Comprehensive analysis of intra- and interpatient evolution of enterovirus D68 by whole-genome deep sequencing

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Worldwide outbreaks of enterovirus D68 (EV-D68) in 2014 and 2016 have caused widespread serious respiratory and neurological disease. To investigate diversity, spread, and evolution of EV-D68 we performed near full-length deep sequencing in 54 samples obtained in Sweden during the 2014 and 2016 outbreaks. In most samples, intrapatient variability was low and dominated by rare synonymous variants, but three patients showed evidence of dual infections with distinct EV-D68 variants from the same subclade. Interpatient evolution showed a very strong temporal signal, with an evolutionary rate of 0.0038 ± 0.0001 substitutions per site and year. Phylogenetic trees reconstructed from the sequences suggest that EV-D68 was introduced into Stockholm several times during the 2016 outbreak and then spread locally. Putative neutralization targets in the BC- and DE-loops of the VP1 protein are slightly more diverse within-host and tend to undergo more frequent substitution than other genomic regions. However, evolution in these loops does not appear to have driven the emergence of the 2016 B3-subclade directly from the 2014 B1-subclade. Instead, the most recent ancestor of both clades was dated to 2009. The study provides a comprehensive description of the intra- and interpatient evolution of EV-D68, including the first report of intrapatient diversity and dual infections. The new data along with publicly available EV-D68 sequences are included in an interactive phylodynamic analysis on `nextstrain.org/enterovirus/d68` to facilitate timely EV-D68 tracking in the future.

Enterovirus D68 (EV-D68) has been recognized as an emerging pathogen after worldwide outbreaks of serious respiratory and neurological disease in 2014 (Holm-Hansen *et al.*, 2016) and 2016 (Barnadas *et al.*, 2017; Dyrdak *et al.*, 2016; Knoester *et al.*, 2017; Messacar *et al.*, 2017; Piralla *et al.*, 2018; Ruggieri *et al.*, 2017; Wang *et al.*, 2017; Wei *et al.*, 2018). The virus was discovered in 1962 in California, USA in four children with pneumonia (Schieble *et al.*, 1967), but has been only sporadically reported until the recent outbreaks (Holm-Hansen *et al.*, 2016). The main clinical presentation of EV-D68 infection is mild to severe respiratory symptoms (Holm-Hansen *et al.*, 2016), but in rare cases EV-D68 can also cause a poliomyelitis-like disease named acute flaccid myelitis (AFM) (Dyda *et al.*, 2018; Messacar *et al.*, 2018). An increase of this otherwise unusual clinical presentation coincided with the outbreak of EV-D68 in 2014. In animal studies, strains from 2014 were more virulent than the prototype Fermon strain isolated in 1969 (Hixon *et al.*, 2017b; Zhang *et al.*, 2018a).

EV-D68, as other enteroviruses, has a genome size of approximately 7.5 kb and codes for a polyprotein, which is processed into four structural (VP1-4), and seven non-structural proteins (2A-C, 3A-D). Phylogenetically EV-D68 is divided into three clades (A, B and C)(Tokarz *et al.*, 2012), and the B clade is further subdivided into three sub-clades or lineages (B1, B2 and B3)(Gong *et al.*, 2016). The 2014 and 2016 outbreaks were caused by viruses belonging to the B1 and B3 lineages, respectively (Barnadas *et al.*, 2017; Dyrdak *et al.*, 2016; Knoester *et al.*, 2017; Piralla *et al.*, 2018; Wang *et al.*, 2017; Wei *et al.*, 2018; Zhang *et al.*, 2016). Relatively little is known about the intrapatient and interpatient evolutionary processes of EV-D68. Similar to other RNA viruses, EV-D68 has been reported to have a high substitution rate with $6.2 \cdot 10^{-3}$ (Tokarz *et al.*, 2012) or $5.12 \cdot 10^{-3}$ (Ny *et al.*, 2017) substitutions per site per year in the highly variable VP1 gene.

Mechanisms of immune protection and immune escape for EV-D68 are not well understood. Neutralizing antibodies have been shown to be protective in animal models (Dai *et al.*, 2018; Hixon *et al.*, 2017a,b; Zhang *et al.*, 2018a,b). Based on homology with the neutralizing sites NIm-IA and NIm-IB on human rhinovirus 14 (HRV-14),

regions on the BC- and DE-loops in EV-D68 have been proposed as putative epitopes for neutralizing antibodies (Liu *et al.*, 2015). However, also other sites in VP1 and other proteins may be of importance. Thus, amino acid variability in regions flanking the VP1 loops were reported to correlate with differences in neutralisation sensitivity between the prototype Fermon strain and strains from 2014 (Zhang *et al.*, 2015). For coxsackie virus B4, it was shown that a deletion in the BC-loop reduced the neutralizing effect of CVB4-specific antisera (McPhee *et al.*, 1994). VP2 and VP3 may also contain neutralizing epitopes as demonstrated in early poliovirus studies (Minor *et al.*, 1986) and as recently reviewed for the Enterovirus A genus (Fang and Liu, 2018). Little is known about the contribution of cellular immunity to the clearance and evolution of EV-D68 (or other enteroviruses).

Here, we report deep sequencing of near full-length genomes on the Illumina next-generation sequencing (NGS) platform and analyze within-sample diversity and EV-D68 evolution on during recent outbreaks. We show that within-host diversity is typically low, but that dual infection is not uncommon suggesting high prevalence during outbreaks. To facilitate exploration of the data and future analyses, we combined the whole genome sequences reported here with publicly available genome and implemented an interactive visualization in `nextstrain` available at nextstrain.org/enterovirus/d68.

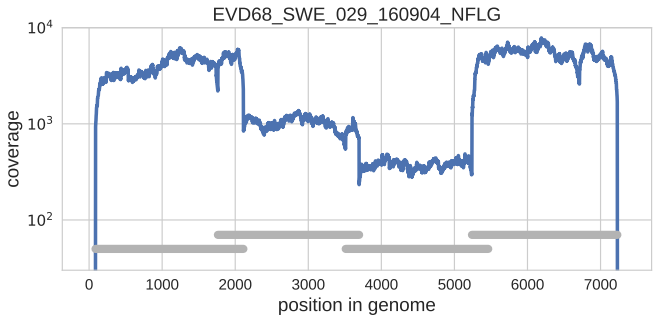

FIG. 1 **Example of coverage across the EV-D68 genome in a representative clinical sample.** Note that the coverage varies somewhat in different parts of the genome due slightly different quantity of amplified DNA from the four overlapping amplicons in the material that was used for sequencing library preparation. The four different amplicons are indicated by the horizontal grey bars.

MATERIALS & METHODS

Samples and sequences

For this study we used 54 nasopharyngeal and 5 other respiratory samples from two earlier studies on EV-D68 outbreaks in Stockholm in 2014 and 2016 (Dyrdak *et al.*, 2016, 2015). The 59 samples were drawn from 54 patients. Six patients had been sampled in 2014, and 48 patients (53 samples) in 2016. Thirty of the patients were female. The mean age was 9.0 years (median 3.6 years, range 2 months – 63 years). Five patients were sampled twice during their acute illness (0, 1, 1, 2 and 7 days apart). Details about the patient demographics are given in table S2.

The collection dates of the samples from the outbreak in 2016 ranged between August 19 to September 13. The samples had tested positive for EV-D68 at the Department of Clinical Microbiology, the Karolinska University Hospital by EV-D68 specific realtime PCR and/or VP4/VP2 sequencing. For details see (Dyrdak *et al.*, 2016, 2015). A few EV-D68-positive samples from the earlier studies were not included in this study because no sample material remained or because the virus levels were very low as evaluated by the cycle threshold (Ct) values in the EV-D68 realtime PCR. The Ct values for the included samples ranged from 13.91 to 33.89 (median 22.27). Details about all patients and samples is given in supplementary table S3. The study was reviewed and approved by the Regional Ethical Review board in Stockholm, Sweden (registration no. 2017/1317-32).

