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Direct whole-genome deep-sequencing of human respiratory syncytial virus A and B from Vietnamese children identifies distinct patterns of inter- and intra-host evolution

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Human respiratory syncytial virus (RSV) is the major cause of lower respiratory tract infections in children <2 years of age. Little is known about RSV intra-host genetic diversity over the course of infection or about the immune pressures that drive RSV molecular evolution. We performed whole-genome deep-sequencing on 53 RSV-positive samples (37 RSV subgroup A and 16 RSV subgroup B) collected from the upper airways of hospitalized children in southern Vietnam over two consecutive seasons. RSV A NA1 and RSV B BA9 were the predominant genotypes found in our samples, consistent with other reports on global RSV circulation during the same period. For both RSV A and B, the M gene was the most conserved, confirming its potential as a target for novel therapeutics. The G gene was the most variable and was the only gene under detectable positive selection. Further, positively selected sites in G were found in close proximity to and in some cases overlapped with predicted glycosylation motifs, suggesting that selection on amino acid glycosylation may drive viral genetic diversity. We further identified hotspots and coldspots of intra-host genetic diversity in the RSV genome, some of which may highlight previously unknown regions of functional importance.

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INTRODUCTION

Human respiratory syncytial virus (RSV) is the most frequently detected virus amongst young children hospitalized with acute respiratory infection worldwide (Lamb *et al.*, 2005), with no effective therapy or approved vaccine currently available. RSV (family *Paramyxoviridae*) is an enveloped, single-stranded, negative-sense RNA virus

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The GenBank/EMBL/DDBJ accession numbers for the genome sequences for RSV A and B are KJ939919–KJ939971. Short reads for all samples were uploaded to the European Nucleotide Archive with accession ID PRJEB6237.

with a ~15.2 kb genome that encodes 11 viral proteins. Two genetically and serologically distinct RSV subgroups, A and B (Coates *et al.*, 1966), co-circulate with varying frequency. Ten RSV A genotypes and 19 RSV B genotypes have been recognized worldwide (Blanc *et al.*, 2005; Dapat *et al.*, 2010; Peret *et al.*, 1998, 2000; Shobugawa *et al.*, 2009; Trento *et al.*, 2010; Venter *et al.*, 2001); genotyping is based on the highly variable G (glycoprotein) gene, which encodes one of two principal surface antigens.

Epidemiological studies have reported periodic shifts in the predominance of RSV A and B (Baek *et al.*, 2012; Dapat *et al.*, 2010). This pattern is thought to be driven by the dynamics of population immunity, where short-lived, subtype-specific herd immunity (predominantly directed against the G protein) over one or two seasons favours dissemination of the alternate subtype in a subsequent season (Botosso *et al.*, 2009). Repeated infections with the same RSV strain within individuals and co-circulation of multiple genotypes suggest the lack of an effective long-term host immune response, and consequently the lack of strong selective pressures, such as those that induce yearly antigenic drift and sequential lineage replacement in influenza virus (Power, 2008). This suggests that RSV is able to evade or modulate the host immune response and several viral proteins have indeed been reported to be involved in this. The highly variable, extensively glycosylated G protein has multiple immune modulation functions (Johnson *et al.*, 1987b, Power, 2008; Roca *et al.*, 2001). The other major surface antigen, the F (fusion) protein, has been shown to block proliferation of peripheral blood lymphocytes (Power, 2008), whilst the NS1 and NS2 nucleocapsid proteins are known to suppress the IFN response (Spann *et al.*, 2004).

Little is known about RSV intra-host genetic diversity over the course of infection or about the immune pressures that drive RSV molecular evolution. A recent case study that examined RSV intra-host genetic variation within a chronically infected infant with severe combined immune deficiency syndrome before and after bone marrow transplantation reported increased diversity, mostly within the G protein, after engraftment, suggesting that adaptive immunity plays an important role in driving viral diversity (Grad *et al.*, 2014).

Many epidemiological questions remain that would benefit from this type of analysis, if done on a larger scale. For example, whilst similar RSV genotypes circulate simultaneously in different geographical regions (Sullender, 2000), it is unclear if specific genetic signatures for each region, and hence region-specific differences in herd immunity, exist. Currently, most published whole-genome RSV sequences are consensus sequences of passaged isolates from the USA and Europe, the majority of which are RSV A (Collins *et al.*, 1987; Connors *et al.*, 1995; Crowe *et al.*, 1996; Firestone *et al.*, 1996; Karron *et al.*, 1997; Lo *et al.*, 2005; Mink *et al.*, 1991; Rebuffo-Scheer *et al.*, 2011; Stec *et al.*, 1991; Tan *et al.*, 2012; Tolley *et al.*, 1996; Whitehead *et al.*, 1998).

