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Research paper

Towards high quality real-time whole genome sequencing during outbreaks using Usutu virus as example

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ABSTRACT

Recently, protocols for amplicon based whole genome sequencing using Nanopore technology have been described for Ebola virus, Zika virus, yellow fever virus and West Nile virus. However, there is some debate regarding reliability of sequencing using this technology, which is important for applications beyond diagnosis such as linking lineages to outbreaks, tracking transmission pathways and pockets of circulation, or mapping specific markers. To our knowledge, no in depth analyses of the required read coverage to compensate for the error profile in Nanopore sequencing have been described. Here, we describe the validation of a protocol for whole genome sequencing of USUV using Nanopore sequencing by direct comparison to Illumina sequencing. To that point we selected brain tissue samples with high viral loads, typical for birds which died from USUV infection. We conclude that the low-cost MinION Nanopore sequencing platform can be used for characterization and tracking of Usutu virus outbreaks.

1. Introduction

During an epidemic, timely availability of genetic information can be crucial in guiding public health measures and research. The fast development of second and third generation sequencing (NGS) approaches allows for in depth analysis into the origin and evolution of the pathogen, and tracking of transmission networks. Depending on the application, current barriers to NGS implementation are high equipment and material costs, time to result, complex methodology for library preparations, the need for bioinformatic expertise, and/or the error rate of base calling (Lesho et al., 2016; Lightbody et al., 2018; Van Nimwegen et al., 2016). Gold standard in the field at the moment are second generation platforms providing ion semiconductor sequencing (Ion Torrent) or sequencing by synthesis (Illumina). Ion Torrent sequencing depends on clonal amplification during an emulsion PCR while Illumina sequencing depends on a clonal bridge amplification to amplify a single molecule into a cluster. The introduction of the handheld third generation nanopore sequencing technology (Oxford Nanopore Technologies MinION sequencing) has opened new possibilities for real-time in field sequencing since it does not require any additional specific amplification steps (Quick et al., 2017; Siddle et al., 2018). Next to the small size of the device, it also comes with a simplified method of library preparation, making it an ideal platform for in field, deployable sequencing. Also, the low purchase price of around €1000 is in sharp contrast with the second generation sequence machines which cost well over €100,000.

While there is consensus in the field that nanopore sequencing for these reasons is a potential game changer for bringing real-time sequencing to preparedness and outbreak research (Faria et al., 2018; Quick et al., 2017; Siddle et al., 2018), the higher error rate (Magi et al., 2016) and the relatively lower throughput of nanopore sequencing compared to second generation platforms potentially limits its applicability. Therefore, this methodology first has to be validated and tested in a controlled environment before steps towards reliable fieldable sequencing can be taken. Our case study was Usutu virus (USUV), an arbovirus in the family *Flaviviridae*. We have developed and validated a multiplex based amplicon-sequencing protocol using two overlapping primer sets to generate full length USUV genomes from bird tissues on the MinION platform. We have compared this methodology to the established Illumina platform and determined the error profile in consensus genome sequencing using different read coverage cut-offs.

USUV has a positive-stranded RNA genome with a genomic length

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of around 11,000 nucleotides which encodes for a single polyprotein. The polyprotein is processed by proteases into structural and non-structural proteins (Pauli et al., 2014). USUV was first identified in Africa in 1959 (Williams et al., 1964) and although the exact timing of the introduction of USUV into Europe is unknown, retrospective analysis of archived blackbird samples demonstrated that the virus was detected for the first time in Italy in 1996 (Weissenböck et al., 2013). After that, the virus was detected in for instance Austria, Hungary, the Czech Republic and Germany (Bakonyi et al., 2007; Engel et al., 2016; Hargitai et al., 2016; Hubálek et al., 2014; Weissenböck et al., 2003) and since 2016 USUV was detected widely throughout Western Europe where it caused epizootic outbreaks (Cadar et al., 2017).