All available genomes of EV-D68 as of 2nd Sept 2018 with a length of at least 6,000 bp on Virus Pathogen Resource (ViPR) (Pickett *et al.*, 2011) were downloaded with metadata. Sub-genogrouping of sequences was done using the Enterovirus Genotyping Tool Version 1.0 (Kroneman *et al.*, 2011).

Near full-length EV-D68 amplification

Primers were designed to cover almost the entire EV-D68 genome in four overlapping fragments (F1–F4) that were approximately 2000 base pairs long (Table S1). An alignment of published EV-D68 genomes was used to find candidate forward and reverse primers targeting highly conserved regions of the EV-D68 genome, with similar melting temperatures, and with minimal tendency for hairpin and primer-dimer formation. Inner nested primers for fragment 1 were designed for template quantification.

RNA was manually extracted using the RNeasy Lipid Tissue Mini Kit (Qiagen Cat. No. 74804). For each nasopharyngeal sample, 200 μl was taken and mixed with 1ml Qiazol, 200 μl chloroform and 5 μl carrier-RNA. After centrifugation and several washing steps, each aliquot was eluted with 100 μl RNase free water and were either stored at -70°C or used directly for PCR amplification.

Each sample was amplified in duplicate by one-step RT-PCR for each of the four overlapping fragments. The RT-PCR mixture contained: 11 μl of RNA template, 0.5 μM of forward and reverse PCR primer, 1 ng/μl random hexamers, 1 μl Superscript III RT/Platinum Taq HiFi Enzyme mix (Invitrogen, Stockholm, Sweden), 25 μl 2x

reaction mix, and RNase free water to a total volume of 50 μl. The PCR cycling profile was: 1x: 50°C, 30 min - 94°C, 2 min (cDNA synthesis), 30 x: 94°C, 15 sec – 50°C, 30 sec - 68°C, 90 sec (PCR-amplification), 1x: 68°C, 5 min - 4°C, ∞ (final extension).

For positive PCR amplifications, duplicates from each of the four fragments 1-4 were pooled and purified with AGENCOURT AMPure XP PCR purification kit and quantified with Qubit assays (Q32851, Life Technologies). The purified samples were diluted to the same concentration and then sent for Next Generation Sequencing (NGS) on the Illumina Hiseq platform at SciLifeLab.

To approximately quantify the number of input EV-D68 RNA templates for sequencing, a dilution series of the RNA (1:10, 1:100, 1:1000) was amplified in duplicate with nested primers for fragment F1. EV-D68 RNA templates were enumerated by the Poisson distribution formula. The outer PCR is described above. The nested PCR mixture contained: 2.5 μl of PCR product from the first PCR, 0.2 μM of forward and reverse PCR primer, 1 unit of Platinum Taq HiFi (Invitrogen, Stockholm, Sweden), 5 μl 10x reaction mix, and RNase free water to a total volume of 50 μl. using 2.5 μl of product from the first (outer) PCR and Platinum Taq High Fidelity. The cycling profile was: 1x: 94°C, 2 min (denaturation) – 30x: (94°C 15 sec – 50°C 30 sec – 68°C 90 sec) (PCR-amplification), 1x: 68°C 6 min – 4°C, ∞ (final extension).

DNA library preparation and sequencing

Amplified DNA was quantified using Quant-iTTM dsDNA High-Sensitivity Assay Kit and Tecan Spark 10M plate reader. 1 μl of DNA (approximately 0.5-2.0 ng/μl) was used in the tagmentation reaction using Nextera chemistry (Illumina) to yield fragments larger than 150 bp. The tagmented library underwent 11 cycles of PCR with single-end indexed primers (IDT Technologies) followed by purification using Seramag beads according to the protocol. The library was quantified using Quant-iTTM dsDNA High-Sensitivity Assay Kit and Tecan Spark 10M plate reader and pair-end (2x101 bp) sequenced to a depth of 100,000 – 1,000,000 reads per sample on a HiSeq 2500 Illumina sequencer. Base calling and demultiplexing was done using bcl2fastq v1.87, without allowing any mismatch in the index sequence.

Read filtering, mapping and analysis

Sequencing reads were trimmed using TrimGalore (Krueger, 2015; Martin, 2011) and mapped against the sequence `KX675261.1` using `bwa mem` (Li and Durbin, 2009). We used the `pysam` wrapper to samtools to generate a pile-up of all reads and quantify insertions

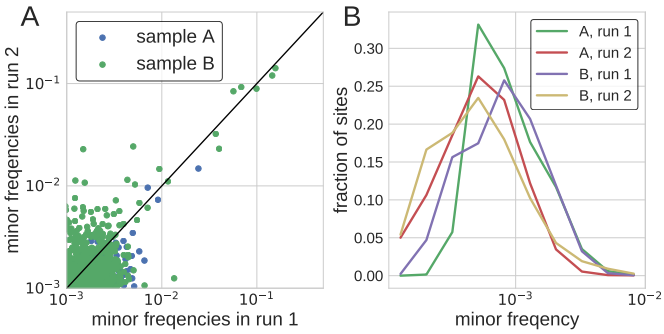

FIG. 2 **Accuracy of minor variant frequencies.** Panel A shows the consistency of minor variant frequencies across two replicate extraction, RT-PCR, and sequencing of two samples. Minor variation in accurately recovered down the frequencies of about 1%. Panel B shows the distribution of the frequencies of non-consensus calls across all sites covered in excess of 2000x. At the majority of sites, no variation above 0.001 is observed. Note that this variation includes within sample variation, RT-PCR errors, and sequencing errors.

relative to the reference. Custom python scripts were used to generate consensus sequences and quantify within host diversity from the pile-ups. All these steps were chained using the workflow engine `snakemake` (Köster and Rahmann, 2012). The entire pipeline is available at `github.com/neherlab/SVVC`. Commit `51ef982` was used to analyze the data. To analyze linkage disequilibrium, we calculated genotype frequencies p_{12} at pairs of positions that were covered by a read pair more than 100x and calculated their correlation $D_{12} = p_12 - p_1p_2$ (aka linkage disequilibrium). This correlation was normalized such that maximal linkage corresponds to $D_{12} = 1$ and complete repulsion to $D_{12} = -1$.

The consensus sequences for successfully sequenced samples have been deposited in GenBank (accession numbers MH674111-MH674166 and MH844544).

Scripts that analyze diversity in pile-ups and generate most of the the figures of this study are available `github.com/neherlab/EV_D68_analysis`.

Quality controls experiments

Quality controls were prepared from two samples (SWE-027 and SWE-037). The Ct values of the two samples were 14.62 and 19.27, respectively. The samples were diluted 1:100 in a pool of virus-negative nasopharyngeal samples and stored at -70C in 200 μl aliquots. The enterovirus RNA concentration of the samples was quantified by limiting dilution in 10 replicas of ten-fold dilutions, from 1:10 to 1:100,000 with an in-house enterovirus realtime-PCR (Tiveljung-Lindell *et al.*, 2009). Based on Poisson calculations the samples were estimated to have 4,400 and 240 EV-D68 RNA copies per μl, respectively, prior to dilution. The two samples were RNA extracted,

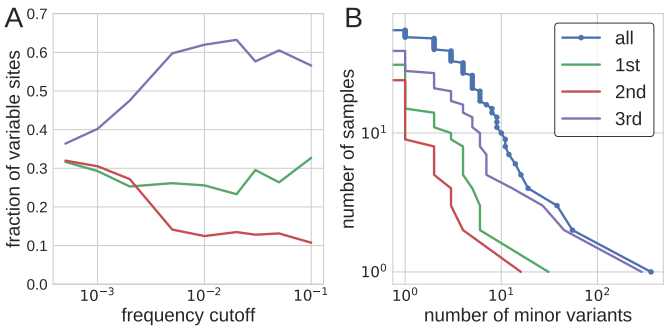

FIG. 3 **Within sample diversity.** Panel A shows the distribution of the number of variable sites in coding regions among different coding positions. At very low frequency cutoffs, variable sites are approximately equally distributed in codons. The distribution rapidly changes once the frequency cutoff exceeds 0.3%, beyond which variable sites are found predominantly at 3rd positions and rarely at 2nd positions in a codon. Panel B shows inverse cumulative distribution of the number of samples that have more than a certain number of sites that are variable at a level of 3%. Separate distributions are shown for all sites and for 1st, 2nd, and 3rd codon position in the coding region of the genome.