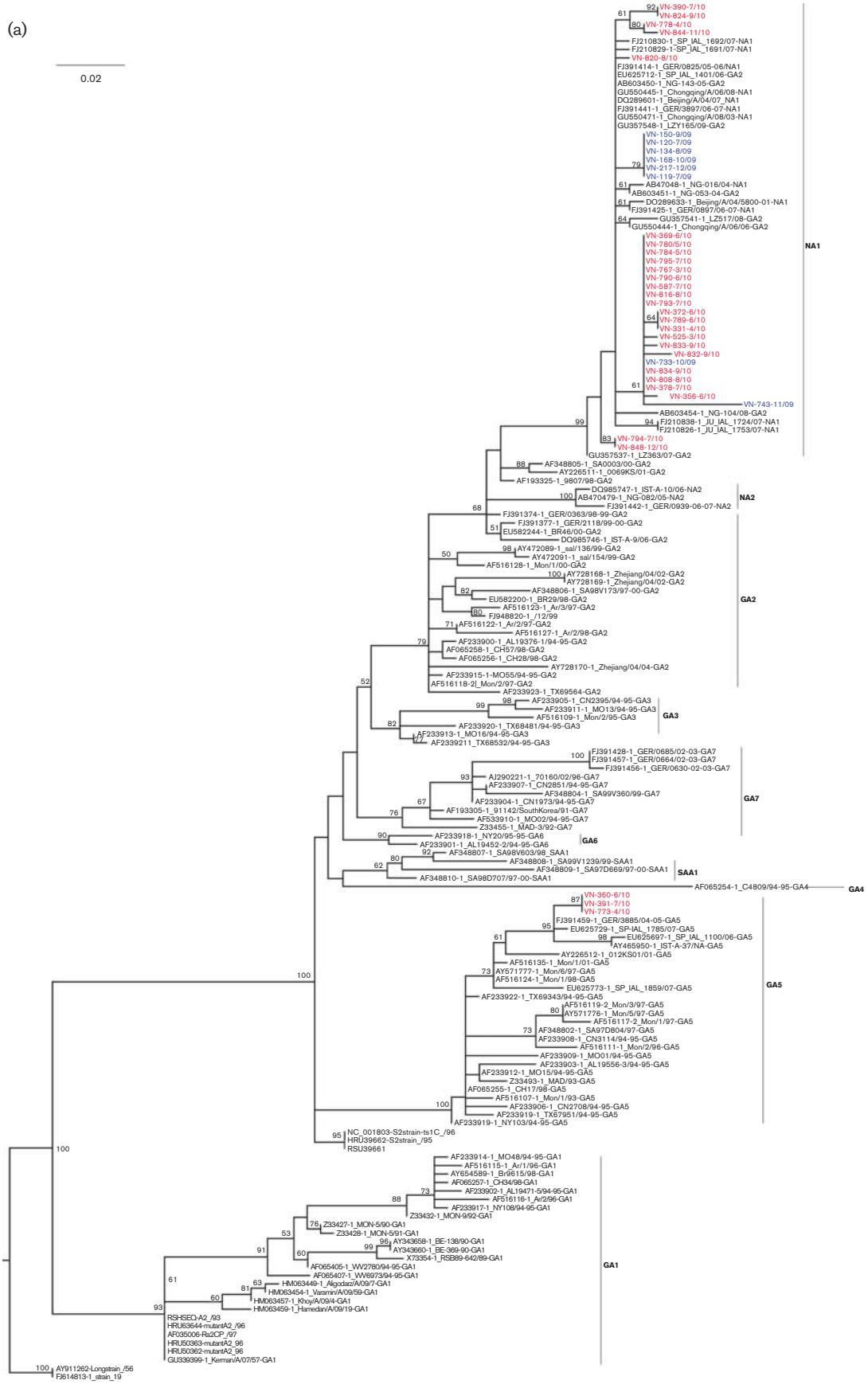
Vietnam is a high-burden country for infant respiratory infection, morbidity and mortality, with RSV as the leading cause amongst hospitalized children (Do *et al.*, 2011; Singh, 2005; Yoshida *et al.*, 2010). Here, we used whole-genome, next-generation Illumina sequencing and a rigorous variant-calling algorithm to characterize RSV inter- and intra-host genetic diversity in clinical samples collected during two consecutive seasons from otherwise healthy children hospitalized in Ho Chi Minh City. This dataset places RSV in Vietnam into the context of global RSV epidemiology and provides important insights into the immune pressures that drive genetic diversity in RSV populations.

RESULTS

RSV in Vietnam in the context of global RSV epidemiology

The global C-terminal G gene phylogeny of 165 and 74 representative sequences indicated that the 37 Vietnamese RSV A sequences formed two distinct genotypes: NA1 (n=34) and GA5 (n=3), with bootstrap values of 99 and 85 %, respectively (Fig. 1a). All RSV A sequences from 2009 were NA1 genotype, whereas NA1 and GA5 co-circulated in 2010. Of the 16 Vietnam RSV B sequences, 13 belonged to the BA genotypes BA9 (n=12) and BA10 (n=1). Of the remaining three sequences, two (VN-125-7/09 and VN-156-9/09) were most closely related to a BA3 reference strain, but were not classified as BA3 due to low bootstrap values, and one (VN-731-10/09) did not cluster with any reported genotype (Fig. 1b). In general, the Vietnamese RSV genomes fit into the current classification system.

We next reconstructed whole-genome phylogenies (data available upon request) to examine the relationship of Vietnamese RSV to published full-length sequences, which at the time of analysis comprised 63 (50 RSV A and 13 RSV B) sequences from the USA and Europe (Collins *et al.*, 1987; Connors *et al.*, 1995; Crowe *et al.*, 1996; Firestone *et al.*, 1996; Karron *et al.*, 1997; Kumaria *et al.*, 2011; Lo *et al.*, 2005; Mink *et al.*, 1991; Rebuffo-Scheer *et al.*, 2011; Stec *et al.*, 1991; Tolley *et al.*, 1996; Whitehead *et al.*, 1998) collected between 1956 and 2010. Of these, 27 had been directly sequenced from clinical samples (Kumaria *et al.*, 2011; Rebuffo-Scheer *et al.*, 2011). The 34 NA1 Vietnamese RSV A whole-genome sequences from 2009 and 2010 formed the same five distinct clusters as on the C-terminal G gene phylogeny. These five clusters grouped together with four genomes (GenBank accession numbers JF920052, JF920053, JF920054 and JF920055) collected in 2010 in Wisconsin, USA, which were previously assigned to genotype GA2 (not NA1), although they were clearly divergent from other GA2 strains (Rebuffo-Scheer *et al.*, 2011). The remaining three GA5 Vietnamese RSV A sequences (VN-360, VN-391 and VN-773) were from the 2010 season



