Examining intra-host genetic variation of RSV by short read high-throughput sequencing

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Abstract

Every viral infection entails an evolving population of viral genomes. High-throughput sequencing technologies can be used to characterize such populations, but to date there are few published examples of such work. In addition, mixed sequencing data are sometimes used to infer properties of infecting genomes without discriminating between genome-derived reads and reads from the much more abundant, in the case of a typical active viral infection, transcripts. Here we apply capture probe-based short read high-throughput sequencing to nasal wash samples taken from a previously described group of adult hematopoietic cell transplant (HCT) recipients naturally infected with respiratory syncytial virus (RSV). We separately analyzed reads from genomes and transcripts for the levels and distribution of genetic variation by calculating per position Shannon entropies. Our analysis reveals a low level of genetic variation within the RSV infections analyzed here, but with interesting differences between genomes and transcripts in 1) average per sample Shannon entropies; 2) the genomic distribution of variation 'hotspots'; and 3) the genomic distribution of hotspots encoding alternative amino acids. In all, our results suggest the importance of separately analyzing reads from genomes and transcripts when interpreting high-throughput sequencing data for insight into intra-host viral genome replication, expression, and evolution.

Introduction

A viral infection involves a replicating and therefore evolving population of viruses. The level and distribution of diversity at a given time after infection will depend on the size and composition of the inoculum, the duration of viral replication, how rapidly viral genetic variation is produced de novo, and the nature of host selective pressures (initial screening via secreted antibodies, the innate immune response, and clearing of infected cells by the cellular immune response). Several studies suggest that respiratory viruses like respiratory syncytial virus (RSV) and influenza undergo mostly neutral evolution within a single host during natural infection (1-3), but few report on the expected levels and distribution of genetic variation and it is unknown to what extent different immune functions might constrain viral evolution.

Here we determined the genetic variation contained within intra-host populations of RSV infecting members of a group of previously described adult hematopoietic cell transplant (HCT) recipients (4-7). Cancer patients undergoing myeloablative conditioning require HCT to restore a healthy supply of resident bone marrow cells, including leukocytes such as T and B lymphocytes, neutrophils and macrophages that play essential roles in the host immune response to viral infections. The majority of HCT recipients considered here were fully engrafted at the time of infection and experienced mostly mild disease (4-7).

We sequenced capture probe-derived (Twist Biosciences, Inc.) RSV cDNAs in nasal wash samples from HCT recipients naturally infected with RSV and separately analyzed reads derived from genomes and transcripts, and assessed both data sets for levels and distribution of variation using calculations of per position Shannon entropy. Shannon entropy provides an elegant metric of variation well suited to analyses of high-throughput sequencing data. We found low levels of total genetic variation within the RSV infections studied here, and interesting differences in the levels and distribution of genetic variation contained within genome- and transcript-derived read sets.

Results

i. Patient sample data

Nasal wash samples were obtained from a previously described cohort of hematopoietic cell transplant (HCT) recipients naturally infected with respiratory syncytial virus (RSV) (Table 1) and were subjected to short read high-throughput sequencing (NovaSeq Illumina). Patients were

68 infected with either of two widely circulating RSV genotypes (A/Ontario or B/Buenos Aires) and
69 shed virus for either less than 14 days or more (Table 1). Shedding time correlated with
70 transplant type (autologous vs. allogeneic), with patients receiving an autologous HSC transplant
71 tending to show shorter viral shedding times and a more robust neutralizing antibody response
72 (Table 1). A nasal wash sample was collected from each patient at the time of study enrollment
73 and approximately weekly for up to 4 weeks (Table 2). A subset of all samples were successfully
74 sequenced (≥ 90% coverage of whole RSV genome at ≥ 1x sequencing depth) and a further
75 subset were sequenced at a depth permitting downstream analyses to be described (Table 2).
76 Additionally, because of the sequencing methodology employed, it was possible to separately
77 analyze reads from genomes and transcripts.

78 *ii. Varying read depth and variation in sequenced RSV genomes and transcripts*

79 We began our analysis by plotting sequencing or read depth across ON and BA reference
80 genomes for data derived from 1) genomes and 2) transcripts (Fig 1). The latter should also
81 reflect the contribution of low-abundance anti-genomes. All 4 data sets show fairly uniform
82 coverage across the RSV genome (Fig 1), with the average read depth from transcripts
83 exceeding that from genomes by approximately 100-fold.

84 In order to begin characterizing the genetic variation supported by the intra-host
85 populations of infecting RSV sequenced here, we adopted an approach based on measuring the
86 Shannon entropy (*H*) of every nucleotide position in our sequencing data set (Fig 2). Plots of per
87 position Shannon entropy across the two reference RSV genomes reveal varying levels of
88 variation across the RSV genome and across samples, with entropy values from genome
89 derived-reads generally exceeding those from transcripts (Fig 2). Calculations of average or bulk
90 Shannon entropy per sample make clear that sequenced RSV genomes show greater variation
91 than sequenced RSV transcripts (Fig 3). Restricting our attention to mean values from day 0
92 samples, genomes show 4-5x more variation than transcripts (Fig 3), although the bulk Shannon
93 entropy is low across samples. For instance, the maximum per sample average Shannon
94 entropy found (=0.11) would in the simplest case of two possible 'alleles' (A or G, say) correspond
95 with a minority 'allele' abundance of just over 2%. Thus, whether analyzing reads from RSV
96 genomes or transcripts, the level of genetic variation supported by an infecting population of
97 RSV within a single host is low in the samples tested.