Usutu virus is mosquito-borne and is recognized as a cause of increased mortality in common blackbirds and great grey owls (Rijks et al., 2016). In Germany, it has been estimated that in habitats that favor USUV-circulation, blackbird populations have been decreased by around 16% as compared to USUV-unsuitable areas (Lühken et al., 2017). In addition to blackbirds, other bird species like owls, song thrushes, common kingfishers, house sparrows, canaries and common starlings have been shown to be susceptible to USUV but the impact on population levels of these species remains to be determined (Becker et al., 2012). Humans and other mammals are considered to be dead-end hosts (Nikolay et al., 2014; Saiz and Blazquez, 2017) but symptoms associated with human USUV infections have been mainly described in immunocompromised individuals (Cavrini et al., 2009; Gaibani et al., 2012; Grottola et al., 2017; Pecorari et al., 2009). However, currently no routine diagnostic testing is performed and the actual number of human USUV infections might be underestimated. USUV also has been detected in 18 blood donors in Austria. Of these donors only one individual described a rash as clinical symptom 1 to 3 days after donation while the other infections were asymptomatic (Aberle et al., 2018).

Seven different USUV lineages (Europe 1–4 and Africa 1–3) have been proposed based on phylogeographical clustering analysis (Bakonyi et al., 2017; Cadar et al., 2017; Calzolari et al., 2017). Several of these lineages are currently circulating in Europe, with evidence for co-circulation in some regions. In Italy between 2009 and 2014 two different lineages of USUV were detected in mosquitoes (Europe 2 and Europe 4) (Calzolari et al., 2017), in Germany three different lineages of USUV were detected in blackbirds in 2016 (Europe 3, Africa 2 and Africa 3) (Sieg et al., 2017) and in France in 2015 the USUV lineages Africa 2 and Africa 3 were detected in mosquitoes from the same region (Eiden et al., 2018). The co-circulation of different USUV lineages is thought to reflect independent introduction events, with further diversification of lineages upon local amplification (Engel et al., 2016). The continued detection of related viruses in subsequent sampling episodes is evidence for local enzootic circulation and/or multiple introduction events. At the moment, no specific protocol for whole genome sequencing for all different USUV lineages has been described yet.

2. Material and methods

2.1. Samples

Brains of two great grey owls that died in two zoos in the Netherlands in September 2016 were submitted to EMC for USUV diagnostics. Both owls were found to be infected with USUV based on a published RT-PCR (Nikolay et al., 2014) with Ct values of 12.94 (Owl_AS201600070) and 13.35 (Owl_AS201600083).

2.2. Usutu virus diagnostics

Brain tissues from the dead owls were homogenized using the Fastprep bead beater (4.0 m/s for 20 s). Samples were spun down for 10 min at 10.000 $\times g$ and after NA extraction, samples were screened for the presence of USUV using an USUV specific RT-PCR as described by Nicolay et al. (Nikolay et al., 2014) and used as input for the USUV multiplex PCR.

2.3. Primer design for USUV whole genome sequencing

All full length USUV sequences available on 22-05-2017 were downloaded from GenBank (Benson et al., 2010) and unique genomes were selected based on a 99% nucleotide identity cut-off using Uclust (Edgar, 2010). Sequences were aligned using muscle (Edgar, 2004) after which the alignment was uploaded to the online primal design scheme (Quick et al., 2017). The amplicon length was set to 500 bps with an overlap of at least 75 bps between the different amplicons. The primers of the 32 resulting amplicons were aligned with all available full-length genomes and manually fine-tuned by changing the position of the primers and/or by including a maximum of 2 degenerative sites while checking for the effect on GC content, melting temperature and self-complementarity using OligoCalc (Kibbe, 2007). If more degenerative sites had to be included in order to match with all available full length USUV sequences a novel primer position was selected manually. The primers were split in 2 different reactions to prevent overlapping fragments. After an initial sequence round the primer concentrations were adjusted to get a more even coverage of the different amplicons. The final selection of primer sequences and primer concentrations is displayed in Supplementary Table 1.