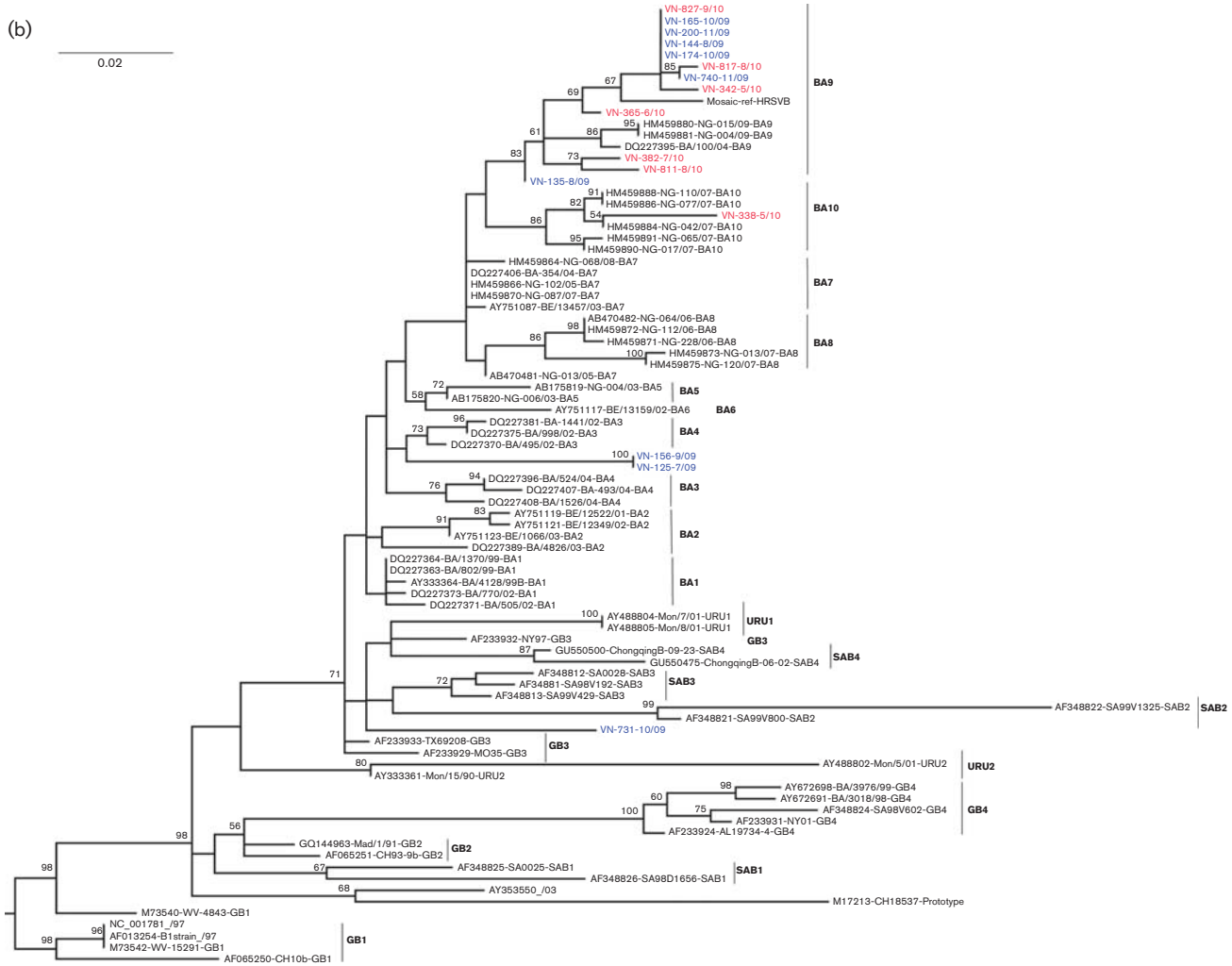


Fig. 1. Maximum-likelihood phylogenetic analysis of the C-terminal second hypervariable region (HVR2) of the G gene of Vietnamese (a) RSV A and (b) RSV B. A discrete Γ distribution was used to model evolutionary rate differences amongst sites (four categories). Branch labels indicate the stability of the branches over 1000 bootstrap replicates. Trees are drawn to scale, with branch lengths measured in the number of substitutions per site. Bar, rate of nucleotide substitutions per site. For RSV A, the cluster of two reference sequences Long and FJ614813 was used as the outgroup; for RSV B, the cluster of three reference sequences NC_001781, AF013254 and M73542 was used. Vietnamese sequences are indicated by 'VN' followed by the month and year of collection; those in blue and in red were collected in 2009 and 2010, respectively. The remaining sequences are from prototype strains and representative genotypes which are indicated with GenBank accession number, place, year of collection and their assigned genotype.

and were related to a 2007 GA5 strain collected in the USA (GenBank accession numbers JF920058) (Rebuffo-Scheer *et al.*, 2011). Of the 16 Vietnamese RSV B genomes, 13 were most closely related to BA strains collected from 2007 to 2010 in Wisconsin (Rebuffo-Scheer *et al.*, 2011). Unlike RSV A, we did not observe clustering by year for RSV B.

RSV inter-host (consensus) genetic diversity

Analysis of inter-host consensus differences revealed the highest overall substitution rate in G and the lowest in M, for both RSV A and B. Using a maximum-likelihood method, evidence of positive selection was only found in the G gene (Table 1), although G and M2-2 showed the highest d*N*/d*S* ratios in both subgroups (Table 2).

We next identified individual G gene codons under selection. Amongst RSV A genomes, a total of 18 possible positively selected sites (6 % of the G gene) were observed (d*N*/d*S*=4.42, $P>50$ %), with seven of 18 of these sites reaching significance ($P>95$ %). Three of these 18 have not been described before as positively selected: aa 214 ($P=82$ %), 244 ($P=70$ %) and 250 ($P=99$ %; all highlighted in green in Fig. 2a). All potentially positively selected sites from

Table 1. Summary of selection pressure analysis

Gene	RSV A (79 sequences)					RSV B (25 sequences)			
	Length (bp)	No. of unique sequences	No. of RB* (position; bp)	Fragment coordinates (if RB >0)	LRT†	Length (bp)	No. of unique sequences	No. of RB* (position; bp)	LRT†
NS1	417	32	0		1	417	14	0	1
NS2	372	26	0		0.88	372	13	0	1
N	1173	53	0		0.73	1173	16	0	1
P	723	45	0		1	723	17	0	1
M	768	39	1			768	14	0	0.50
				1–168	1				
				169–768	1				
SH	192	25	0		1	195	11	0	0.65
G	891	57	1			936	21	0	0.002
				1–570	0.23693				
				571–891	2.87×10^{-10}				
F	1722	62	1		1	1722	20	0	0.29
				1–543	1				
				544–1722	1				
M2-1	582	38	0		1	585	17	0	0.73
M2-2	264	29	0		−1	270	10	0	0.74
L	6495	74	5			6498	23	0	1
				1–741	1				
				742–1599	0.63128				
				1600–2805	0.39852				
				2806–3924	1				
				3925–5574	1				
				5575–6495	1				

*Recombination breakpoints (RB) predicted by SBP and GARD.
†Likelihood ratio test (LRT) is significant if <0.05.

RSV A were located in the C-terminal second hypervariable region (HVR2) of the G gene (Fig. 2a). Amongst RSV B genomes, five potentially positively selected sites (2 % of the G gene) were found ($dN/dS=6.67$, $P>50$ %). Two of these have not been described before: aa 159 ($P=99$ %) and ($P=88$ %; highlighted in green in Fig. 2b). Consistent with an earlier report (Parveen *et al.*, 2006), many serine and threonine residues in HVR2 (34 and 42 for RSV A and B, respectively, *G* score >0.5) were predicted to be *O*-glycosylated and three sites were predicted to be *N*-glycosylated (Fig. 2). We hypothesize that host immune pressure could result in selection on amino acid glycosylation, which is well known to influence the antigenicity of the G protein. In support of this, nine of 18 positively selected RSV A sites (aa 226, 244, 250, 258, 274, 279, 289, 290 and 297) were found next to *O*-glycosylation motifs; in addition, aa 289 was both positively selected and predicted to be *O*-glycosylated in all NA1 sequences, and aa 250 was positively selected and predicted to be *N*-glycosylated in all GA5 sequences (Fig. 2a). In contrast, none of the RSV B potentially positively selected sites were predicted to be *O*- or *N*-glycosylated (Fig. 2b).