98 We also observed that the more variable genomes showed a general drop in bulk entropy
99 over time, while transcript entropies appeared more stable (Fig 3). There are exceptions to the
100 decline in genome entropies over time: one patient showed a bulk entropy maximum at day 14,
101 and a few showed sharp increases ($\Delta H \approx 0.02$) over 2 to 5 days (Fig 3). The former patient shed
102 RSV for longer than 14 days, and most cases showing an increase in bulk genome entropy over
103 any window of time came from longer shedders (Fig 3).

104 *iii. Distribution of hotspots and estimates of functional variation*

105 Our initial analysis of per position and bulk Shannon entropies from genome- and transcript-
106 derived reads showed low levels of genetic variation within intra-host populations of infecting
107 RSV. However, bulk or average per sample Shannon entropies mask the existence of positions
108 showing exceptionally high variation. Thus, we decided to analyze our data for such 'hotspots'
109 ($H \geq 0.3$) and to determine their distribution across the RSV genome. For this analysis, we
110 restricted our attention to genome- and transcript-derived data sets showing at least 10x
111 coverage across 90% of the reference genome. In both data sets, a minority of positions show
112 a Shannon entropy high enough to be considered hotspots (Fig 4). However, consistent with the
113 differences observed between sequenced RSV genomes and transcripts, RSV genomes are
114 ~20-fold more enriched for such hotspots (~3.7% vs. 0.2% of all positions analyzed per sample).
115 In addition, the genomic distribution of hotspots is much more uniform across non-coding and
116 coding sequences in genome- than transcript-derived reads and variation in the latter has a
117 strong tendency to cluster in non-coding sequences (Fig 4).

118 We further analyzed these hotspots for obvious functional variation by determining
119 whether hotspots within coding sequences encoded alternative amino acids. Interestingly,
120 whether from genomes or transcripts, approximately 50% of all hotspots identified encoded
121 alternative amino acids (Fig 5). The number and distribution of these sites varied from sample
122 to sample, and more highly in transcript- than genome-derived reads (Fig 5).

123 Per position Shannon entropies were recalculated for the amino acid (AA) sequences
124 derived from nucleotide hotspots within coding sequences (Fig 6). For both genome- and
125 transcript-derived read sets, the level of variation for any given AA hotspot varies greatly from
126 sample to sample, but genome-derived reads show a much greater number of and more highly
127 distributed AA hotspots than transcript-derived reads (Fig 6).

128

129 **Discussion**

130 Our study revealed generally low levels of genetic variation with interesting differences between
131 genome- and transcript-derived read sets from intra-host populations of RSV infecting a cohort
132 of adult HCT recipients.

133 The 100-fold difference observed in average read levels between genomes and
134 transcripts is consistent with the expectation established from in vitro measurements. These
135 results suggested our sequencing data were minimally biased to different regions of the RSV
136 genome and to either of the two major species of viral nucleic acid present during RSV infection
137 (transcripts and genomes). However, reads mapping to the G gene are slightly more abundant
138 in both genomes and transcripts, especially those derived from RSV/A/ON infections. This
139 appears consistent with multiple studies showing higher than expected levels of the G gene (8-
140 11), especially G gene mRNA. However it may also reflect a subtle sequencing bias of unknown
141 origin, as it is present in both genome- and transcript-derived reads. There is also a noticeable
142 bump in reads mapping to the NS2 gene from RSV/B/BA genomes. This fluctuation appears
143 specific to RSV/B/BA genomes and may reflect a larger proportion of variant genomes (perhaps
144 partly or fully defective viral genomes) containing the NS2 gene along with a subset of the
145 remaining RSV genes. This might also reflect a subtle sequencing bias.

146 Although both genome- and transcript-derived read sets showed a number of high
147 entropy positions across reference genomes in different samples, the vast majority of positions
148 showed little variation. For example, and as mentioned previously, the largest average or bulk
149 Shannon entropy calculated for a single sample was 0.11, which equals a minority 'allele'
150 abundance of just over 2% assuming the simplest case of only two possibilities (A or G, say).
151 The average bulk Shannon entropy for a given sample is closer to 0.03 for genomes and 0.01
152 for transcripts. The former value corresponds to a minority 'allele' abundance of around 0.5%
153 (again assuming only two possible 'alleles'). Nevertheless, genome sequences clearly contained
154 greater variation than transcripts (approximately 4-5x more) and bulk genome entropies from
155 different patients showed more interesting dynamics, generally dropping over time, while bulk
156 transcript entropies were more stable. This might reflect a purifying selection of viral genomes
157 within the host while the greater stability of the lower transcript entropies may be a consequence

158 of a time-independent error rate for transcribing RSV polymerases. There were exceptions to
159 the decline in genome entropies over time, with most samples showing an increase over any
160 time interval coming from patients who shed RSV for ≥ 14 days (vs. < 14 days). This might
161 reflect, albeit very subtly, the somewhat greater permissiveness of these hosts.

162 Genome- and transcript-derived reads showed interesting differences in the number and
163 distribution of variation 'hotspots' ($H \geq 0.3$). We chose a Shannon entropy of ≥ 0.3 to identify
164 variation hotspots because $H = 0.3$ corresponds with a rather large minority 'allele' abundance
165 of 10% assuming two possibilities (A and G, say). Genomes showed approximately 20-fold more
166 hotspots than transcripts, and the distribution of hotspots was much more uniform across non-
167 coding and coding sequences in genome- than transcript-derived reads. Indeed, variation in the
168 latter had a strong tendency to cluster in non-coding sequences, especially when considering
169 the density of hotspots (i.e., the number of hotspots within a given region divided by the number
170 of positions within that region). Indeed, transcript hotspots appear to be a non-random subset of
171 genome hotspots, potentially indicating the contribution of transcriptionally mute defective viral
172 genomes to our sequencing data (12, 13).