PCR amplified and sequenced twice in two separate runs. The number of EV-D68 RNA templates in each PCR reaction was approximately 48,400 and 2,640 respectively.

Phylodynamics analysis

Sequences generated in this study were combined with sequences and metadata of all near-complete EV-D68 genomes available on Virus Pathogen Resource as of 2018-09-02. The combined data set was analyzed using nextstrain (Hadfield *et al.*, 2018) and snakemake (Köster and Rahmann, 2012). The `augur` pipeline was run using the aligner MAFFT (Katoh *et al.*, 2002), the tree-builder (Nguyen *et al.*, 2015), and the phylodynamic package TreeTime (Sagulenko *et al.*, 2018). The repository detailing the analysis pipeline is available at `https://github.com/neherlab/enterovirus_nextstrain`.

Five sequences where excluded from the phylodynamic analysis because they did not fit a molecular clock model or because they appeared problematic. The resulting analysis is visualized with `auspice` and is available at `nextstrain.org/enterovirus/d68`.

Mixed effects model of evolution (MEME) analysis (Murrell *et al.*, 2012) using the DataMonkey package (Weaver *et al.*, 2018) was used to analyze for episodic diversifying selection.

RESULTS

Fifty-four of 59 samples yielded a coverage of at least x100 in all four fragments and were included in further analyses. Of the excluded samples, two did not yield sequence in any fragment (Ct 30.99 and 33.89) and three samples lacked complete sequence in one of four fragments (Ct 20.58, 22.95, and 32.31). All five excluded samples were from 2016. Fig. 1 shows the coverage plot for a representative sample. Details about patient characteristics and sequencing coverage in all samples is presented in supplementary tables S2 and S3.

Reproducibility of identification of intra-sample single nucleotide variants (iSNVs)

In addition to consensus sequences, deep population sequencing delivers information about within-sample diversity. Low genome template input, biased amplification, RT-PCR errors and sequencing errors can all potentially skew or inflate within sample diversity estimate (Zanini *et al.*, 2017). To control for such artifacts, we processed two high-titer patient samples in duplicate (see Methods). The accuracy of the detection and quantification of iSNVs was evaluated by comparing results from the two runs (Fig. 2). Minor variants down to below 1% were consistently detected in both runs. Also, the estimated frequency of individual iSNVs showed good consistency between the two runs at frequencies above approximately 1% (Panel A), but a few iSNVs had markedly higher frequencies in one of the replicates (run 2 of sample B).

Analysis of intra-sample variability in all 54 patient samples also provided information about our ability to detect true biological variation. Fig. 3A shows the distribution of sites with variation above a certain frequency cutoff among the three codon positions. The distribution was approximately even between codon sites at very low cutoffs below 10^{-3} and then rapidly changed around approximately 0.3% before plateauing at 1%. Five patients were sampled twice during their acute illness. Fig. S3 shows that there was reasonable agreement in detection of iSNVs above the 1% level in these longitudinally collected samples, especially in patient 037 and less so in patient 012.

Collectively our results show that a majority of iSNVs at frequencies $> 1\%$ represent true biological variation, whereas variation $< 0.1\%$ mostly is due to amplification and sequencing errors. Between 0.1% and 1% an increasing proportion of variation represents true iSNVs. The level of accuracy achieved is comparable to what we reported earlier for a similar sequencing strategy for HIV-1 (Zanini *et al.*, 2017, 2016) and other viruses (Grubaugh *et al.*, 2018).

Intrapatient variability

Fig. 3 A displays the distribution of iSNVs among 1st, 2nd and 3rd codon positions in all 54 patient samples

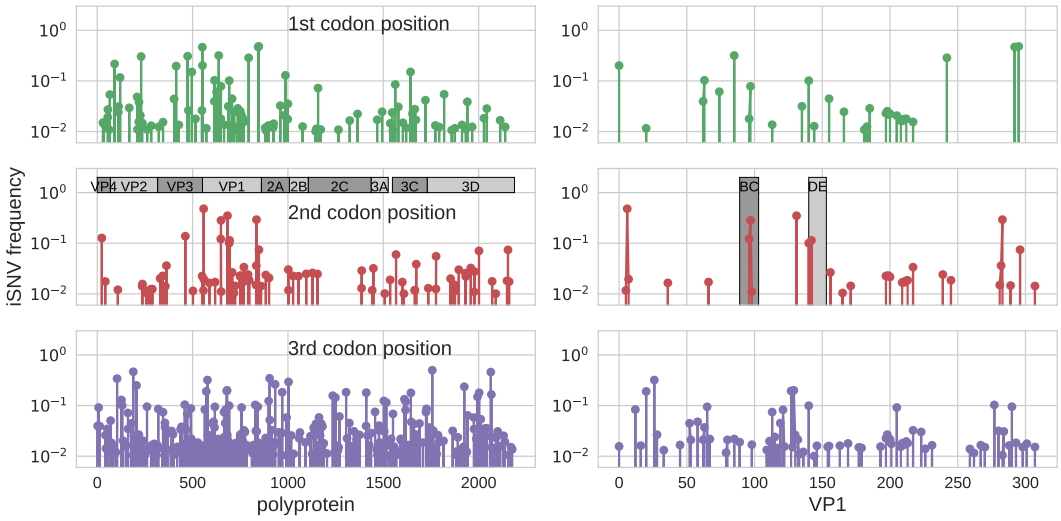

FIG. 4 **EV-D68 intrapatient variability across the genome and codon positions.** The three rows show how intrapatient variability is distributed across the EV-D68 genome for 1st, 2nd, and 3rd codon position, respectively. The panel on the left show iSNVs along the entire polypeptide, the panels on the right zoom into VP1 and highlight the BC and DE loop. These panels include data from all samples other than the three dual infected samples, see main text.

as a function of the frequency cut-off. This distribution changes rapidly from being almost even to being dominated by 3rd positions as the cut-off increases beyond 0.3%. At levels > 1% approximately 60% of the variable sites occurred at the 3rd codon position, slightly less than 30% in the 1st codon position, and slightly more than 10% in the 2nd codon position. Thus, intrasample variability above 1% likely represents true biological variation and is mostly synonymous. Five patients were samples twice with intervals ranging from a few hours to 7 days. Frequencies of iSNVs within these individuals where largely concordant across two consecutive when these samples were obtained less than 2 days apart, see Fig. S3.

Fig. 3B shows that most (44 of 54) samples had fewer than 10 sites that displayed variability above a level of 3%. However, three samples had more than 20 variable sites, which suggested dual infections (see below). In the remaining 51 samples both synonymous and non-synonymous were mostly scattered across the genome Fig. 4. However, there was some clustering of minor variation in 1st and 2nd codon positions in the structural proteins (VP4 – VP1) including putative targets for neutralizing antibodies in BC- and DE-loops of VP1 (Liu *et al.*, 2015). The details about complete amino acid substitutions and minor variation in these regions are shown in supplement table S4 and S5.