Amongst Vietnamese RSV A sequences, predicted *N*-glycosylation patterns in the G protein HVR2 varied by genotype (Fig. 2a). Potential *N*-glycosylation sites at aa 237 and 250 were present in GA5, but not NA1 sequences, and a third site at aa 294 was observed in all three GA5 strains, but only in four of 37 NA1 strains (Fig. 2a). We similarly found three polymorphic *N*-glycosylation sites amongst the RSV B sequences, two of which (aa 230 and 296) were conserved across genotypes. A substitution was found in the third site (aa 310) in BA3 strains (Fig. 2b). No specific *O*-glycosylation patterns were observed amongst different genotypes for either RSV subgroup.

No distinctive patterns between RSV A and B were found in non-coding regions (NCRs) or intergenic non-coding regions (IGSs) (data available upon request). 5′-NCRs were generally shorter and more conserved than 3′-NCRs, and IGSs were more variable than either NCR. Whilst gene start sequences were well conserved and identical for both RSV A and B, gene end sequences showed much higher variation (Table 3).

RSV intra-host genetic diversity

Low-frequency intra-host single nucleotide variants (SNVs) in the viral genome were identified using the

Table 2. Rates of synonymous (d*S*) and non-synonymous (d*N*) mutations in Vietnamese RSV A and B

Gene	RSV A					RSV B				
	Mean d*S*	Mean d*N*	d*N*/d*S*	Total sites	Substitutions per site*	Mean d*S*	Mean d*N*	d*N*/d*S*	Total sites	Substitutions per site*
NS1	0.11	0.003	0.03	420	0.006	0.04	0.003	0.08	420	0.004
NS2	0.13	0.003	0.03	375	0.005	0.06	0.003	0.04	375	0.005
N	0.11	0.002	0.02	1176	0.005	0.04	0.0008	0.02	1176	0.003
P	0.09	0.003	0.03	726	0.005	0.07	0.001	0.02	726	0.005
M	0.10	0.0005	0.01	771	0.004	0.03	0.0007	0.03	771	0.002
SH	0.13	0.004	0.03	195	0.005	0.04	0.007	0.18	198	0.002
G	0.10	0.04	0.39	897	0.020	0.07	0.02	0.33	960	0.008
F	0.11	0.006	0.05	1725	0.008	0.04	0.03	0.06	1725	0.004
M2-1	0.10	0.0061	0.05	585	0.005	0.06	0.004	0.06	588	0.005
M2-2	0.05	0.03	0.48	267	0.009	0.04	0.009	0.26	273	0.004
L	0.07	0.003	0.05	6498	0.006	0.05	0.002	0.03	6501	0.003

*Substitutions per site was based on maximum-likelihood phylogeny trees of each coding gene.

sensitive, quality-aware variant caller LoFreq (Wilm *et al.*, 2012). It was found that 22 % of SNVs were present as the dominant variant in other samples. SNVs were further aggregated to identify regions of the genome with an excess (hotspot) or depletion (coldspot) of intra-host diversity (Wilm *et al.*, 2012) (Fig. 3a, b). Despite sharing some key features, RSV A and B exhibited distinct patterns of intra-host diversity.

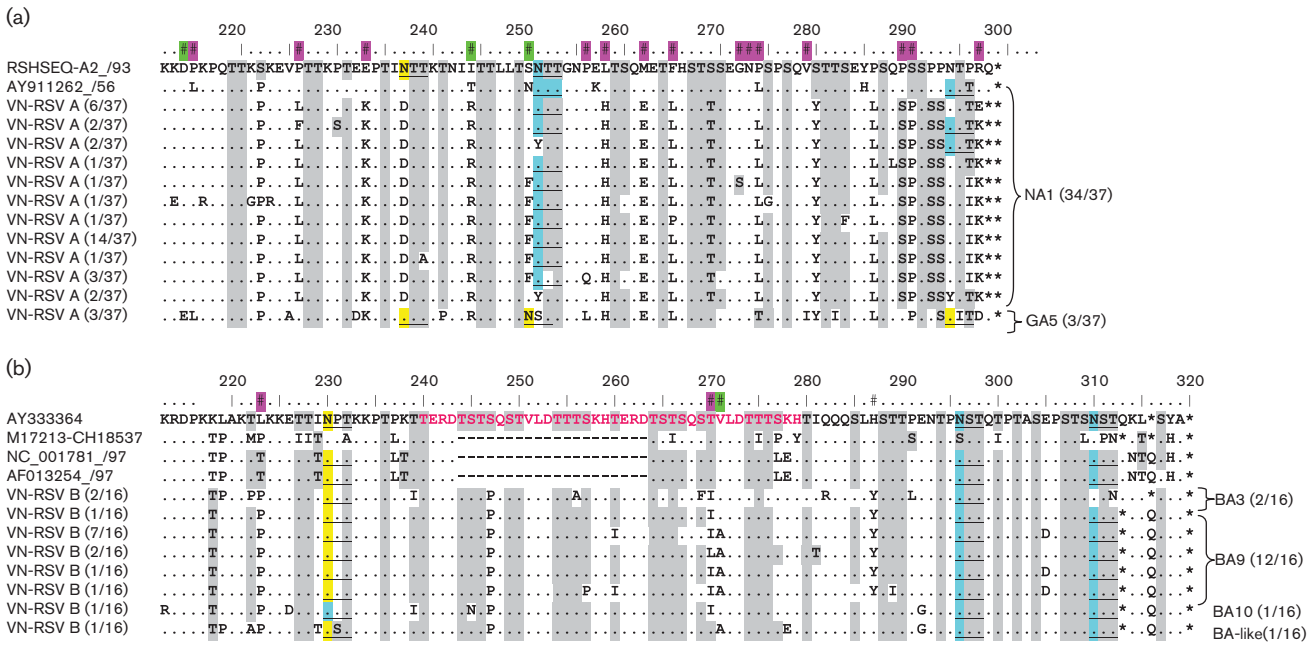


Fig. 2. Predicted positively selected and glycosylated sites in the C-terminal HVR2 of the G glycoprotein from Vietnamese (a) RSV A and (b) RSV B sequences. Hash symbols (top row) indicate positions identified as positively selected (purple, previously identified positions; green, newly detected positions). Potential *N*-glycosylation sites, e.g. NXT motifs, where X is not a proline, are underlined; asparagines strongly predicted to be *N*-glycosylated are highlighted in yellow (default threshold of >0.5 and supported by nine networks), whilst potentially predicted asparagine sites are highlighted in blue (default threshold of >0.5, but not clearly supported by networks); potential *O*-glycosylation sites (KPX motif) are highlighted in grey. Red text indicates residues previously reported to belong to an immunogenic region or antibody epitope. Dots indicate identical residues and asterisks indicate stop codons. Only one representative of each sequence group is shown and labelled 'VN'; the number of times the sequence was detected is indicated in parentheses.