173 We further analyzed variation hotspots for clear functional variation by determining
174 whether hotspots within coding sequences encoded alternative amino acids. Approximately 50%
175 of all hotspots identified encoded alternative amino acids whether from genomes or transcripts.
176 We thus estimated that the percentage of all coding sequence positions in the RSV genome
177 encoding alternative amino acids was ~2% from sequenced genomes and ~0.1% from
178 transcripts within our data. As observed throughout this study, and consistent with the presence
179 of defective viral genomes (12, 13), the variation contained within transcripts is a subset of that
180 contained within genomes.

181 Here we made use of the ability to separately analyze genome- and transcript-derived
182 reads from high-throughput sequencing data to characterize the levels and distribution of genetic
183 variation contained within natural infections of RSV. Future studies might involve patient
184 populations containing greater differences in host immune status to better search for an immune-
185 mediated effect on the magnitude, distribution, and evolution of viral genetic variation within
186 single infections. It would also be ideal to collect data from a larger number of patients and more
187 densely through time – Grad et al. sequenced 26 samples over more than 2 months from a

single infant infected with RSV (14) – while optimizing sample collection for the generation of high-quality sequencing data. Finally, subjecting samples to long read sequencing in order to resolve variant viral genomes including defective viral genomes would be highly informative.

Methods

i. Study population

A group of previously described hematopoietic cell transplant (HCT) recipients with laboratory-confirmed RSV infection and negative chest radiography findings were identified from 2012 to 2015 (4-7). Patients were enrolled as part of a ribavirin efficacy trial within 72 hours of RSV diagnosis. Longitudinal nasal wash (NW) samples were collected at enrollment (i.e., day 0), day 2-7, and weekly up to 29 days post-enrollment. The study protocol was approved by the institutional review boards of Baylor College of Medicine and the University of Texas MD Anderson Cancer Center. Written informed consent was obtained from all participants.

ii. Sample preparation and sequencing

Viral RNA was extracted from NW samples using the Mini Viral RNA Kit (QIAGEN Sciences, Maryland, USA) on the automated platform QIAcube (QIAGEN, Hilden, Germany) according to the manufacturer's instructions. Pooled cDNA libraries were hybridized with biotin-labeled probes from the RSV Panel (Twist Biosciences, Inc.) at 70°C for 16 hours according to (15). The RSV probe set size was 23.77 Mb and was designed based on 1,570 publicly available genomic sequences of RSV isolates. In this probe set there are 87,025 unique probes of 80 bp length which cover 99.79% of the targeted isolates. Captured virus targets were incubated with streptavidin beads for 30 minutes at room temperature. Streptavidin beads bound with virus targets were washed and amplified with KAPA HiFi HotStart enzyme. The amount of each cDNA library pooled for hybridization and post-capture amplification PCR cycles (12–20) were determined empirically according to the virus Ct values. In general, between 1.8 to 4.0 µg of pre-capture library were used for hybridization with the probes; post-capture libraries were sequenced on an Illumina NovaSeq S4 flow cell to generate 2x150 bp paired-end reads.

iii. Sequencing data preparation

Cleaned RNA sequence was called using the VirMap pipeline (16). Sample sequences were aligned to custom RSV reference genomes using Bowtie2 (17). Samtool's mpileup (18) command was used for read pileup creation for each sample. A custom Python (Python Software Foundation, www.python.org) script was utilized to transform the pileup output into a tabular form.

iv. Analysis of sequencing data

The sequencing data from each sample was separated into two subsets, genomic and transcriptomic. Unless otherwise noted, a minimum sequencing depth of 10 reads was required at each position across 90% of the reference genome (RSV/A/ON or RSV/B/BA) for each sample to be used in downstream analyses.

v. Viral sequence Shannon entropy calculations

Shannon entropy (*H*) was defined within each sample and at every genomic position as:

$$H_i = \sum_{(K=A,C,G,T)} - p_{(I,K)} \cdot ln(p_{(I,K)})$$

i = sample identified; has a dimension of rows = # genomic positions with coverage ≥ 10 reads and columns = 1

p = proportion of base = the number of counts for a given base divided by the total counts at a given genomic position

I = genomic position

A, C, G, and T= nucleotide base type

Analyses were conducted in R 3.4.4 (R Foundation for Statistical Computing, Vienna, Austria) unless otherwise stated.

vi. Detecting non-synonymous changes and calculating amino acid Shannon entropies

To predict the amino acid (AA) representation across coding sequences from genome- and transcript-derived reads, we assumed a uniform sequencing error rate of nt substitution and only analyzed nt composition (i.e., did not consider insertions/deletions or associated frameshift

mutations). Nucleotide counts, binned as the four possible nt bases (A, C, G, T), were determined at each coding position within each sample. The AA abundance was calculated at each position within a codon; if neighboring positions within a codon showed more than one nt base, the majority base(s) was used to determine the AA assignment.