2.4. Multiplex PCR for USUV whole genome sequencing

The multiplex PCR for MinION sequencing was performed as previously described (Quick et al., 2017). In short, random hexamer primers (Invitrogen) were used for reverse transcription using ProtoScript II (NEB, cat. no. E6569) after which the USUV specific multiplex PCR was performed in 2 separate reactions (A and B) using Q5 Hot Start High-Fidelity DNA Polymerase (NEB, cat no. M0493).

For Illumina sequencing the KAPA HyperPlus library preparation kit was used after which the samples were sequenced on an Illumina MiSeq sequencer (paired end, 250 bp). MinION sequencing was performed according to manufacturer's instructions using the 1D Native barcoding genomic DNA Kit (Nanopore, EXP-NBD103 and SQK-LSK108) on a FLO-MIN106 flowcell. For Nanopore sequencing, 12 samples were multiplexed on one flow cell.

2.5. Data analysis for Illumina sequencing

Adapters were removed using trimmomatic (Bolger et al., 2014) after which primers were trimmed and reads were quality controlled to a minimal length of 75 nt and a median PHRED score of 30 using QUASR (Watson et al., 2013). The amplicon coverage was normalized to 50 using BBNorm (Bushnell et al., 2019) after which a *de novo* assembly was performed using SPAdes (Bankevich et al., 2012). Raw, quality controlled reads were mapped back against the obtained consensus genome using Geneious (Kearse et al., 2012) to curate the consensus sequence.

2.6. Data analysis MinION sequencing

Raw sequence data was demultiplexed using Porechop (https://github.com/rrwick/Porechop). Primers were trimmed and reads were quality controlled to minimal length of 150 and a median PHRED score of 10 using QUASR (Watson et al., 2013). First a reference based alignment against the randomly selected USUV strain S.nebulosa-7890/Fra/2016 (KY128481) was performed in Geneious (Kearse et al., 2012). The consensus genome was extracted and compared to the non-redundant database using Blastn (Altschul et al., 1990), after which the most closest relative sequence was selected and used for a second reference based alignment using the quality controlled reads. This consensus genome was extracted and homopolymeric regions were manually checked and resolved consulting close reference genomes.

2.7. Phylogenetic analysis

All available full length USUV genomes available in GenBank on 27-10-2018 were collected (Benson et al., 2010) and aligned with the newly obtained USUV sequences using muscle (Edgar, 2004). The alignment was manually checked for discrepancies after which IQ-TREE (Nguyen et al., 2015) was used to perform phylogenetic analysis under the GTR + I + G4 model as best predicted model using the ultrafast bootstrap option.

2.8. Accession numbers

The genomic sequences of the Usutu viruses sequenced in this study have been deposited in the GenBank database under the accession numbers MK796168 and MK796169.

3. Results

Brain tissues from two USUV positive owls (Owl_AS201600070 and Owl_AS201600083) were selected for the development and validation of a specific multiplex RT-PCR for whole genome sequencing of USUV using Nanopore sequencing. They have been send in for USUV diagnostics and we have used these samples to compare the performance of the Nanopore sequencing platform to the more robust Illumina platform. A 500 bp amplicon primer set was developed to enable better amplification of highly degraded RNA or for samples with a lower viral load (Quick et al., 2017). These amplicons were subsequently fragmented to an average size of around 250 bp for Illumina sequencing. For Nanopore sequencing no fragmentation steps were performed. Both sequence technologies resulted in an identical consensus genome with a length of 10,932 nt which encompassed the entire coding region of USUV (Genbank accession numbers *xxx*). The average USUV read length was 159 nt and 162 nt for Illumina sequencing and 462 nt and 545 nt for Nanopore sequencing. The maximum USUV sequence read length was 232 nt and 238 nt for Illumina sequencing and 2531 nt and 3082 nt for Nanopore sequencing (Table 1). When multiplexing 12 samples on one flowcell, a minimal genome coverage of 273× was obtained for sample AS201600070 and of 6744× for sample AS20160083 using Nanopore sequencing. For sample AS201600070, Illumina sequencing poorly covered amplicon 31, resulting in a gap of 38 nucleotides which were replaced by “N's”. Furthermore, the consensus sequences generated by MinION sequencing were identical to the consensus sequences obtained by Illumina sequencing. Phylogenetic analysis showed that both USUV genomes cluster within the Africa 3 lineage (Fig. 1).

