/2013	HA, EPI538834	VNR (HA sequence)	Pailin	29	M	26-Oct-13	7-Nov-13	7-Nov-13	Fatal	Event based

[a] Numbers in parentheses were the case numbers during 2013.
[b] C1, first passage in cell culture; E1, first passage in embryonated eggs; VNR, virus not recovered.
[c] Sequence generated directly from clinical specimen when the virus was not recovered after inoculation in MDCK cells or embryonated chicken eggs.
[d] Province where case was identified.
[e] Ages are in years except where otherwise noted.
[f] NA, antiviral treatment not received.
[g] ILI, influenza-like illness; SARI, severe acute respiratory infection.

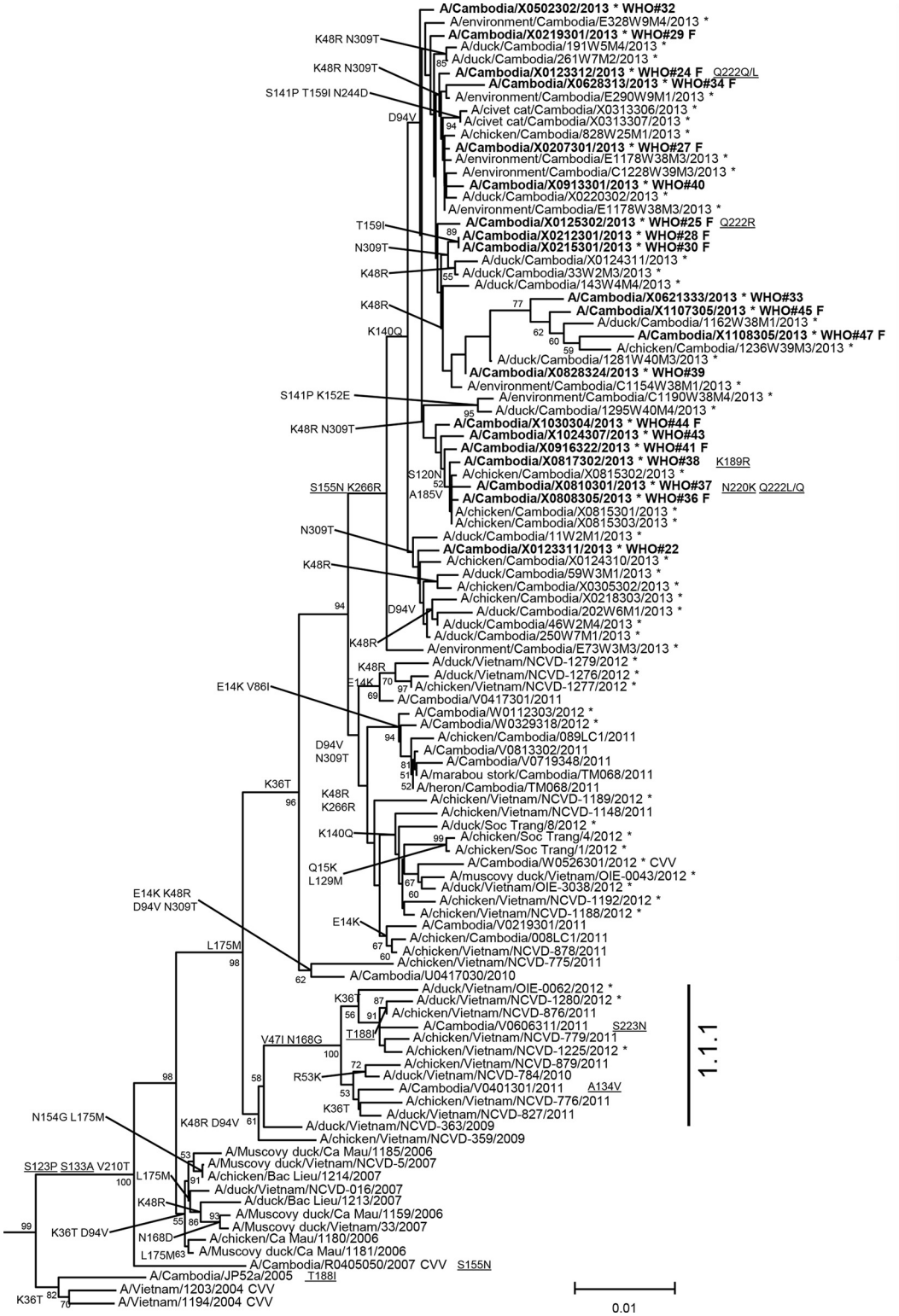


FIG 1 Neighbor-joining phylogenetic tree of the HA genes of clade 1 highly pathogenic avian influenza A(H5N1) viruses constructed in MEGA5. The nearest reassortant WHO candidate vaccine viruses (CVV) for each group of clade 1 are denoted by CVV at the end of the strain name. Viruses collected in 2012-2013 are denoted with an asterisk. Sequences were aligned using MUSCLE, and amino acid differences at branch nodes indicate shared HA1 substitutions relative to the reference strain, A/Vietnam/1203/2004. Mutations to the right of a strain name indicate amino acid changes found only in that individual virus. Underlined amino acid substitutions indicate previously recognized molecular markers and/or markers of note as listed in the H5N1 Genetic Changes Inventory (24). Branches on the tree with HA sequences from human cases are in bold. Bootstraps greater than 50 generated from 1,000 replicates are shown at branch nodes. The scale bar represents the number of nucleotide substitutions per site.