247 **Figures and tables**

	RSV shedding time (days)		
	< 14	≥ 14	p-value*
Age (y), mean ± st. dev.	53.2±14.0	46.3±18.5	
% female (n)	57.1 (12)	42.1 (8)	
Race			
% White (n)	45.0 (9)	65.0 (13)	
% Black (n)	15.0 (3)	5.0 (1)	
% Hispanic (n)	30.0 (6)	30.0 (6)	
% Asian (n)	10.0 (2)	0.0 (0)	
Transplant type			
% autologous (n)	45.0 (9)	10.0 (2)	significant
% allogeneic (n)	55.0 (11)	90.0 (18)	significant
Time from HCT (days), median (range)	169 (6-945)	100 (5-1067)	
Acute RSV Nt Ab titer (log2)	7.0	6.2	
Convalescent RSV Nt Ab titer (log2)	10.2	8.2	significant

248

249

250 **Table 1. Demographics of RSV-infected HCT recipients.** A group of previously described
251 hematopoietic cell transplant (HCT) recipients with laboratory-confirmed RSV infection and negative
252 chest radiography findings were identified and enrolled as part of a larger efficacy study within 72 hours
253 of RSV diagnosis (4-7). Patients shed RSV for either less than 14 days or more. Shedding time
254 correlated with transplant type (autologous vs. allogeneic), with patients receiving an autologous HSC
255 transplant tending to show shorter viral shedding times and a greater neutralizing antibody response at
256 convalescence (i.e., 14-60 days after hospitalization).
257

a.

Sequencing: 90% coverage at 1x depth

Infecting virus	RSV shedding time (days)	# subjects	Samples per subject, mean [range]	Duration of illness covered (days), mean [range]
RSV/A/ON	< 14	7	1.6 [1-2]	3.4 [0-5]
	≥14	9	2.7 [2-5]	12.8 [5-25]
RSV/B/BA	< 14	8	2.0 [1-3]	2.9 [0-5]
	≥14	7	3.4 [2-6]	12.3 [5-28]

b.

Sequencing: 90% coverage at 10x depth

Infecting virus	RSV shedding time (days)	Read type (Gen/Trx)	# subjects	Samples per subject, mean [range]	Duration of illness covered (days), mean [range]
RSV/A/ON	< 14	**Gen**	4	1.0 [1-1]	1.7 [0-5]
		Trx	7	1.6 [1-2]	3.0 [0-5]
	≥14	**Gen**	7	1.6 [1-4]	2.6 [0-14]
		Trx	9	2.7 [2-5]	11.2 [5-25]
RSV/B/BA	< 14	**Gen**	5	1.4 [1-2]	1.8 [0-4]
		Trx	8	2.0 [1-3]	2.9 [0-5]
	≥14	**Gen**	5	1.4 [2-6]	2.6 [0-11]
		Trx	7	3.4 [2-6]	12.3 [5-28]

Table 2. Basic sample sequencing information for subgroups of HCT recipients and different read types. HCT recipients were naturally infected with either of two widely circulating RSV genotypes (A/Ontario [A/ON] or B/Buenos Aires [B/BA]) and shed virus for either less than 14 days or more. A nasal wash sample was collected from each patient at the time of study enrollment (i.e., day 0) and approximately weekly for four weeks. A subset of all samples were successfully sequenced at ≥ 90% coverage of whole RSV genome and ≥ 1x sequencing depth; a further subset were sequenced at a depth permitting downstream analyses to be described (≥ 90% coverage of whole RSV genome at ≥ 10x sequencing depth). Additionally, because of the sequencing methodology employed, it was possible to separately analyze reads from genomes and transcripts (Gen and Trx, respectively). **(a)** Basic summary information for samples sequenced at 1x read depth across ≥ 90% of the reference RSV genome (RSV/A/ON or RSV/B/BA). **(b)** Basic summary information for samples sequenced at 10x read depth across ≥ 90% of the reference RSV genome (RSV/A/ON or RSV/B/BA).