Next to homopolymeric regions, the expected random error profile of MinION sequencing was clearly visible in the sequence data and therefore a custom iteration was written to randomly generate subsets of reads to determine the amplicon coverage required before a reliable consensus sequence could be generated. As full genome sequencing in addition to diagnosis of a condition can also be used for tracking of cases or clusters during outbreak investigations, understanding the error frequency of the final sequences is important. To that point, all MinION reads mapping to a 500 bp amplicon were collected and used to generate 1000 random subsets using the random function in Python. Subsets of either 20, 50, 75, 100, 150 or 200 reads were randomly sampled and used to generate a consensus genome by reference-based assembly. The number of discrepancies from the original consensus sequences was counted. This process was repeated three times to get a reliable estimate (Table 2). A genome coverage of 100 reads resulted in highly robust sequences, with only one erroneous position out of the 3000 × 390 positions inspected (0.00005%). This means that one in every 1,170,000 nucleotides is called erroneously which corresponds to one miscalled nucleotide position in every 106 USUV whole genomes sequenced. A virus sequenced with a coverage of 20, 50 or 75 reads resulted in respectively 402, 34 or 12 erorous positions in every 1,170,000 nucleotides sequenced (0.0344%, 0.0030% and 0.0010%). These coverage cut-offs correspond to respectively 3.75, 0.32 or 0.11 wrongly called nucleotides per whole USUV genome sequenced.

4. Discussion and conclusion

Whole genome sequencing has been shown to be a powerful tool in outbreak scenarios (Arias et al., 2016; Faria et al., 2018; Grubaugh et al., 2007; Quick et al., 2017). However, preferably this should be done as fast as possible to enable rapid risk assessment and outbreak control. Here, we describe a multiplex PCR protocol for whole genome USUV sequencing on the Nanopore platform, which provides a rapid turnaround time but is less reliable due to the high reported error rate. We challenged the platform and studied reliability of consensus sequence in relation to depth of coverage. We show that – despite the error frequency – when the input levels of RNA are high enough, robust consensus genomes can be generated with quality equal to that of Illumina sequencing but with a fast turnaround time and at substantially lower cost. While multiplexing 12 samples on one flowcell we have calculated the cost to be around €50 per USUV genome sequenced when using the Nanopore technology as compared to around €220 per USUV genome sequenced when 12 samples would be multiplexed and sequenced on the Illumina Miseq machine. These costs per generated USUV genome might differ per institute and do not include the initial investments costs to buy the sequence machines. A minimal read coverage of 100× resulted in no more than one erroneous nucleotide position in every 106 USUV whole genomes sequenced, while using the lower read coverage of 20×, around 3–4 bases might be called wrong per full length USUV sequence (0.0344% error rate). This error rate might be problematic for very detailed research questions like contact tracing or the identification of transmission clusters where single nucleotide mutations are important (Qiu et al., 2015). However, for more general research questions like for instance to determine if a certain virus is part of an ongoing cross-species transmission chain or if there is a particular strain which perhaps is suddenly sustained by human-to-human transmission, this reported error range (0.0344%) is acceptable and will not influence the conclusions.

The error profile in Nanopore sequencing requires different analytical tools for sequence analysis. Instead of performing a *de novo* assembly, which is commonly used for Illumina data, a reference-based alignment can be performed. Although this process is in general faster and requires less computational power, a major limitation of using

Table 1
Platform comparison for USUV whole genome sequencing.