Table 3. Gene start and gene end sequences of RSV A and B

Dots indicate identical nucleotides and dashes indicate deleted nucleotides (compared with consensus sequences).

Gene	Gene start	No. of polymorphism sequences amongst RSV A and B	Gene end	No. of polymorphism sequences amongst RSV A and B
Consensus sequence*	**GGGGCAAATA**		**AGTTAATATAAAA**	
NS1	..........†		.....	
NS2	.....		..... T.TA..–	37/37 RSV A
			..... A.T..A..–	16/16 RSV B
N	..........†		.....A.....A	34/37 RSV A
			..... C.A.....A	3/37 RSV A
			.....C.A ....TA	16/16 RSV B
P	..........†		..... CA.A.....	36/37 RSV A
			....CA.A ...AAG	1/37 RSV A
			.... A.CA.A	15/16 RSV B
			..... A.CA.A.G..	1/16 RSV B
M	..........†		.....A.....–	23/37 RSV A
			..... C.A..	1/37 RSV A
			.....A..	13/37- RSV A
			..G ..... ATA.–	16/16 RSV B
SH	.....	37/37 RSV A	.....TA.....	34/37 RSV A
	.....T.....	16/16 RSV B	..... CTA.....	3/37 RSV A
			..... T.TA.....	16/16 RSV B
G	.....G†		..... C.T..A..–	36/37 RSV A
			..... C.T.TA.–	1/37 RSV A
			–..... T.CA...	3/16 RSV B
			–.....T.TA	13/16 RSV B
F	..........†		..... TATA.–	37/37 RSV A
			.....CATA	15/16 RSV B
			.....CATA...G	1/16 RSV B
M2	..........†		..... T.TA..–	37/37 RSV A
			.....TCTA–	16/16 -RSV B
L	... A...A–†		..... T.TA..–	37/37 RSV A
			.....T..A ....–	16/16 RSV B

*Consensus sequences of gene start and gene ends for both RSV A and B.
†Identical gene start sequences for both RSV A and B.

RSV A and B share characteristic coldspots in the region flanked by the nucleoprotein gene N and the phosphoprotein gene P, including the intergenic region, highlighting their conserved functions. N binds viral RNA by forming a groove between the N- and C-terminal domains (Tawar *et al.*, 2009); P is known to form homo-tetramers and the corresponding α-helical oligomerization domain is covered by another coldspot, also shared between RSV A and B (aa 104–163) (Llorente *et al.*, 2006, 2008). Similarly, shared coldspots in the polymerase gene L overlap with conserved region V of the polymerase, which is known to have mRNA capping activity and is conserved across non-segmented negative-sense RNA viruses (Li *et al.*, 2008). Finally, a shared coldspot was also detected on the F gene, which is a drug and vaccine target (Dormitzer *et al.*, 2012; Swanson *et al.*, 2011). This coldspot covers two of the three subunits that form the 'head' of the structure (Fig. 3c) and are exposed on the protein surface. It extends towards the α-helical regions that form the 'stalk' of the trimeric protein, but does not overlap with the α-helical regions that are mainly responsible for the complex structural rearrangements that F undergoes during cell entry, suggesting greater genomic plasticity in this region. In both RSV A and B, the P and M genes had the least intra-host diversity, as measured by the fraction of the gene covered by coldspots. Overall, a larger fraction of the RSV B genome was covered in coldspots; despite this, a subtype-specific coldspot was detected in the N terminus of the M gene in RSV A.

Although hotspots in the SH and G genes were found in both RSV A and B, the specific locations of these hotspots differed by subgroup (Fig. 3a, b). Both genes are non-essential for viral replication *in vitro* (Karron *et al.*, 1997) and could therefore be more likely to acquire SNVs without negative effects. RSV A has a large number of novel hotspots in the L gene, clustered within the *S*-adenosyl-L-methionine-dependent methyl-transferase domain of the polymerase. Novel hotspots were also observed in RSV A NS1 and N genes, potentially