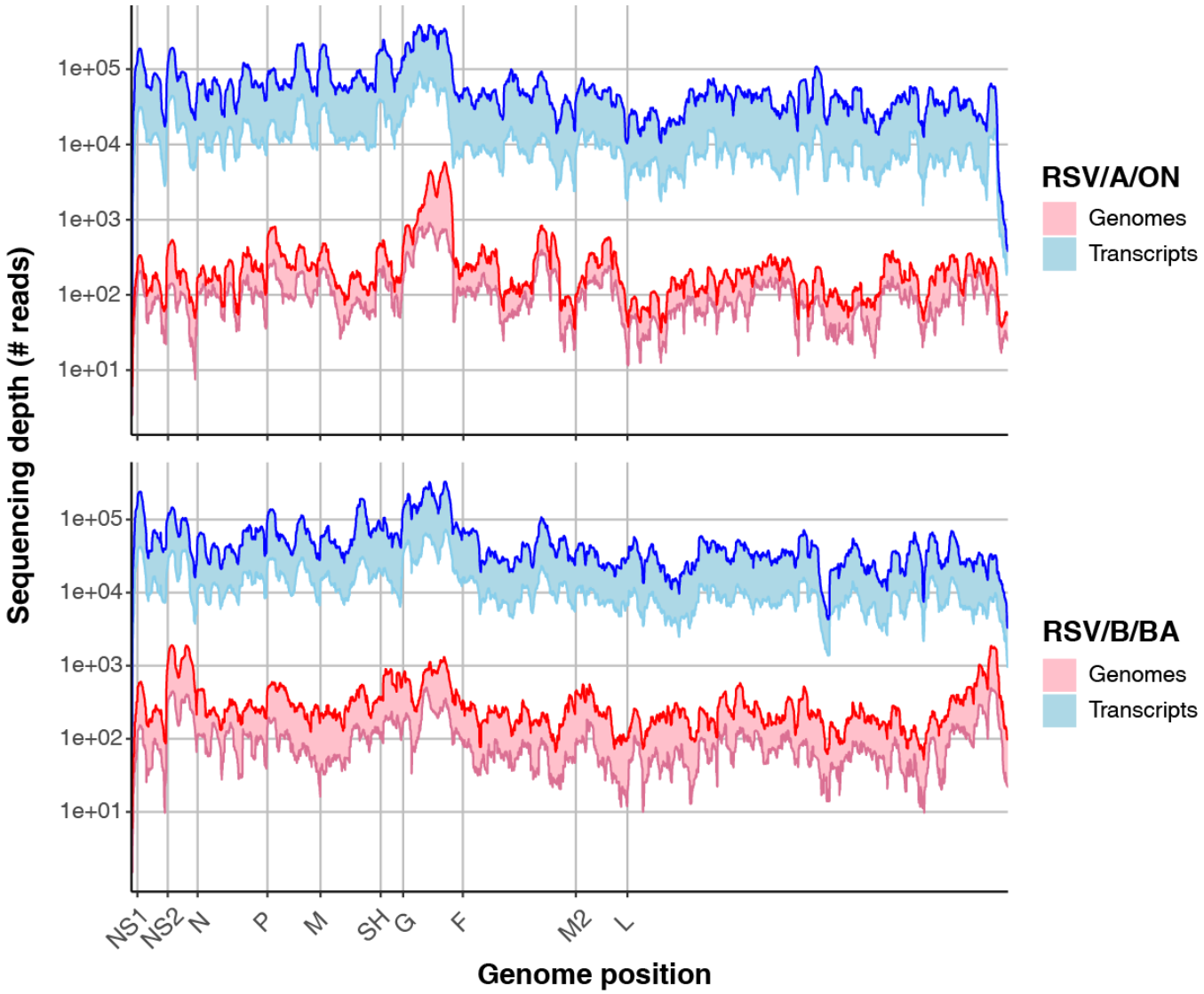


Fig 1. Transcripts exceed genomes by ~100x and reads derived from both show fairly uniform coverage of reference RSV genomes. The interquartile range of per position sequencing depth from genome- (in red) and transcript-derived read sets (in blue) is plotted along the RSV genome for both RSV/A/ON (top plot) and RSV/B/BA references (bottom plot). Darker lines represent the upper bounds of Q3 and Q1.

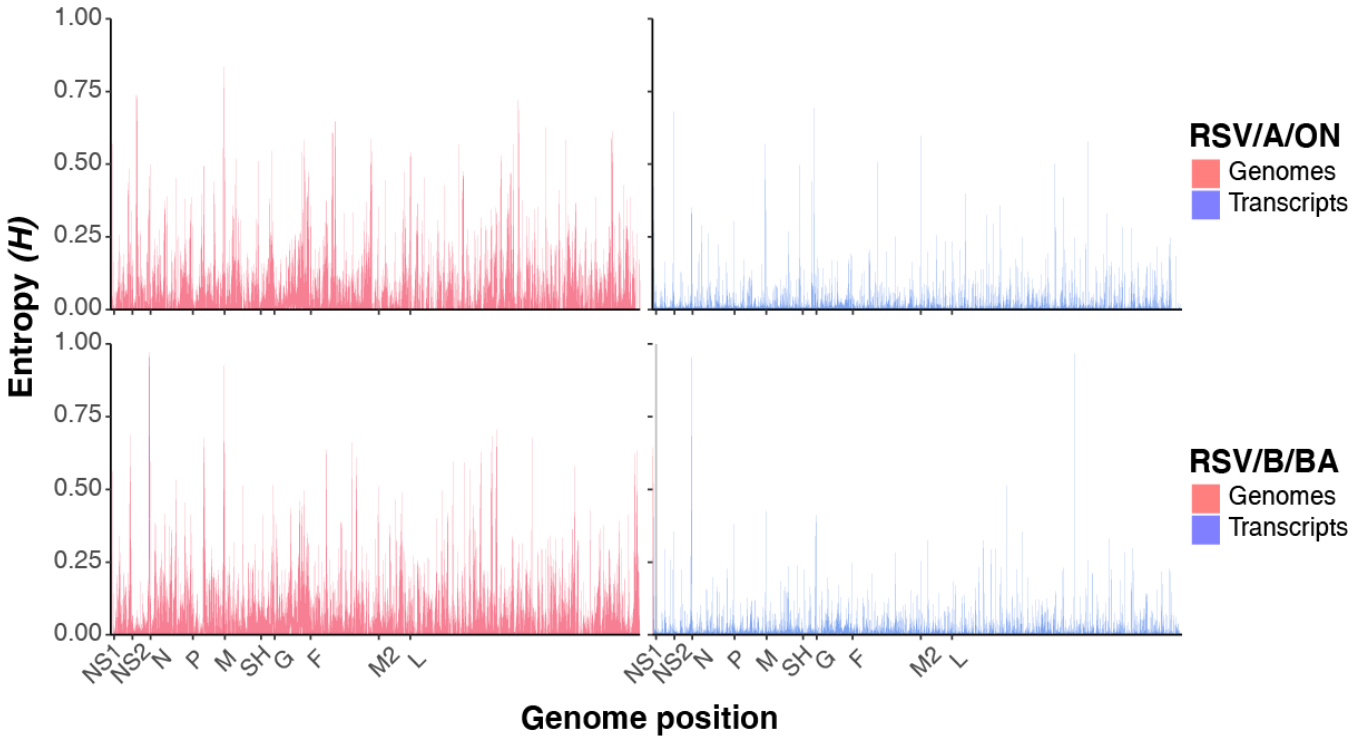


Fig 2. Genomes are more variable than transcripts but both contain highly variable positions located across the RSV genome in different samples. The interquartile range of per position Shannon entropy (*H*) from genome- (in red) and transcript-derived read sets (in blue) is plotted along the RSV genome for both RSV/A/ON (top plot) and RSV/B/BA references (bottom plot).