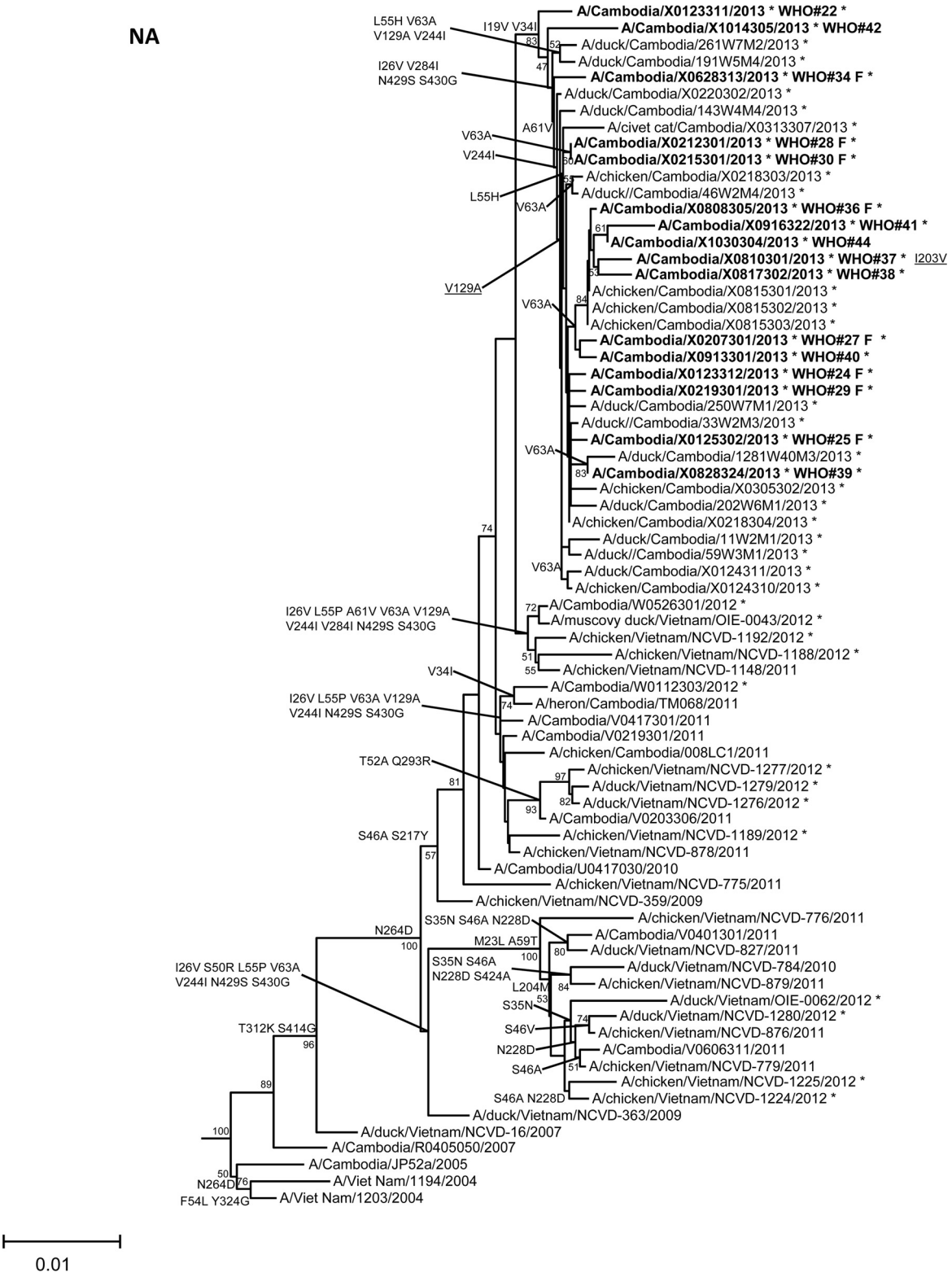


FIG 2 Neighbor-joining phylogenetic tree of the neuraminidase (NA) and internal genes of highly pathogenic avian influenza A(H5N1) viruses constructed in MEGA5. Viruses collected in 2012-2013 are denoted with an asterisk. Sequences were aligned using MUSCLE; amino acid differences at branch nodes indicate substitutions relative to reference strain A/Vietnam/1203/2004 for the NA gene and A/Hubei/1/2010 for the internal genes. Mutations to the right of each strain name indicate amino acid changes found only in that individual virus. Underlined amino acid substitutions indicate previously recognized molecular markers and/or markers of note as listed in the H5N1 Genetic Changes Inventory (24). Branches on the tree with sequences from human cases are in bold. Bootstraps greater than 50 generated from 1,000 replicates are shown at branch nodes. The scale bar represents the number of nucleotide substitutions per site.