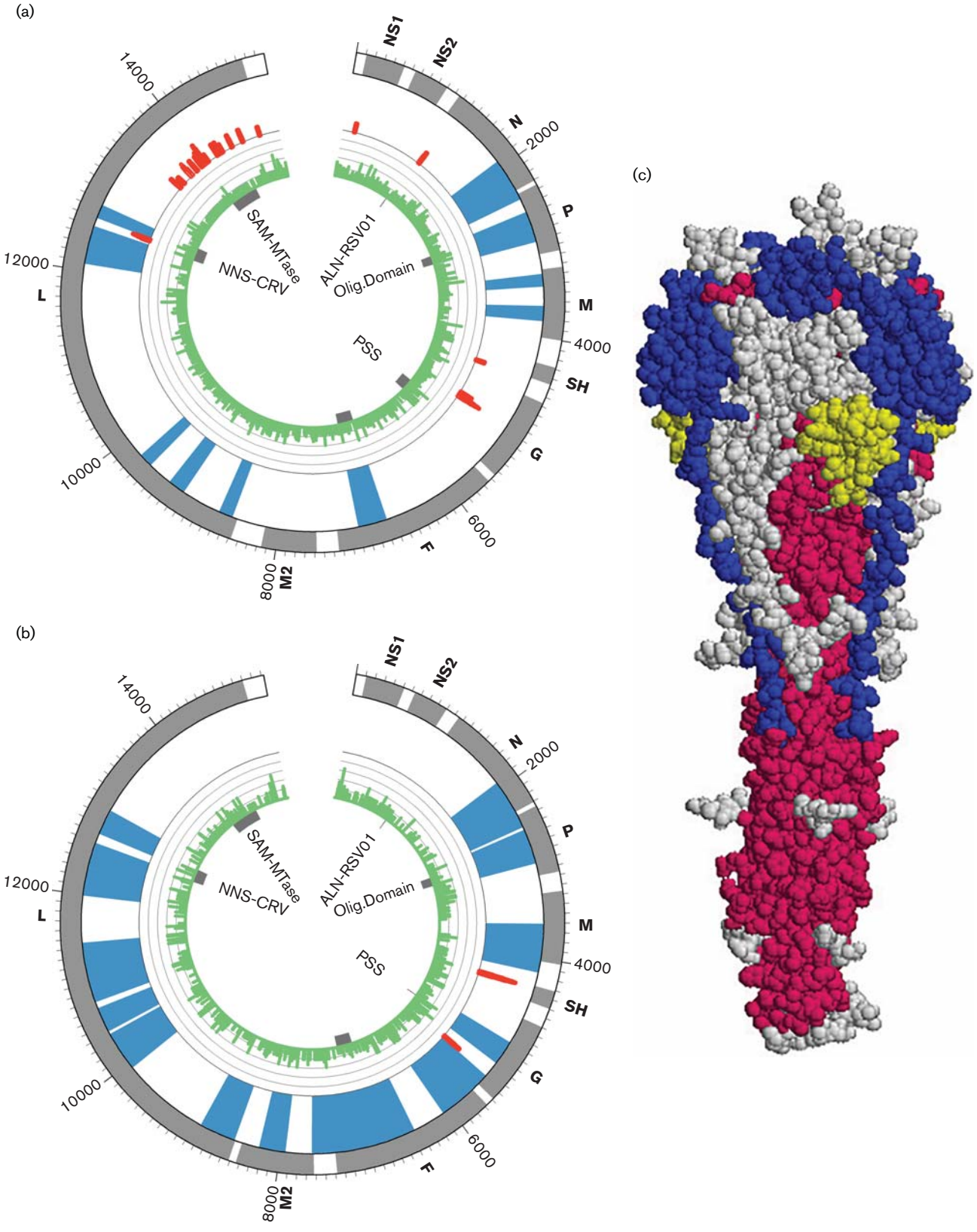


Fig. 3. Distribution of mutational hotspots and coldspots across the RSV genome and on the F glycoprotein. Circos plots (Krzywinski *et al.*, 2009) with genomic features for (a) RSV A and (b) RSV B. Blue wedges, regions depleted in intra-host variants (coldspots); red bars, regions with significant excess of intra-host variants (hotspots; height proportional to the number

of times they were identified); green bars, sequence conservation in the corresponding multiple sequence alignment measured as Shannon entropy. ALN-RSV01, location of therapeutic small interfering RNA of the same name; Olig.Domain, oligomerization domain of P. PSS, positively selected sites; NNS-CRV, conserved region V of non-segmented negative-sense RNA viruses; SAM-MTase, the *S*-adenosyl-L-methionine-dependent methyl-transferases domain of the polymerase. (c) Three-dimensional model of the RSV-F post-fusion trimer (based on Protein Data Bank ID: 3RKI); also marked as grey box in (a) and (b). Red, α-helical regions; blue, predicted coldspots; yellow, motavizumab epitope.

indicating uncharacterized functional regions. Interestingly, the N 5′-end hotspot is just 60 bp downstream of the region targeted by the therapeutic small interfering RNA ALN-RSV01 (Alvarez *et al.*, 2009), which is currently in clinical trials.

RSV A sample numbers were sufficient to allow us to conduct a hotspot analysis separately for severe and non-severe cases. Intriguingly, we found a hotspot unique to severe cases located at the mucinoid I region of the G gene (aa 114–141), whilst hotspots uniquely found in non-severe cases were located in NS1 and NS2, the IGS between the SH and G genes, and L gene regions (Fig. 3a, b).

DISCUSSION

Here, we report the development and application of a high-throughput sequencing strategy to sequence Vietnamese RSV whole genomes directly from clinical samples, enabling the study of inter- and intra-host genetic diversity. Overall, the data highlight evolutionary dynamics of individual genes and their impact on RSV fitness, specifically in the context of immune evasion.

The dominant Vietnamese RSV genotypes (RSV A NA1 and RSV B BA9) were similar to those circulating during the same period in Cambodia, Canada and the USA, consistent with previous observations of global dissemination since their first description in 2008–2009 (Arnott *et al.*, 2011; Dapat *et al.*, 2010; Eshaghi *et al.*, 2012). Recently, a novel genotype (ON1) containing a 72 bp duplication in the HVR2 of the G gene (aa 283) has become dominant in Canada, whilst being reported only sporadically in India, South Korea, Malaysia (2010–2011) and China (2012) (Choudhary *et al.*, 2013; Cui *et al.*, 2013; Eshaghi *et al.*, 2012; Khor *et al.*, 2013; Lee *et al.*, 2012). We did not observe this genotype amongst our 37 RSV A sequences from 2009 to 2010, nor amongst 331 Vietnamese RSV G gene sequences from a 2010–2011 nosocomial cohort of hospitalized children with acute respiratory infection (unpublished data). This suggests that its occurrence in Asia is sporadic and that it has not yet spread widely. In contrast, the BA genotype of RSV B with a duplication of 60 bp at HVR2 of the G gene (at aa 239 using GenBank accession number AY333364 as reference) (Fig. 2b) spread globally within a short period of time and is now continuously evolving with regular detection of new BA variants (Arnott *et al.*, 2011; Eshaghi *et al.*, 2012; Rebuffo-Scheer *et al.*, 2011; Salter *et al.*, 2011; Trento *et al.*, 2003, 2010). In our study, all Vietnamese RSV B sequences were BA-like genotypes containing this 60 bp duplication and the majority (12/16) were classified as BA9. Novel subgenotypes (BA7–BA10) have been reported from 2006 to 2010 in different regions in the world (Dapat *et al.*, 2010). One RSV B sequence (VN-731) of the BA-like genotype appears to be a novel variant of BA10, based on 96 % nucleotide similarity in the G gene and specific amino acid substitutions observed in the prototype BA10 strain (AY333364). The data suggest that the emergence and evolution of novel subgenotypes is an ongoing phenomenon.