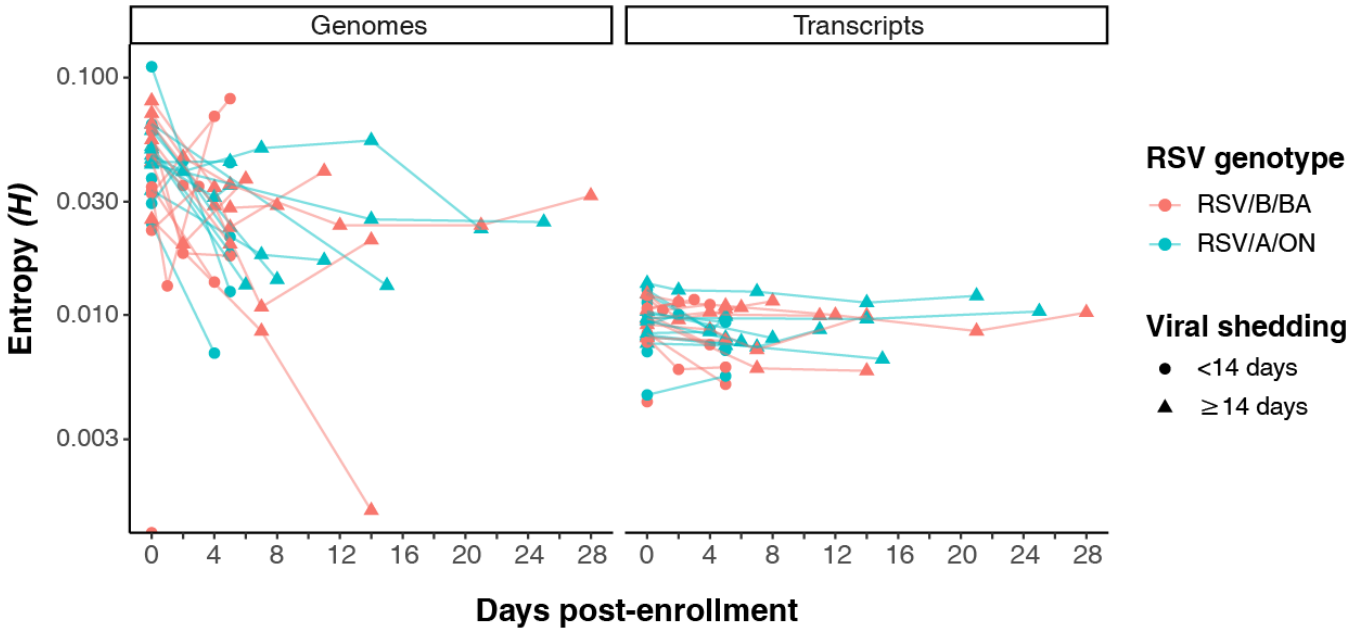


Fig 3. Per sample average or bulk Shannon entropies of genomes and transcripts differ in magnitude and dynamics. Plots of per sample average Shannon entropy (*H*) vs. day of sample acquisition. All per position Shannon entropies for single samples were averaged for genome- (left plot) and transcript-derived read sets (right plot). RSV/A/ON data in blue; RSV/B/BA data in red; data from subjects who shed RSV for < 14 days in closed circular points; data from subjects who shed RSV for ≥ 14 days in closed triangular points.