Evidence of dual infections

As mentioned above, three samples (SWE-007, SWE-045, and SWE-046) drawn from three different patients showed more than 20 iSNVs. In all three samples most minor variants were detected at a frequency around 10% (Fig. 5). This suggested the presence of two distinct variants; a major variant constituting around 90% of the virus population in the sample and a second minor variant constituting around 10% of the virus population. To investigate the origin of these minor variants we constructed a "minor" consensus sequence for each sample, by exchanging the strict majority rule consensus nucleotide by the minor variant at positions where they exceeded a frequency of 1%. A phylogenetic tree analysis showed that the major and minor sequences were monophyletic in all samples except the three samples with indications of dual infections. For these samples the major and minor consensus sequences occupied different positions in the tree, see Supplementary figure S1. Importantly, these minor variants did not cluster closely to any of the other study samples, arguing against sample-to-sample cross-contamination. To further investigate these putative dual infections we analyzed the linkage of iSNVs (Fig. 5, right). Almost all iSNVs occurred in complete linkage with neighboring iSNVs, which provides strong evidence for dual infection, rather than rapid evolution of intrapatient variation starting from a single transmitted variant.

Phylodynamics of EV-D68

The interpatient evolution and phylogenetic relationship of the EV-D68 variants from Sweden and 509 published EV-D68 sequences was investigated using the `nextstrain` platform available at `https://nextstrain.`

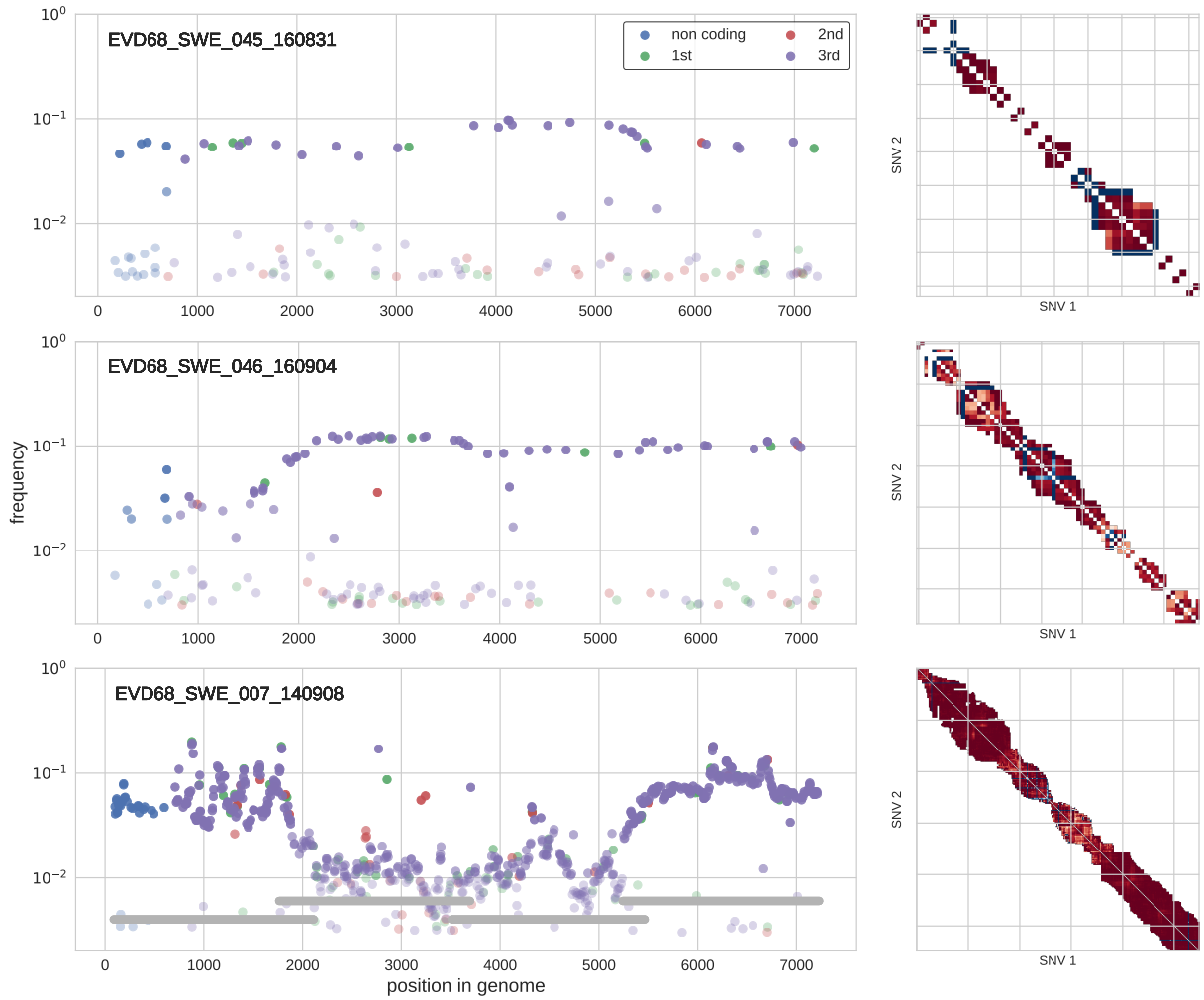

FIG. 5 **Minor iSNVs across the EV-D68 genome for three samples with putative dual infections.** Three samples had more than 20 highly covered sites with minor variants in excess of 3% frequency. The majority of these variants were at 3rd positions and had similar frequencies across the amplicons indicated by grey bars in the lower panel. iSNVs in three amplicons (amplicon 1 of sample SWE_046 and amplicons 2&3 of sample SWE_007), however, had were found at substantially lower frequency, possibly due to primer mismatches. The three panels on the right show linkage disequilibrium between iSNVs (>1%) close enough to each other that they were covered at least 100 times by the same sequencing read. Almost all of these variants at in complete linkage (dark red). A number of iSNVs just above 1% in sample 045 and 046 are likely variants in the background of the dominant variant. Those are in complete 'anti-linkage' with neighboring iSNVs are $\sim$ 10% (dark blue).

`org/enterovirus/d68` (Hadfield *et al.*, 2018), which allows users to interactively explore the dataset. A screen shot is displayed in Fig. 6, left.

The `nextstrain` phylodynamic analysis showed that all Swedish EV-D68 variants from the 2016 outbreak belonged to the B3 lineage of EV-D68, which agrees with our earlier findings based on partial VP2/VP4 gene sequences (Dyrdak *et al.*, 2016). Within the B3 lineage our new Swedish sequences were interspersed with international sequences, primarily from the US, but monophyletic clusters of Swedish sequences were also observed. These findings indicate that during the worldwide 2016 outbreak EV-D68 was introduced into Sweden several times and that these separately introduced variants thereafter spread locally.

Interestingly, the tree indicated that the 2016 B3 subclade did not evolve directly from the 2014 B1 subclade. Instead, the most recent common ancestor (MRCA) of the B1 and B3 subclades was estimated to have existed in first half of 2009 (90% CI Dec 2008 - Jul 2009). Many of the proximal sequences in the B1 and B3 subclades were sampled in East Asian countries, but this should be interpreted with caution since sampling probably was incomplete and biased. A tangle-tree analysis suggested recombination within subclades, but not between clades or subclades (Suppl fig S2), but also this finding should be interpreted with caution since there was limited within subclade variation.