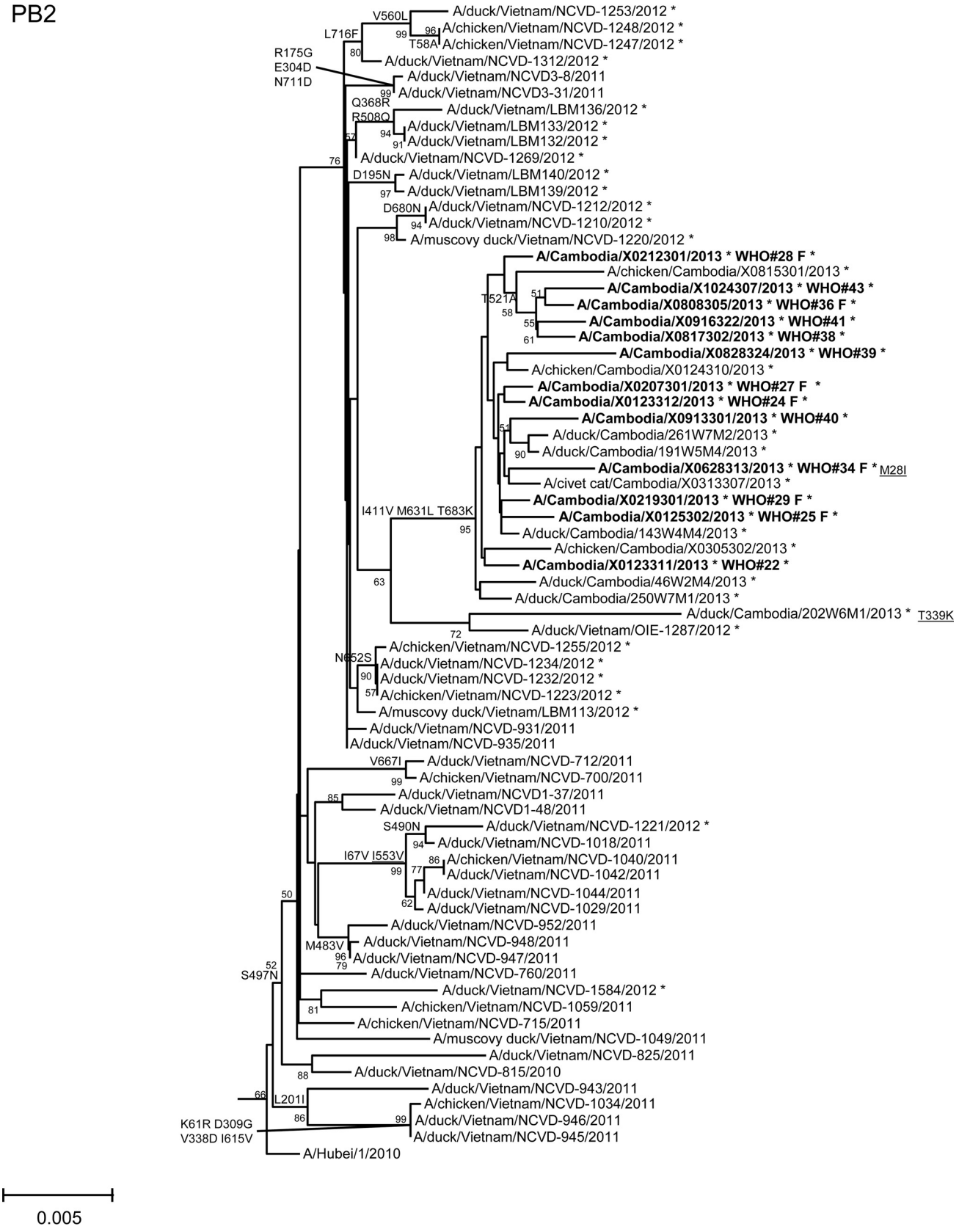