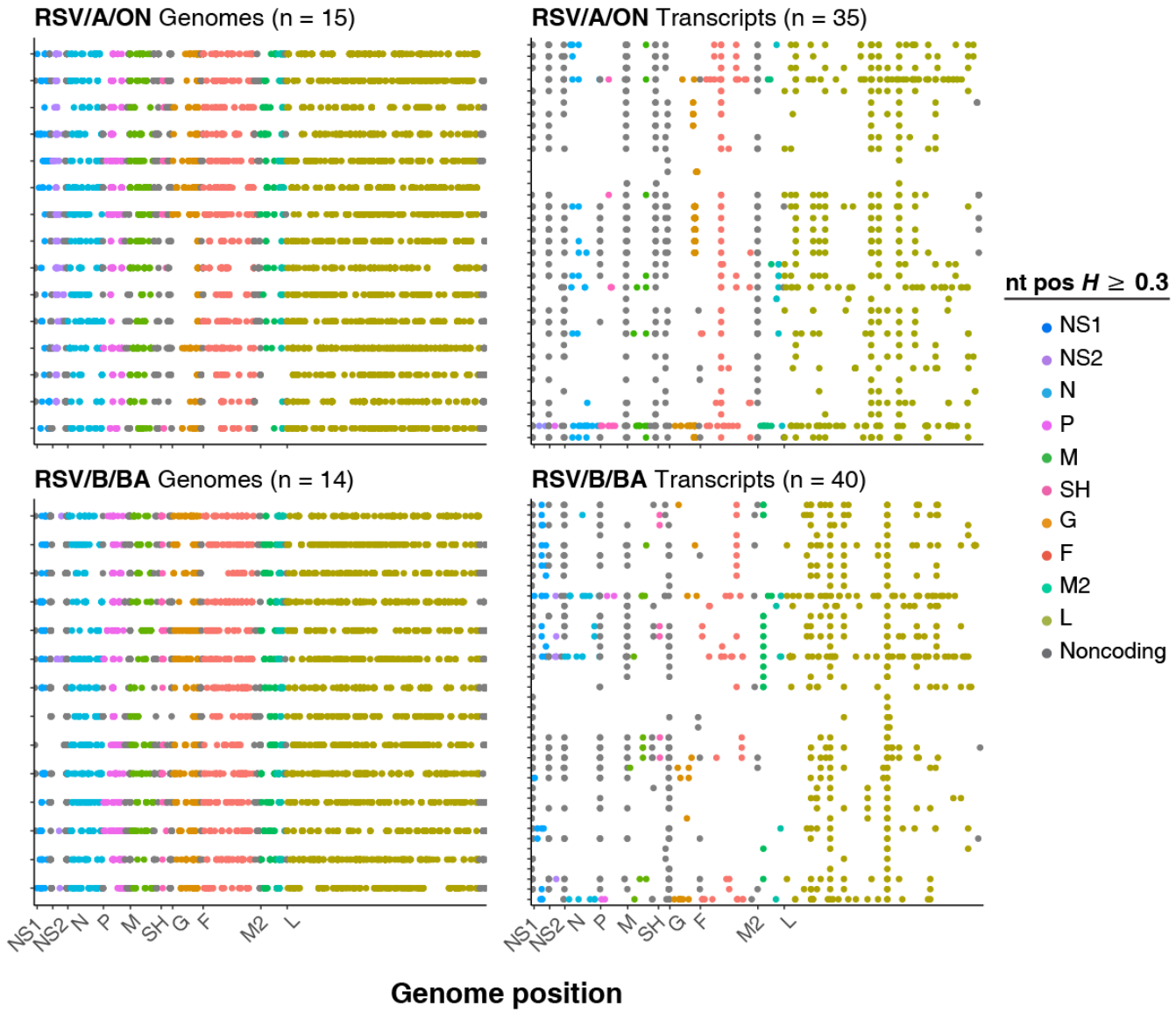


Fig 4. 'Hotspots' of variation are more numerous and densely distributed across the RSV genome in genome-derived reads than transcripts. Hotspots or single positions showing exceptionally high variation (Shannon entropy (H) ≥ 0.3) are plotted across the genome for both genome- (left plots) and transcript-derived read sets (right plots) from RSV/A/ON (top plots) and RSV/B/BA (bottom plots) infections. Each plot contains data for the number of samples indicated in parentheses. Each line in each plot shows the genomic distribution of hotspots for a single sample. Hotspots are colored according to their position within either 1) different noncoding sequences or 2) the 10 coding sequences of RSV.