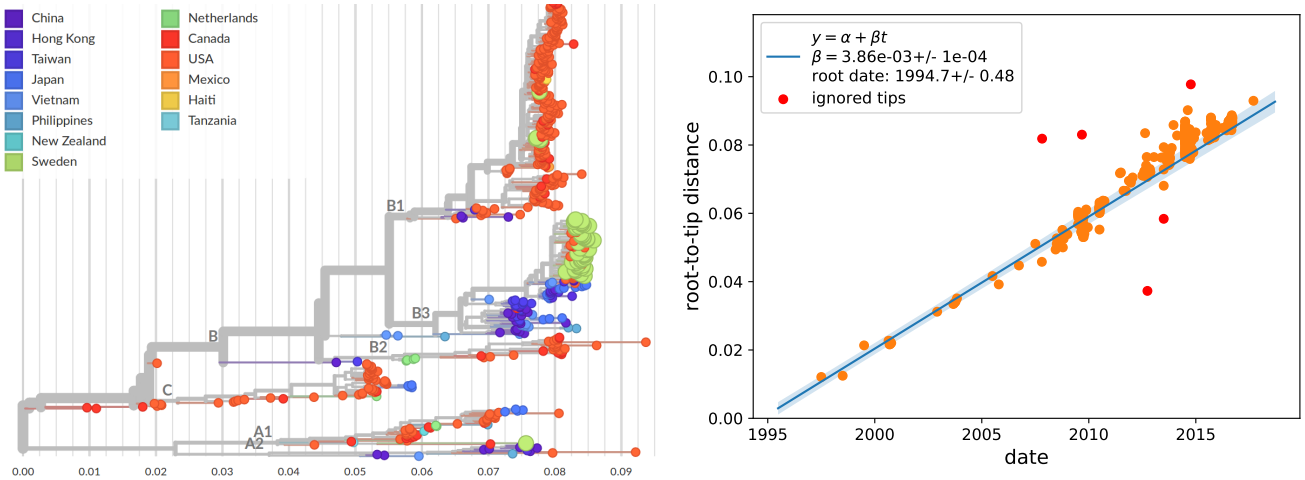

FIG. 6 **Phylogenetic analysis.** The left panel shows a phylogenetic tree as rendered by nextstrain with the new sequences from Sweden colored in green. The right panel shows a scatter plot of root-to-tip distance vs time indicating an extremely strong temporal signal with an evolutionary rate of $\mu = 0.0038 \pm 0.0001$ substitutions per site and year.

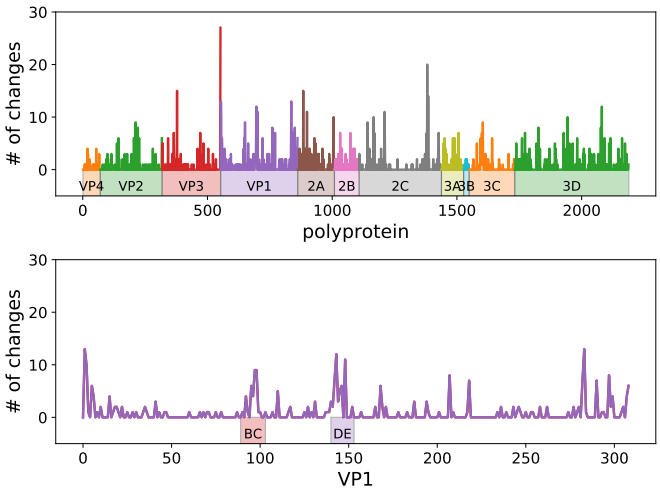

FIG. 7 **Interpatient diversity of EV-D68.** Amino acid substitution events on the phylogeny in Fig. 6A were enumerated using `nextstrain`. Top panel shows the number of such events for each codon along the entire coding sequence. Bottom panel shows events in VP1, where peaks were observed in the BC- and DE-loops as well as both ends of the protein.

Interpatient evolution

A plot of root-to-tip distances of EV-D68 sequences vs. time showed an extremely strong temporal signal (Fig. 6, right). We estimated an evolutionary rate of $3.8 \times 10^{-3} \pm 10^{-4}$ substitutions per site and year for the entire genome and $4.2 \times 10^{-3} \pm 4 \times 10^{-4}$ for the VP1 region, which is slightly lower than previous estimates (Ny *et al.*, 2017; Tokarz *et al.*, 2012)). 87% of all mutations were synonymous (85% on terminal branches).

We used `nextstrain` to investigate diversity across the EV-D68 genome in the sample containing the genomes reported in this study and genomes available in Genbank. Fig. 7 shows that amino acid substitution events were observed in all EV-D68 proteins and varied between 0 and 27 events per codon. The five positions with the highest number of substitution events were: codon 234 in protein VP3 (27 events), 273 in 2C (20 events), 22 in 2A (15 events), 60 in VP3 (15 events), and 277 in 2C (14 events). None of these positions have been reported to be neutralizing epitopes. Codon 277 in 2C, as well as codon 1 in VP1 showed evidence of episodic diversifying selection as per MEME analysis ($p < 0.05$).

The BC- and DE-loops in the VP1 protein, showed relatively high diversity, especially in positions 95, 97, 98, 142, 143 and 148) suggesting that immune selection may have impacted on EV-D68 evolution (see Fig. 7, bottom panel and for details see `nextstrain`).

We also used `nextstrain` to track on which branches in the EV-D68 phylogeny amino acid substitutions had evolved and been fixated. The branches leading to the MRCA of the B1 and B3 clades had many amino acid substitutions, including substitutions at codons 90, 95, 97, 98, 145 and 148 in the BC- and DE-loops. In contrast, no amino acid substitutions in the loops were observed on branches separating the B1 and B3 subclades. Similarly, only very few substitutions in the loops were observed within the B1 and B3 subclades.

DISCUSSION

We have performed a comprehensive investigation of the intra- and interpatient evolution of EV-D68 using new deep near full-length sequences from 54 Swedish patients sampled during the 2014 and 2016 outbreaks and available database sequences. The new data and previ-

ously published sequences, are available on `nextstrain` to facilitate real-time tracking and further study of EV-D68 evolution and spread. Intrapatient variability was mostly low and dominated by rare synonymous mutations consistent with deep sequencing studies on influenza virus infections (Debbink *et al.*, 2017). But three patients showed evidence of dual infections. The phylodynamic analysis indicated EV-D68 was introduced into Stockholm several times during the 2016 outbreak and then spread locally.

Three patients showed clear evidence of dual infection with two different EV-D68 variants. Even though dual infection with different enteroviruses was reported early on for poliovirus (PV) and non-polio enteroviruses (NPEV) (Melnick *et al.*, 1951; Parks *et al.*, 1967) and more recently for NPEVs (Isaacs *et al.*, 2018), this is to our knowledge the first report of dual infections with EV-D68. Dual infections during the 2016 outbreak are not completely surprising in view of the high EV-D68 incidence during the outbreak (Dyrdak *et al.*, 2016) and the fact that the present study showed multiple introductions of the B3 subclade into Stockholm. We also found indications of intra-subclade recombination, but not between clades or subclades. Similar findings have been reported in three recent studies (Ny *et al.*, 2017; Tan *et al.*, 2015; Yip *et al.*, 2017). It is expected that recombination primarily would occur within subclades since recombination requires co-infection with two or more virus variants and viruses that circulate during an outbreak or season tend to be closely related.

The EV-D68 genome was amplified in four overlapping amplicons that were sequenced on the Illumina HiSeq platform. Our results indicate that variation down to a frequency of 1% represented "true" iSNVs, which were reproducibly detected and quantified. In contrast, most variation below 0.1% represented noise from cDNA synthesis, PCR and sequencing. It would be interesting to compare the performance of our sequencing protocol with the pan-enterovirus protocol recently published by (Isaacs *et al.*, 2018). In the 54 samples that were sequenced to sufficient depth we observed that most nucleotides were conserved. Among iSNVs detected above the 1% level, most occurred at the 3rd codon position, i.e. were usually synonymous. The finding that intrapatient variation was limited and mostly synonymous was expected since infection usually appeared to have been established by a single virion and EV-D68 is an acute infection where is limited time for the virus to diversify within the infected host.