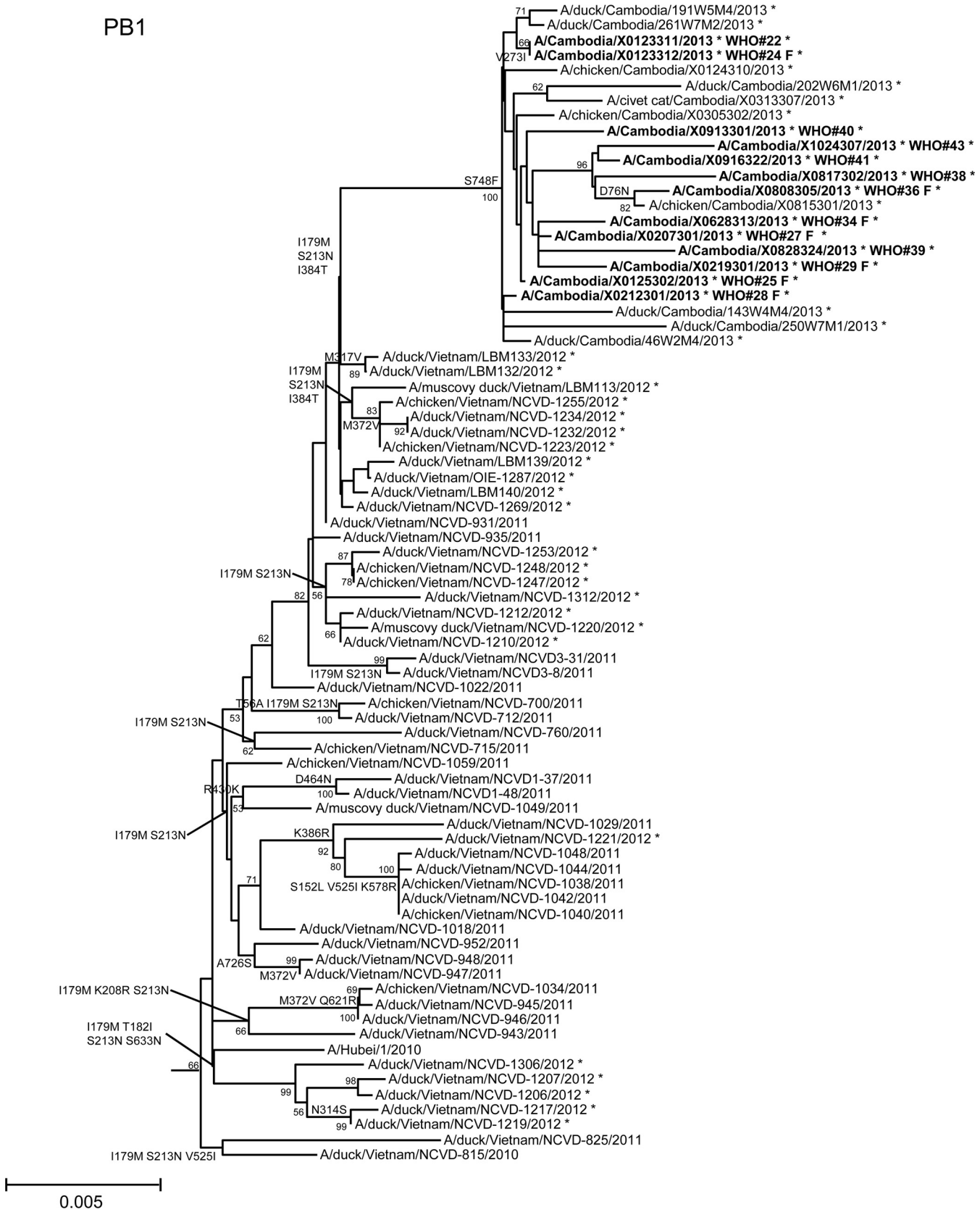