The G gene is the most variable in the genome and encodes a surface glycoprotein that carries host cell receptor binding sites and neutralizing antibody epitopes (Escribano-Romero *et al.*, 2004; Johnson *et al.*, 1987a, b; Krusat & Streckert, 1997). Thus, immune pressure on this gene is likely to play a key role in driving the evolution of RSV genotypes described above. In our RSV A dataset, positively selected sites on the G protein were strongly associated with known antibody epitopes, as described in escape mutants of specific mAbs (aa 226, 265, 274 and 290) (García *et al.*, 1994; Martínez *et al.*, 1997; Rueda *et al.*, 1991) or in WT strains (aa 214, 215, 226, 265 and 272) (Cane, 1997; Cane & Pringle, 1995; García *et al.*, 1994). For example, sites P215L and P226L/F, which belong to an immunogenic region of G (Olmsted *et al.*, 1989), were identified to be under positive selection in three of 37 and 34 of 37 Vietnamese RSV A sequences, respectively. Substitutions F265L and P274L/T, located within epitope 25G (Cane & Pringle, 1995; García *et al.*, 1994, Rueda *et al.*, 1991), were under positive selection in 36 of 37 and six of 37 Vietnamese RSV A sequences, respectively. Moreover, amino acid substitution R297E/K/D, demonstrated to influence the integrity of multiple overlapping strain-specific epitopes (Rueda *et al.*, 1995), was positively selected in all our sequences. Further epitope mapping and site-directed mutagenesis studies are required to confirm the effect of specific substitutions. In contrast, none of the positively selected RSV B sites were associated with previously described epitopes (Martínez *et al.*, 1997); there is, however, much less information on RSV B epitopes. Interestingly, positively selected sites in RSV B, such as aa 227, 257, 276, 291 and 293, were associated with the major division of the RSV B phylogenetic tree into two branches (Botosso *et al.*, 2009).

Glycosylation dramatically influences the antigenicity of the G protein (Palomo *et al.*, 1991) and can thus contribute to immune evasion by masking or creating antigenic sites, abolishing G protein recognition by carbohydrate-specific

antibodies (Melero *et al.*, 1997; Palomo *et al.*, 2000), or enhancing the reactivity of certain antibodies (Palomo *et al.*, 2000). These influences might help explain the poor immune memory for RSV. In our RSV A data, predicted glycosylation sites were located next to nine of 18 positively selected sites and overlapped with two, suggesting possible selection on amino acid glycosylation. There is a need to further explore a possible correlation between glycosylation patterns and seasonal shifts in subgroups that have been observed in many previous studies, including ours (data not shown). Note that these studies (including our previous study) have shown strong seasonal peaks of RSV prevalence during the rainy season from May to October (Do *et al.*, 2011).

Our analysis of intra-host diversity showed similarly high rates of genetic variation in the G gene of RSV A and B (Fig. 3), which overlaps with the positive selection findings from our inter-host analysis (Table 1). Intriguingly, a hotspot region was also identified in the SH gene of both RSV A and B strains (and one in the L gene for RSV A) which was not detected to be under positive selection. This could potentially be due to the small number of sequences analysed for positive selection signatures. Alternatively, this could suggest a more direct role for the nucleic acid in viral survival that does not impact selection at the protein level. Our analysis also identified several coldspots that correlate well with known functionally important regions in RSV, suggesting that this approach could complement traditional methods (e.g. multiple sequence alignments to detect sequence conservation) for identifying new functional regions and drug targets. Overall, the large number of coldspots detected is consistent with the observation that RSV is genetically very constrained and has replicated largely unchanged over the past 50 years (Tan *et al.*, 2012).

Our inter- and intra-host analysis indicated that the M gene was the most conserved in the genome, consistent with its biological function as a major structural protein involved in viral replication, assembly and interaction with host cells (Kumaria *et al.*, 2011; Rebuffo-Scheer *et al.*, 2011; Tan *et al.*, 2012). The presence of highly conserved M gene regions in both RSV A and B clinical samples (Fig. 3a, b) makes it a promising candidate for vaccine and drug development. Coldspots in M overlap with leucine-rich nuclear export signals (aa 195–206 and 46–60); *in vitro* inhibition of nuclear export by leptomycin has been shown to block virus assembly and RSV virion production (Ghildyal *et al.*, 2009). Other previously explored drug targets within the RSV genome include highly conserved regions of the N and F proteins (ALN-RSV01/RSV-604 and motavizumab, respectively) (Dormitzer *et al.*, 2012; Empey *et al.*, 2010). Regions containing signals that direct viral mRNA transcription or antigenome synthesis, such as the 3′ extragenic leader and the gene start and gene end sequences, were also found to be conserved in our study (Table 3) (Collins *et al.*, 1991; Fearns *et al.*, 2000; Kuo *et al.*, 1996, 1997; Mink *et al.*, 1991; Moudy *et al.*, 2003).

Some studies have suggested that intra-host variation correlates with disease severity (Vignuzzi *et al.*, 2006, 2008); however, our phylogenetic analysis of consensus sequences did not reveal any such clustering. Nevertheless, the identification of unique hotspots in the mucinoid I region of the G protein and in the central region of the L protein in severe RSV cases is intriguing, although the limited number of severe RSV cases (n=6) in this study precludes statistical analysis. Our focus on patients with high viraemia also limits our ability to explore factors that could be involved in viral attenuation, such as hotspots that differentiate between patients with high versus low viral loads.

We were also able to compare the evolution of RSV A and B. Similar to Rebuffo-Scheer *et al.* (2011), rates of synonymous and non-synonymous substitution, the rate of nucleotide substitutions per site, and the number of positively selected sites per gene and in total were higher in RSV A than in RSV B. In contrast, others have shown that RSV B evolves faster than RSV A (Martínez *et al.*, 1999; Matheson *et al.*, 2006). This discordance could be explained by the larger number of sequences from subgroup A collected over a longer period and emphasizes the need for further studies on RSV B.

METHODS

Ethics. This study was approved by the Institutional Review Board of Children's Hospitals 1 and 2, the Scientific and Ethical Committee of the Hospital for Tropical Diseases, Ho Chi Minh City, Vietnam and the Oxford University Tropical Research Ethical Committee, Oxford, UK. Written informed consent was obtained from parents or legal guardians of children before enrolment into the study.

Collection, preparation and deep-sequencing of clinical samples. Nasopharyngeal swabs were collected from 301 RSV-positive children enrolled in a study with 632 enrolled patients on acute lower respiratory infections between May 2009 and December 2010 at the two largest paediatric referral hospitals in southern Vietnam: Children's Hospitals 1 and 2 in Ho Chi Minh City. Swabs were placed in 1 ml viral transport medium (WHO, 2006), kept at 4 °C for a maximum of 24 h, and then aliquoted and stored at −80 °C. A shift in the predominant subgroup from B to A was seen between the 2009 and 2010 seasons (data not shown). Severe cases were defined as patients hospitalized in the paediatric intensive care unit requiring supplementary oxygen/mechanical ventilation or having a peripheral capillary oxygen saturation (SpO_2) <92 %.