The phylodynamic analysis showed that the splits between EV-D68 clades and subclades occurred several years ago. For instance, the MRCA of the B1 and B3 subclades was estimated to have existed in 2009. Enterovirus infections, like influenza, has a seasonal pattern. However, in contrast to influenza, which infects people of all age groups and has a the ladder-like phylogenetic pattern indicates a strong immune selection, EV-D68 infects predominantly children and its phylogeny does not suggest continuous immune escape (Grenfell *et al.*, 2004). In line with this, the branches leading to the B1 and B3 subclades did not have amino acid substitutions in the BC- and DC-loops, which are putative targets for neutralizing antibodies. However, it should be acknowledged that there is limited knowledge about targets for humoral and cellular immunity in EV-D68.

Our phylodynamic analysis suggested that the recent global outbreaks of EV-D68 might have been preceded by low-level circulation of EV-D68 in East Asia. However, this pattern should be interpreted with caution since the number of available full-length EV-D68 sequences is low and the sequences have not been systematically collected in time or space. We hope that the `nextstrain` platform will allow for continuous collection and sharing of EV-D68 sequence data, which will be needed to obtain a better understanding of how new successful EV-D68 variants evolve and spread. Better knowledge about humoral and cellular immunity against EV-D68 is also needed.

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SUPPLEMENTARY INFORMATION

TABLE S1 Primers for near full-length sequencing of EV-D68 in four overlapping fragments (F1-F4). The nested primers for F1 were used for template quantification.

Primers for fragments 1-4 (F1-4) and nesting primer (F1)	
Primers (length)	Sequence
F1 (2108 bp)	
E68_F1_fw_63-87	TCG GTA CCT TTG TAC GCC TGT TTT A
E68_F1_rev_2146-2170	TAC TAG ATT GYA RTC CAA ART CCC A
F2 (1962 bp)	
E68_F2_fw_1772-1796	CAA CTC CAG ARA TGC ACA TIC CAG G
E68_F2_rev_3757-3733	GTT CCA TAR CAT CAG TAT CTA ACC A
F3 (2001 bp)	
E68_F3_fw_3524-3548	ACC CRA TTT GYT TTG AAG GYC CAG G
E68_F3_rev_5524-5500	TGT AAA TGR TTT CTC CAA CAG ATG C
F4 (2015 bp)	
E68_F4_fw_5252-5276	CCA CCT TTG TAT CAA TAG CIG GTG T
E68_F4_rev_7291-7267	CTA ACC ATT TCC GTC TAA GAC TAG A
Nesting	
F1 (1547 bp)	
E68_F1_fw2_303-327	CGT GTG TAG CTT GGG TCG ATG AGT C
E68_F1_rev2_1849-1825	TAA TCT CCA TCA TTG ATT CCA CTT G

TABLE S2 Breakdown of patient age, sex, and sample material.

Age (years)	$n =$
< 1	8
1–5	27
6–18	10
> 18	9
Total	**54**
Sex	
F	30
M	24
Total	**54**
Sample material	
Nasopharyngeal (aspirate)	31
Nasopharyngeal (secretion)	23
Tracheal (secretion)	3
Other respiratory	2
Total	**59**

TABLE S3: Details about study samples.

Case no.	Sampling date	GenBank ID	Coverage	CT EV-PCR	CT rhino-PCR
001_14	2014–09–05	MH674113	$\geq 10^3$	33.80	0
002_14	2014–09–08	MH674115	$\geq 10^3$	31.47	0
004_14	2014–09–16	MH674116	$\geq 10^3$	25.59	0
005_14	2014–08–14	MH674111	$\geq 10^3$	24.00	24.86
006_14	2014–08–14	MH674112	$\geq 10^3$	16.77	19.67
007_14	2014–09–08	MH674114	$\geq 10^3$	17.62	17.40
007_16	2016–09–09	MH674135	$\geq 10^3$	20.97	26.99
008_16	2016–09–09	MH674136	$\geq 10^3$	30.94	37.45
009_16	2016–09–11	MH674137	$\geq 10^3$	31.8	32.54
010_16	2016–09–09	MH674160	$\geq 10^3$	22.94	26.09
011_16	2016–09–09	MH674161	$\geq 10^3$	21.20	22.86
012_16	2016–09–11	MH674163	$\geq 10^3$	21.97	23.75
012_16	2016–09–13	MH674164	$\geq 10^3$	23.33	24.46
013_16	2016–09–14	MH674165	$> 10^3$	23.93	24.67
017_16	2016–08–23	MH674118	Missing	20.58	23.92
019_16	2016–08–24	MH674119	$\geq 10^3$	19.26	19.55
020_16	2016–08–25	MH674120	$\geq 10^3$	21.15	22.20
021_16	2016–08–28	MH674121	$\geq 10^3$	15.73	18.52
021_16	2016–09–04	MH674129	$\geq 10^2$	31.68	0
022_16	2016–08–28	MH674122	$\geq 10^3$	31.60	0
023_16	2016–08–30	MH674123	$\geq 10^3$	23.76	24.95
024_16	2016–08–30	MH674124	$\geq 10^3$	15.75	17.81
024_16	2016–08–31	MH674154	$\geq 10^3$	19.91	21.23
025_16	2016–08–30	MH674125	$> 10^3$	25.63	27.51
026_16	2016–08–31	MH674126	Missing	22.95	23.76
027_16	2016–08–29	MH844544	$\geq 10^3$	18.62	20.38
028_16	2016–09–04	MH674128	$\geq 10^3$	22.34	23.90
029_16	2016–09–04	MH674130	$\geq 10^2$	20.46	23.00
030_16	2016–08–19	MH674140	$\geq 10^3$	21.90	24.49
031_16	2016–08–22	MH674141	$\geq 10^3$	13.91	14.99
032_16	2016–08–25	MH674142	$\geq 10^3$	16.39	16.37
033_16	2016–08–26	MH674143	$\geq 10^3$	25.63	25.58
034_16	2016–08–29	MH674146	$\geq 10^3$	20.98	23.20
035_16	2016–08–29	MH674147	$\geq 10^2$	27.82	29.35
036_16	2016–08–29	MH674148	$\geq 10^3$	18.71	19.95
037_16	2016–08–29	MH674149	$\geq 10^3$	19.27	21.56
037_16	2016–08–30	MH674150	$\geq 10^3$	23.37	25.55
038_16	2016–08–30	MH674151	$\geq 10^3$	20.20	21.91
039_16	2016–08–31	MH674153	$\geq 10^3$	17.57	19.78
039_16	2016–08–31	MH674127	$\geq 10^3$	20.79	20.91
040_16	2016–09–01	MH674155	$\geq 10^3$	15.34	15.52
041_16	2016–09–01	MH674156	$\geq 10^3$	26.74	28.64
042_16	2016–09–02	MH674157	$\geq 10^3$	22.27	22.97
044_16	2016–09–10	MH674162	$\geq 10^2$	28.77	30.29
045_16	2016–08–31	MH674152	$\geq 10^3$	24.43	25.70
046_16	2016–09–04	MH674131	$\geq 10^3$	22.15	24.76
047_16	2016–09–13	MH674139	$\geq 10^2$	28.70	21.22
048_16	2016–08–22	MH674138	$\geq 10^3$	28.69	28.62
049_16	2016–08–23	MH674117	$\geq 10^3$	19.77	21.53
050_16	2016–08–29	MH674145	$> 10^3$	20.68	22.88
051_16	2016–09–05	MH674132	Missing	32.31	33.59
052_16	2016–09–08	MH674159	$\geq 10^3$	25.57	0
053_16	2016–09–16	MH674166	$\geq 10^3$	27.38	28.48
054_16	2016–09–07	MH674133	$\geq 10^3$	25.59	27.44
055_16	2016–09–07	MH674134	$\geq 10^3$	21.94	25.74
056_16	2016–09–05	MH674158	$\geq 10^3$	17.81	19.30
057_16	2016–08–29	MH674144	$\geq 10^3$	18.62	20.38
058_16	2016–09–16	N/A	Fail	33.89	0
059_16	2016–09–11	N/A	Fail	30.99	25.98

Note that some patients were sampled at two different time points. Coverage: $\geq 10^3$ = at least x1000 over all amplicons; $\geq 10^2$ = at least x100 over all amplicons; Missing = at least one amplicon with $< 10^2$ coverage; Fail = No amplicon. EV-PCR = realtime-PCR for enterovirus. RV-PCR = realtime-PCR for rhinovirus.