FIG 2 continued



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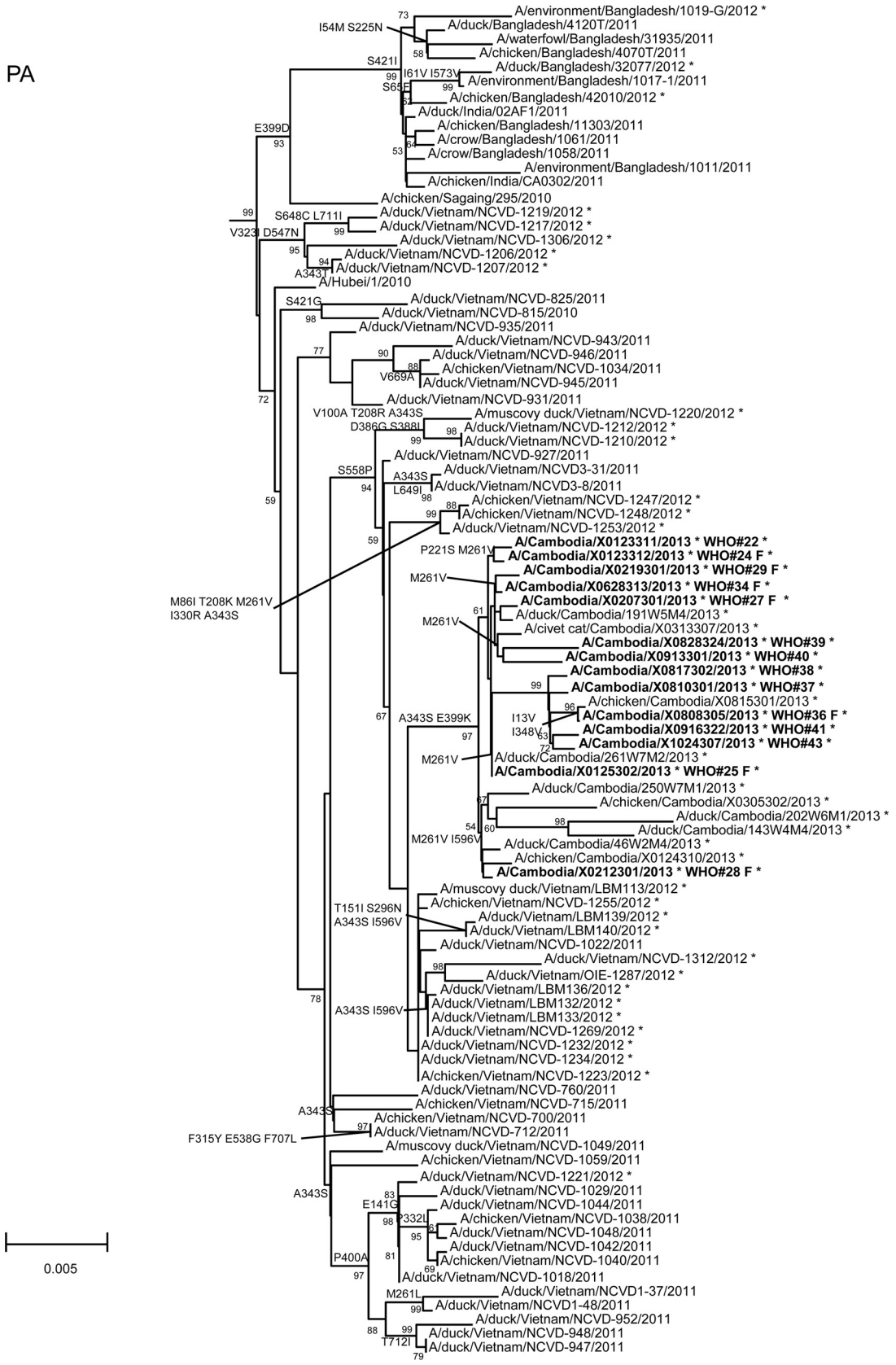