	USUV Ct value	QC reads	%USUV reads	Min coverage	Max coverage	Average USUV read length	Max USUV read length
Illumina							
Owl_AS201600070	13.35	597,690	86.87%	0×	17,463×	159 nt	232 nt
Owl_AS201600083	12.94	575,850	91.96%	3081×	15,429×	162 nt	238 nt
MinION							
Owl_AS201600070	13.35	390,652	96.06%	273×	42,398×	462 nt	2531 nt
Owl_AS201600083	12.94	498,203	92,05%	6744×	60,375×	545 nt	3082 nt

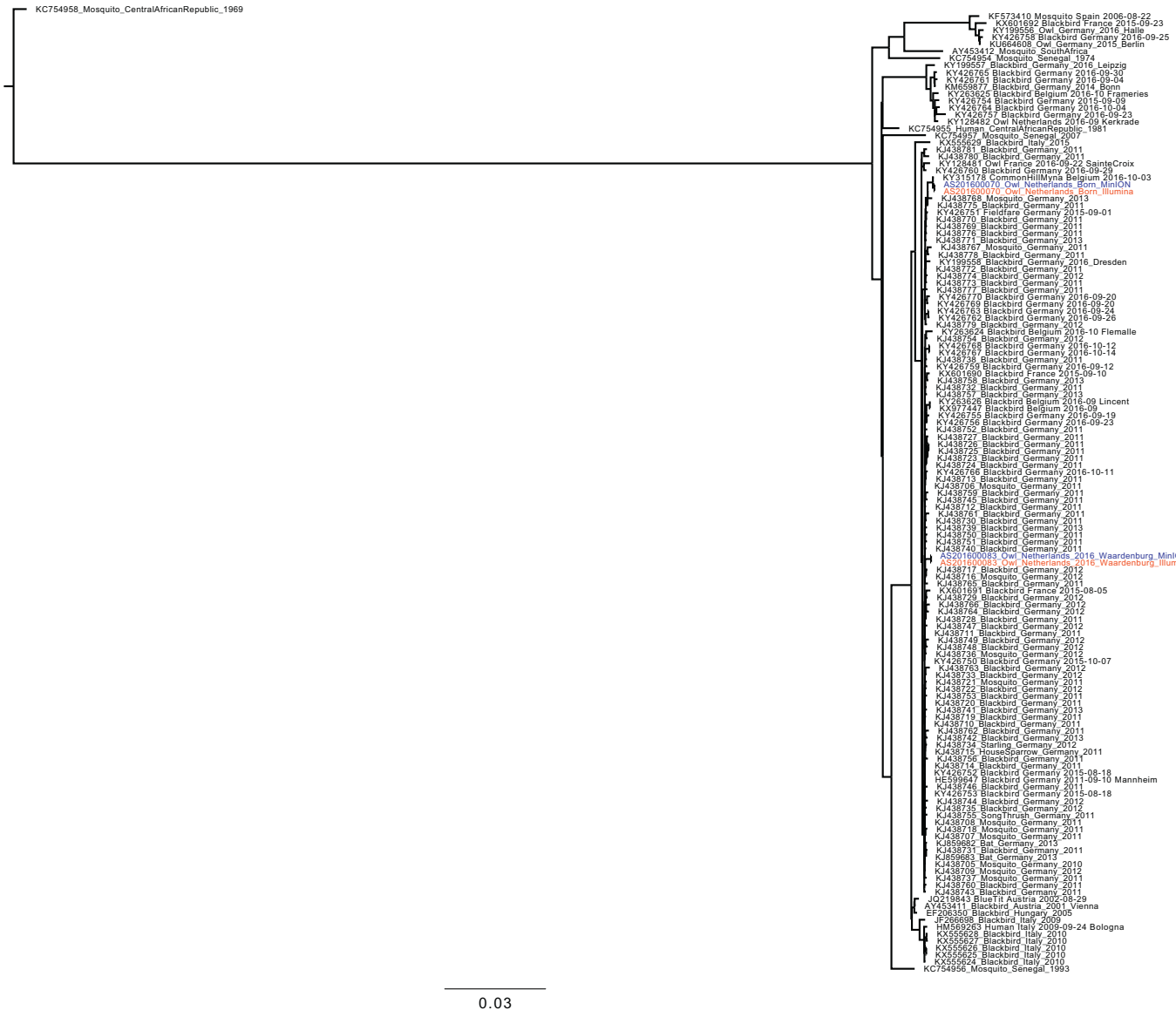

Fig. 1. Phylogenetic analysis of the whole genome sequences USUV. The tree was constructed under GTR + I + G4 model as best predicted model using the best-fit model prediction option in IQ-TREE. The scale bar represents the amount of nucleotide substitutions per site.