After viral RNA extraction with a QIAamp Viral RNA Mini kit (Qiagen), 53 out of 301 samples were selected for whole-genome sequencing. These showed high viral loads, as determined by quantitative reverse transcription-PCR (Do *et al.*, 2012), and encompassed two consecutive transmission seasons. More details of the sequencing protocol (primer sequences, read statistics and example coverage plots) are available upon request. In all, 53 RSV-containing samples (37 RSV A and 16 RSV B) were selected for analysis as representative of the total (summary information of all sequenced samples is available upon request) and comprised 18 % (53/301) of the enrolled RSV-positive patients. All sequences have been uploaded to GenBank and next-generation sequencing data have been uploaded to the European Nucleotide Archive.

Assembly of full-length consensus sequences. For each sample, we computed consensus/master genome sequences with iCORN (Otto *et al.*, 2010), which iteratively maps reads against a reference

sequence and extracts a new reference. As an initial reference, we used a mosaic sequence created from Sanger-sequenced fragments (from samples VN-217 and VN-144 for RSV A and B, respectively), which covered the genome only partially and filled the gaps with RefSeq NC_001803 for RSV A and the recently sequenced JN_032120 (Rebuffo-Scheer *et al.*, 2011) for RSV B. The latter was preferred over RefSeq NC_001781 because it contains a 60 base duplication in the G gene, which we also identified in *de novo* assembled contigs otherwise too short to be useful (data not shown). Reads of each sample were mapped against each sample's consensus sequence with RazerS (Weese *et al.*, 2009). Reads overlapping PCR primer positions were removed and base-quality recalibration was performed with GATK (McKenna *et al.*, 2010) ignoring sites with >1 % variation.

Phylogenetic analyses. For genotyping, maximum-likelihood phylogenetic trees of the hypervariable region of the G gene of RSV A and B were reconstructed from an assembled database of 165 and 74 sequences, respectively (database available upon request). RSV genotypes were assigned based on the maximum-likelihood trees and relationship to sequences of representative genotypes if bootstrap support was >70 % (Arnott *et al.*, 2011; Dapat *et al.*, 2010; Gaunt *et al.*, 2011; Venter *et al.*, 2001).

To study the relationships of Vietnamese RSV to other whole genomes available in GenBank, we used MUSCLE 3.8 (Edgar, 2004) to generate alignments with sequences from Rebuffo-Scheer *et al.* (2011) ($n=25$ for RSV A, $n=9$ for RSV B), Kumaria *et al.* (2011) ($n=14$ for RSV A), 11 reference RSV A sequences (GenBank accession numbers U39661.1, NC_001803.1, AF035006.1, U39662-S2, U50363.1, U50362.1, U63644.1, M74568.1, AY911262.1, FJ948820.1 and FJ614813.1) (Collins *et al.*, 1987; Connors *et al.*, 1995; Crowe *et al.*, 1996; Firestone *et al.*, 1996; Lo *et al.*, 2005; Mink *et al.*, 1991; Stec *et al.*, 1991; Tolley *et al.*, 1996; Whitehead *et al.*, 1998) and four reference RSV B sequences (GenBank accession numbers AF013255.1, AF13254.1, AY353550.1 and NC_001781.1) (Karron *et al.*, 1997).

For each subgroup, separate alignments of protein-coding sequences (NS1, NS2, N, M, P, G, F, SH, M2 and L), NCRs and IGSs were generated using BioEdit 7.0.9.0 (Hall, 1999). Maximum-likelihood phylogenies were reconstructed for the whole genome and protein-coding sequences using the GTR + Γ4 model of nucleotide substitution determined by ModelTest 3.7 (Posada & Crandall, 1998) in RAxML 7.0.4 (Stamatakis, 2006) with 1000 bootstrap replicates. Phylogenies were viewed with FigTree (http://tree.bio.ed.ac.uk/software/figtree/).

Substitution rates and positive selection analysis. Protein-coding sequence alignments were analysed with SNAP (Korber, 2000) to estimate overall substitution rates, and the rates of synonymous (d*S*) and non-synonymous substitutions (d*N*) (Nei & Gojobori, 1986). Percentages of conserved nucleotides in each NCR for RSV A and B subgroups were calculated and compared with reference viruses with GenBank accession numbers NC_001803 (RSV A) and NC_001781 (RSV B). The HyPhy software package (Pond *et al.*, 2005) was used to identify individual codons within the protein-coding sequence evolving under positive selection. Duplicate sequences were removed from multiple sequence alignments. Recombination breakpoints were predicted using a combination of SBP and GARD programs, and used to split the multiple sequence alignments into recombination-free subalignments, which were analysed for positive selection (Nielsen & Yang, 1998) using the Nielsen–Yang (NY98) method. In subalignments with evidence for positive selection, a Bayesian calculation for posterior probabilities was used to identify individual codons under selection (Yang *et al.*, 2005); sites with a posterior probability of $P>0.5$ for having d*N*/d*S*>1 were identified as possibly under positive selection; those with high posterior probabilities ($P>0.95$) were identified as significant.

Variation in coding genes and predicted glycosylation sites. The deduced amino acid sequences of the C-terminal HVR2 of the G protein were compared with the following references (GenBank accession number): the prototype A2 (M11486), BA4128/99B (AY33364), Long (AY911262), CH18537 (M17213), B1 (NC_001781) and BA1 (AF013254). Potential *N*- and *O*-glycosylation sites in the C-terminal HVR2 of the G protein were identified with the online services NetOGlyc 3.1 and NetNGlyc 1.0 (http://www.cbs.dtu.dk/services/).

Analysis of intra-host genetic variation. Low-frequency SNVs present in the viral population in each sample were predicted with the sensitive, quality-aware variant caller LoFreq (Wilm *et al.*, 2012). We only considered variants passing a *P*-value threshold of <5 % after multiple testing correction (Bonferroni). See https://github.com/CSB5/2015-do-hrsv for a list of all predicted intra-host variants. To identify mutational hotspots per sample, a scanning window approach was used (window size of 20 and overlap of 5 nt) to look for an excess of SNVs compared with the genome-wide mean (binomial test; Bonferroni-corrected *P*-value <0.05). For coldspots, SNVs were pooled from all samples and SNV-free windows of significantly large size were detected (binomial test, Bonferroni-corrected *P*-value <0.05). Variant calling and hotspot/coldspot analysis followed the recipes described and validated in Wilm *et al.* (2012).

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