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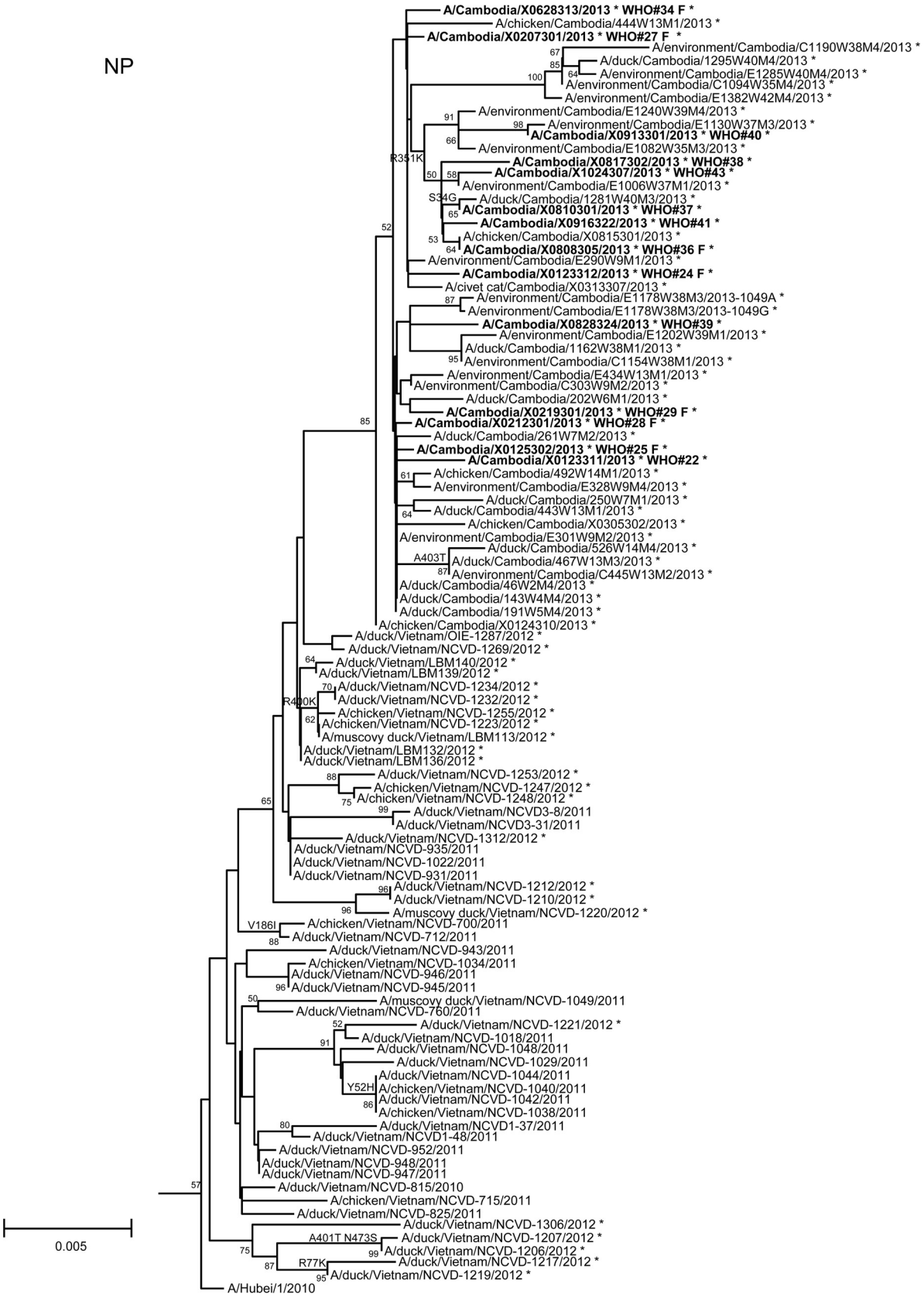