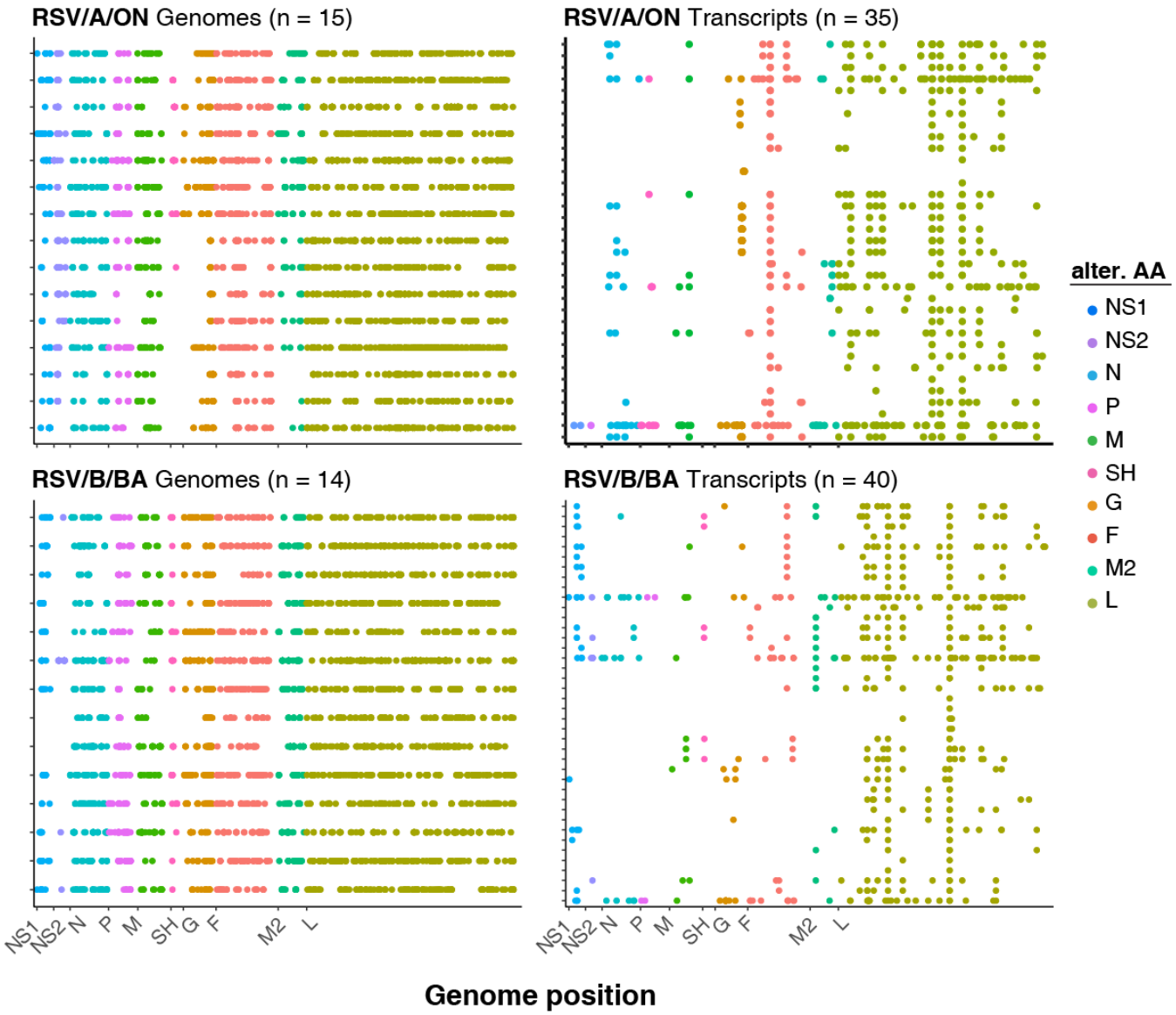
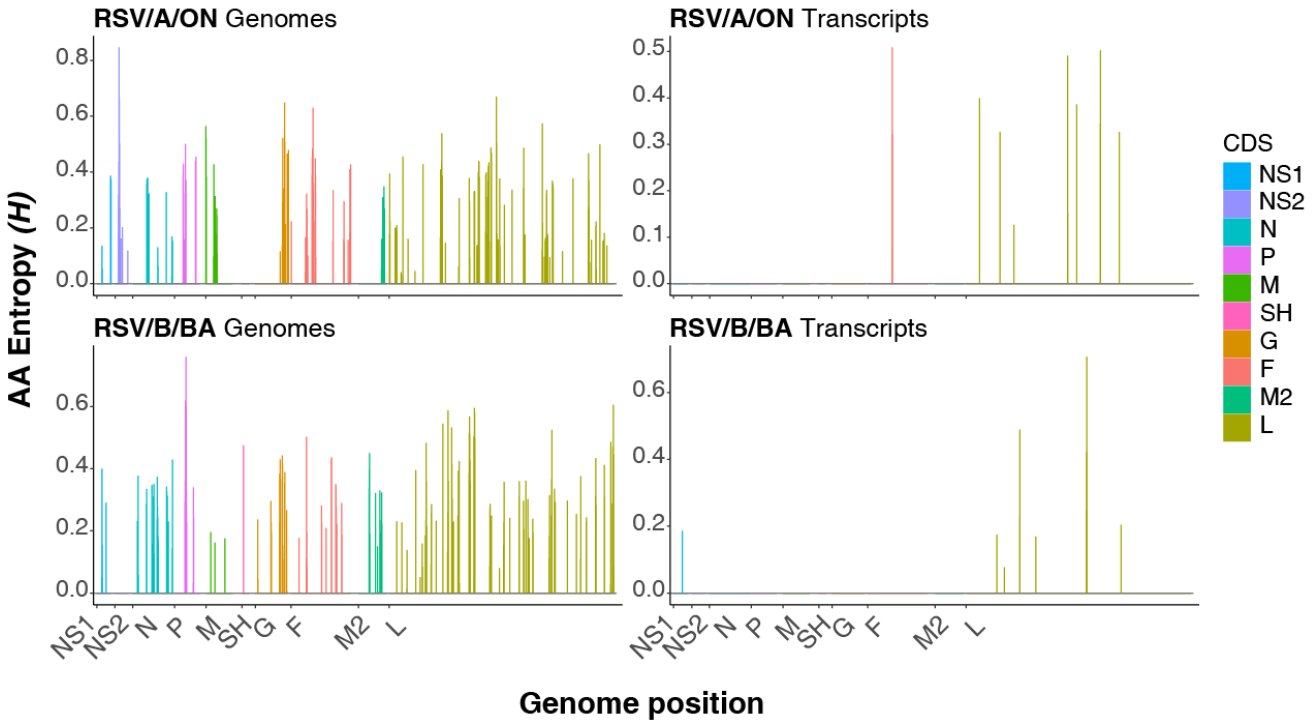


Fig 5. 'Hotspots' of functional variation are more numerous and densely distributed across the RSV genome in genome-derived reads than transcripts. Hotspots or single positions showing exceptionally high variation (Shannon entropy (H) ≥ 0.3) and encoding alternative amino acids are plotted across the genome for both genome- (left plots) and transcript-derived read sets (right plots) from RSV/A/ON (top plots) and RSV/B/BA (bottom plots) infections. Each plot contains data for the number of samples indicated in parentheses. Each line in each plot shows the genomic distribution of hotspots encoding one or more alternative amino acids for a single sample. Hotspots are colored according to their position within the 10 coding sequences of RSV.



317

318 **Fig 6. Amino acid (AA) Shannon entropies are higher across the RSV genome in genome-**
319 **derived reads than transcripts.** The interquartile range (IQR) of per position amino acid Shannon
320 entropy (*H*) from genome- (left plots) and transcript-derived read sets (right plots) is plotted along the
321 RSV genome for both RSV/A/ON (top plots) and RSV/B/BA references (bottom plots). Bars showing the
322 IQR of AA *H* are colored according to their position within the 10 coding sequences of RSV.

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