TABLE S4 Amino acid sequence and substitutions in the putative targets for neutralizing antibodies in the BC-loop in the VP1 protein (codon 89–105, consensus sequence KDHTSSAAQADKNFFKW).

Sample			Codon position and consensus amino acid			
			96	98	99	100
Case no.	Collection date	Sequence variant	A	Q	A	D
007_16	2016–09–09	Major	-	-	T	-
007_16	2016–09–09	Minor	-	-	T	-
012_16	2016–09–11	Major	-	-	T	-
012_16	2016–09–11	Minor	-	-	T	-
012_16	2016–09–13	Major	-	-	T	-
012_16	2016–09–13	Minor	-	-	T	-
021_16	2016–09–04	Minor	-	R	-	-
022_16	2016–08–28	Major	-	-	T	-
022_16	2016–08–28	Minor	-	-	T	-
023_16	2016–08–30	Major	-	-	T	-
025_16	2016–08–30	Minor	-	-	-	H
027_16	2016–09–01	Major	-	-	T	-
027_16	2016–09–01	Minor	-	-	T	-
028_16	2016–09–04	Minor	-	R	-	-
031_16	2016–08–22	Major	-	-	T	-
031_16	2016–08–22	Minor	-	-	T	-
033_16	2016–08–26	Minor	-	-	-	H
036_16	2016–08–29	Major	-	-	T	-
036_16	2016–08–29	Minor	-	-	T	R
038_16	2016–08–30	Minor	-	-	V	-
039_16	2016–08–31	Minor	-	R	-	-
053_16	2016–09–16	Major	-	-	T	-
053_16	2016–09–16	Minor	-	-	T	-
054_16	2016–09–07	Major	T	-	-	-
054_16	2016–09–07	Minor	T	-	-	-

TABLE S5 Amino acid sequence and substitutions in the putative targets for neutralizing antibodies in the DE-loops in the VP1 protein (codons 140–152 consensus sequence: NGSSNNTYVGLPD).

Sample			Codon position and consensus amino acid	
			144	147
Case no.	Collection date	Sequence variant	S	T
007_16	2016–09–09	Minor	-	A
010_16	2016–09–09	Major	-	A
010_16	2016–09–09	Minor	-	A
012_16	2016–09–11	Major	N	-
012_16	2016–09–11	Minor	N	-
012_16	2016–09–13	Major	N	-
012_16	2016–09–13	Minor	N	-
013_16	2016–09–14	Major	-	A
013_16	2016–09–14	Minor	-	A
023_16	2016–08–30	Major	N	-
023_16	2016–08–30	Minor	N	-
035_16	2016–08–29	Major	-	A
035_16	2016–08–29	Minor	-	A
036_16	2016–08–29	Major	N	-
036_16	2016–08–29	Minor	N	-
050_16	2016–08–29	Major	-	A
050_16	2016–08–29	Minor	-	A
055_16	2016–09–07	Major	G	-
055_16	2016–09–07	Minor	G	-

Fig. S1 Tree with minor sequences in dually infected patients

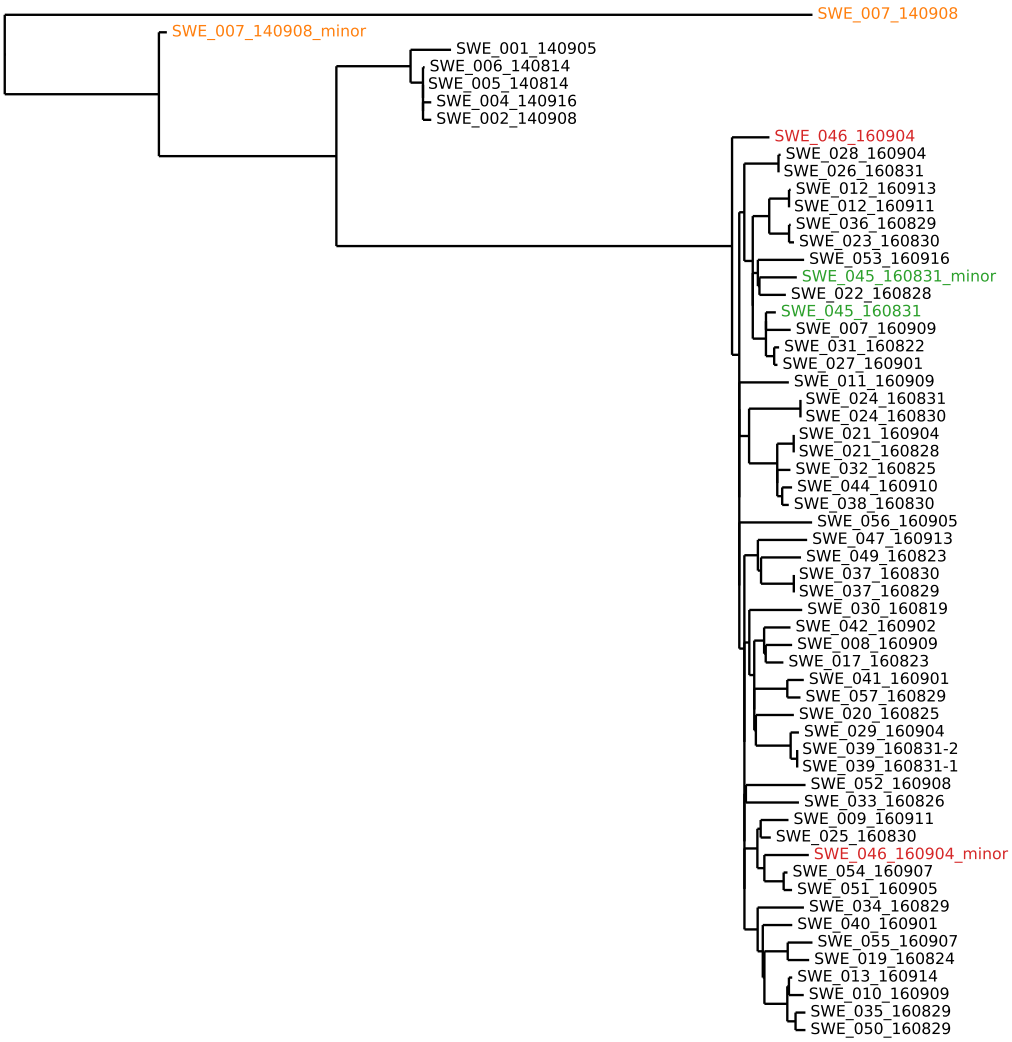

FIG. S1 **Phylogenetic placing of minor variants in dually infected patients.** The figures hows a mid-point rooted ML tree of all consensus sequences obtained in this study along with minority sequences from three samples from dually infected individuals. The minor variants don't cluster tightly with any other sample but rather look like representative samples from the populations. Minor variant sequence of the the SWE_007_140908 is slightly problematic since variants in the amplicons 2 and 3 cannot be cleanly separated from sequencing error.