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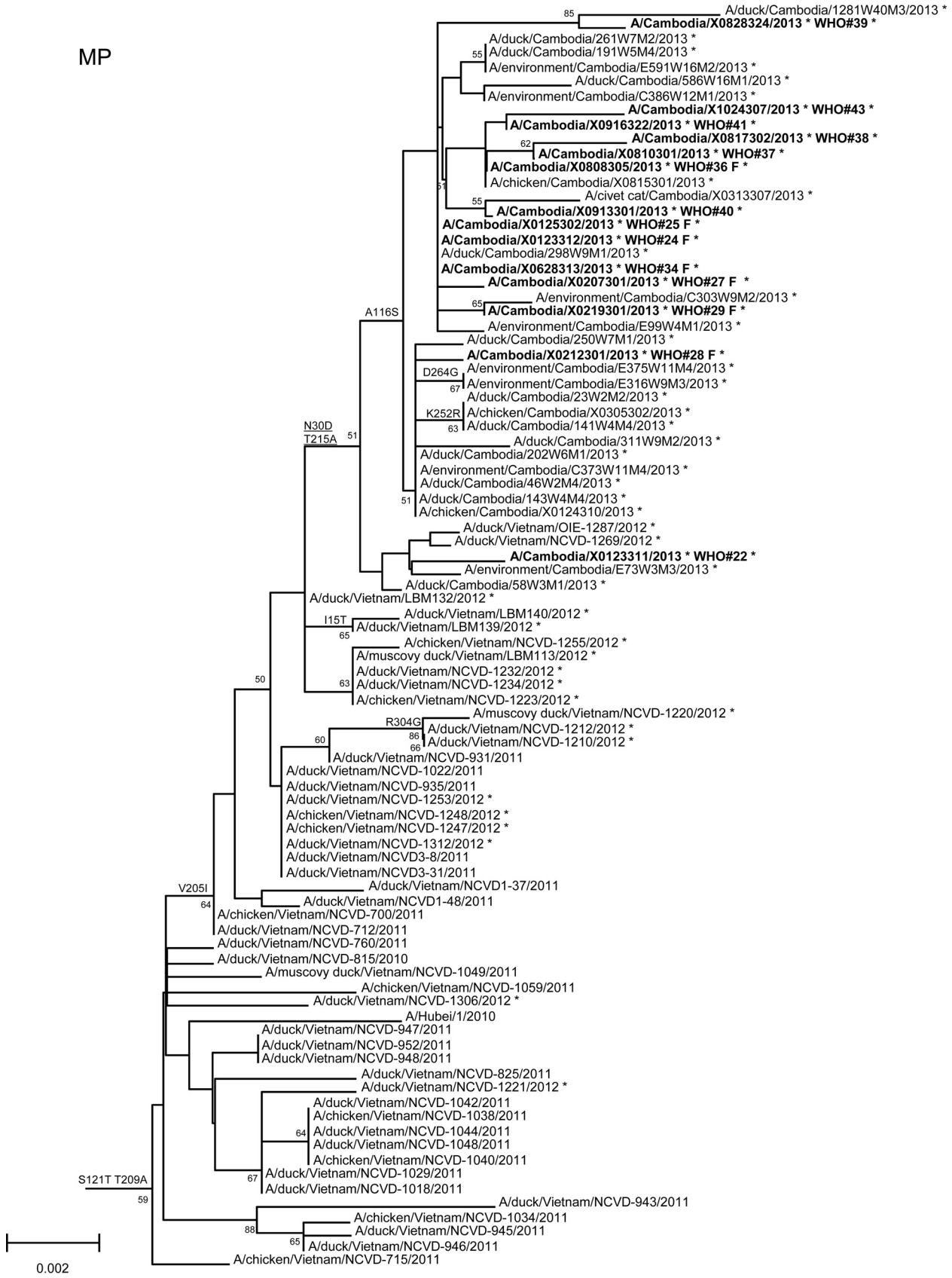