Table 2
Error rate in the consensus sequence (390 nt) after MinION sequencing with different reads coverages. Each iteration consisted of 1000 times random sampling.

Coverage	Errors iteration 1	Error rate iteration 1	Errors iteration 2	Error rate iteration 2	Errors iteration 3	Error rate iteration 3
20×	154	0.0395%	120	0.0308%	128	0.0328%
50×	11	0.0028%	11	0.0028%	12	0.0031%
75×	3	0.0008%	3	0.0008%	6	0.0015%
100×	0	0.0000%	0	0.0000%	1	0.0003%
150×	0	0.0000%	0	0.0000%	0	0.0000%
200×	0	0.0000%	0	0.0000%	0	0.0000%

reference-based alignments is that a close reference sequence needs to be available. Nevertheless, for tracking virus outbreaks – where the pathogen in question is known – this approach can be used, in combination with the amplicon based whole genome sequencing approach that is biased against divergent viruses. A next level of ambition would be to move to metagenomic sequencing, combined with approaches to reduce the error rate of Nanopore sequencing to reliably perform metagenomic sequencing in low resource settings.

An important aspect of successful real-time sequencing is the quality of the primersets designed for the generation of the amplicons. The USUV primers were generated according to the recently described protocol (Quick et al., 2017), but have been manually fine-tuned to enable targeting of all currently known USUV strains. The read coverage per amplicon sequenced varied in the two selected USUV strains, possibly due to the degenerative primers of which some might perform better than others for different USUV strains. For Illumina sequencing one amplicon was not completely sequenced, while this amplicon was sequenced with a depth of 237× with Nanopore sequencing. This difference can likely be explained by the differences in the library preparations for different sequence platforms. Illumina sequencing requires an additional DNA fragmentation step (Head et al., 2016), while the third generation Nanopore sequencing platform can directly sequence amplicons of different lengths.

Currently, the throughput of the Nanopore sequencer is lower than that of Illumina sequencing. Illumina sequence machines can sequence between 4 million (MiniSeq) and 10,000 million reads per run

(NovaSeq S4 chip), while the Nanopore platform can sequence up to around 8 million reads. However, typically, in an outbreak investigation, the number of samples for sequencing is limited, and the lower throughput platform actually is favorable as it can be run at limited cost with small sample numbers. Upscaling of the sequence effort can be achieved by starting several runs in parallel or by using the GridION or PromethION machine, although these machines are not portable and cannot easily be used in field applications. The platform choice therefore depends on the specific questions asked, the access to equipment, the time to result required, and the costs of sequencing. With the small instrument size and relatively low equipment costs, MinION sequencing has shown to be promising for outbreak sequencing in terms of costs speed, ease of use and portability.

One of the most important advantages of nanopore sequencing is that every molecule is sequenced regardless of its length which obviates the need for fragmentation of the sequence library. Next to this simplified library preparation methodology, Nanopore sequencing also produces results in real-time which facilitates quick decision making which can be crucial to guiding public health measures and research. We show that the higher error rate of nanopore sequencing has a minor impact on sequence consensus for samples yielding high coverage sequences, to a level that is compatible with more in-depth analyses on relatedness of genomes. Whether this can be achieved depends greatly on the target virus: we used USUV positive samples from deceased birds that contained high viral loads and would not hesitate to use nanopore sequencing routinely for characterization and tracking of USUV outbreaks based on dead bird surveillance. In contrast, levels of viremia for infections in humans can be quite low, as observed for instance in Zika virus infection or West Nile virus infection, making whole genome sequencing more challenging. In addition, in live bird surveillance most often low USUV viral loads can be detected in throat swabs. Therefore, the choice of sequence platform is highly dependent on the model organism, viral loads and research questions.

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