Fig. S2 Tangle-tree analysis for possible recombinants.

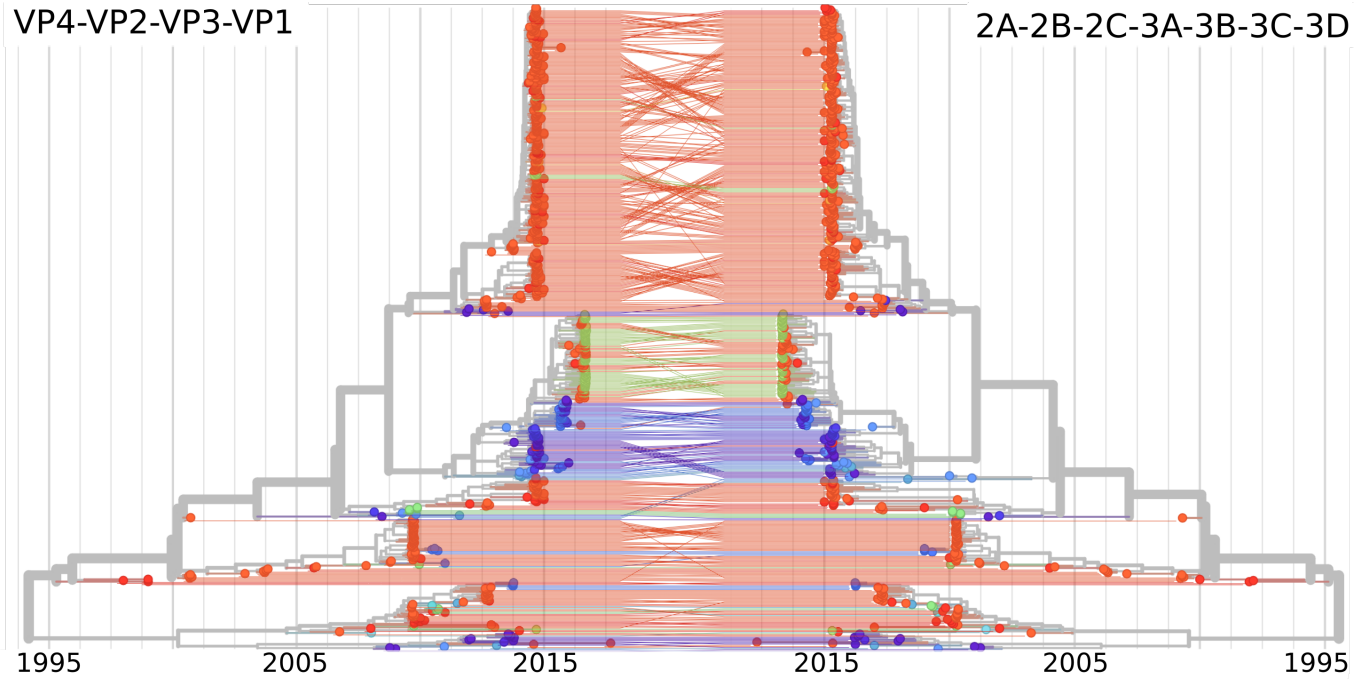

FIG. S2 **Tangle-tree analysis for possible EV-D68 recombinants.** Phylogenetic trees of sequences coding for VP4-VP1 (5' region) and 2A-3D (3' region) are congruent at the level of major clades, suggesting little inter-clade recombination. Trees were calculated using the nextstrain analysis pipeline and visualized using nextstrain's auspice.

Fig. S3 Minor variant consistency of in repeatedly sampled individuals.

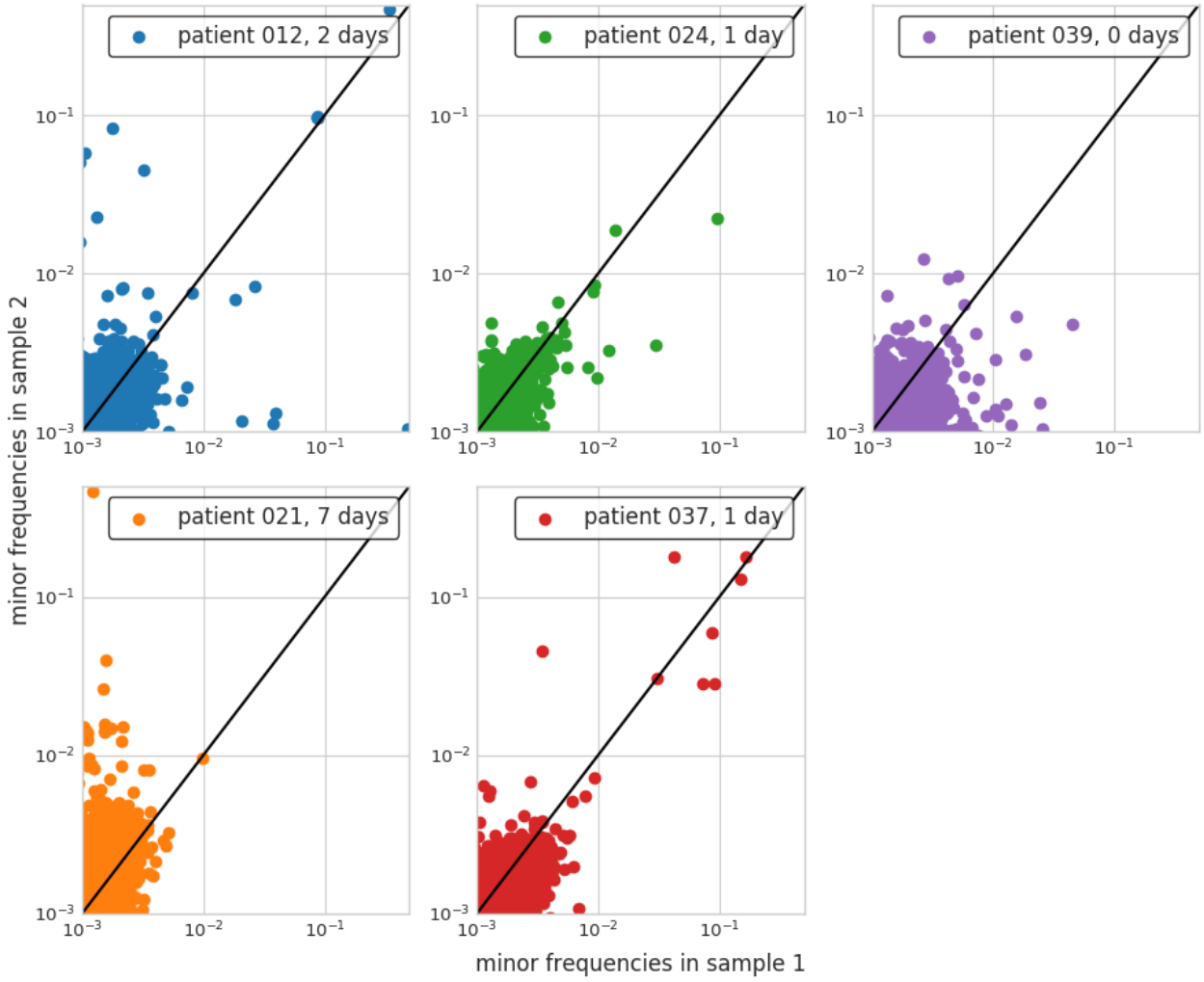

FIG. S3 **Minor variant consistency in repeatedly sampled individuals.** Each panel shows a scatter plot of minor variant frequencies in two samples from the same individual, sampled between 0 and 7 days apart. As expected, most iSNVs are at frequencies below one percent but some samples harbor iSNVs in the range of 10%. Their frequencies are largely concordant for samples 1 day apart, but deviate in the samples that are 2 or 7 days apart.