FIG 2 continued

FIG 2 continued

of these viruses (NST to NNT; underlining indicates the mutation within the motif). In addition, several individual viruses from human cases had other HA mutations previously linked to increased binding to α2,6 host cell receptors (K189R, Q222L) and enhanced respiratory droplet transmission in a ferret model (N220K with Q222L) (5, 6, 11). Virus sequenced from the clinical specimen of case 24 had a mixed base population of Q222Q/L. Another virus isolated from Cambodian case 25 had a mixed base population of Q222R/Q. A clinical specimen collected from case 37 (A/Cambodia/X0810301/2013) had both the N220K and Q222L/Q mutations, and the K189R mutation was identified in the virus infecting case 38 (Fig. 1). Although all clade 1.1.2 viruses detected to date, including 2013 Cambodian viruses from poultry, civet cats, and environmental samples, share three previously recognized HA markers (S123P, S133A, S155N), other mutations associated with mammalian adaptation were not found in the nonhuman samples, but viruses from the recently identified human cases in Cambodia had four to five amino acid residues in the HA associated with alteration of receptor-binding specificity.

In order to assess additional potential markers of mammalian adaptation, we analyzed the other influenza virus protein sequences and annotated amino acids of note on phylogenetic trees (Fig. 2). The neuraminidase (NA) of all 2013 viruses had a V129A mutation, which has been shown to reduce susceptibility to zanamivir (14). Virus isolated from case 37 had a mutation associated with reduced susceptibility to oseltamivir (NA I203V) (15). Unlike the HA and NA genes of the 2013 viruses, which were phylogenetically related to those of previous clade 1.1 viruses belonging to genotype Z, matrix (M) and internal protein genes analyzed from all animal, human, and environmental samples clustered with clade 2.3.2.1a viruses circulating in Vietnam in 2012. This finding indicated that all of these viruses represent a novel, previously undescribed genotype resulting from reassortment of clade 1.1.2 viruses with clade 2.3.2.1a viruses. The M1 protein sequences of all viruses possessed mutations associated with increased virulence in mice (N30D and T215A), which are common to the majority of circulating strains of H5N1 viruses (16). Interestingly, replacement of the clade 1.1 (genotype Z) M gene, which had two highly conserved markers of adamantane drug resistance (M2 L26I and S31N), with the clade 2.3.2.1a gene that possesses neither mutation indicates a noteworthy change for possible antiviral treatment options (17). None of the 2013 Cambodian viruses had mutations in polymerase genes associated with mammalian host adaptation. One polymerase basic 2 protein (PB2) mutation (R368Q) identified in all 2013 viruses and two other mutations (M28I, T339R) found in individual viruses were shown to alter polymerase activity and enhance virulence in mice when found in combination with other mutations (18–20). Finally, the NS1 protein of all viruses had four molecular markers (P42S, L98F, I101M, N200S) of enhanced virulence in mice and decreased the antiviral response commonly found with circulating H5N1 viruses (21–23).

This study provides the first identification of two HA mutations (220K and 222L) in a clinical sample collected from a human infection following exposure to poultry. These are two of the four mutations described in the ferret-transmissible H5 mutant (6). There was no evidence that either of these cases resulted from or led to human-to-human transmission, as indicated by follow-up investigations with close contacts of the human cases after these and other cases were detected.

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Some of the authors are employed by the U.S. government, and this work was done as part of their official duties.

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