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Protocols

Serotyping of foot-and-mouth disease virus using oxford nanopore sequencing

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ABSTRACT

Foot-and-mouth disease virus (FMDV), belonging to the family of Picornaviridae, infects mostly cloven-hoofed animals and leads to huge economic losses. Since there is no cross-protection between the seven serotypes of FMDV, effective vaccination relies on the knowledge of the serotype causing the outbreak. The most common methods of serotyping are antigen ELISAs and amplification-based sequencing. Serotype-specific PCR methods exist but have limitations due to emerging mutants within serotypes. Sequencing is a promising technology, but currently suffers from cumbersome procedures and long turnaround times. In this study, we have established a novel sequencing protocol relying on nanopore sequencing and offline BLAST search. The procedure was completed in 5 h including RNA extraction, reverse transcription, second-strand synthesis, barcoding, sequencing and data analysis, which did not require a bioinformatician. In total, 12,193 sequence files were obtained. The offline BLAST search to the P1 region revealed the most successful categorization of the seven FMDV serotypes (specificity: 98.3%) over whole genome (24.8%), P2 (23.6%) and P3 (21.4%). In conclusion, our protocol enables rapid and reliable FMDV serotyping. The whole procedure can be conducted with a mobile suitcase laboratory, which is easy to use at the point of need in endemic countries.

1. Introduction

Foot-and-mouth disease virus (FMDV) is a highly infectious positive single-stranded RNA virus belonging to the family of Picornaviridae. The virus affects mainly cloven-hoofed animals causing huge economic losses including high mortality in young stock, reduced herd fertility and productivity, trade restrictions as well as high cost for control programs. FMDV has seven serotypes (SAT1-3, Asia-1, A, O and C). Vaccines are available, but there is no cross-protection between the serotypes and tremendous variability within them. Thus, identifying the serotype and strain is crucial to select an appropriate vaccine to confine outbreaks (Knight-Jones and Rushton, 2013; Tekleghiorghis et al., 2014). Antigen ELISA with serotype-specific polyclonal sera is the most common method of serotyping FMDV (Roeder and Le Blanc Smith, 1987). However, the ELISA has limited sensitivity and is prone to cross-reactions between serotypes. Type-specific RT-PCRs targeting the VP1-encoding genome region are also applied for serotyping, both as conventional and real-time PCR is highly sensitive and specific, but most typing assays are tailored to a pool of viruses circulating in a defined region and may not be able to identify emerging strains or new viruses of unknown origin that are introduced into free areas (Bachanek-Bankowska et al., 2016). Another promising way of serotyping is sequencing of the FMDV genome, which also gives more insight into the virus evolution and allows tracing the origin and spread of an outbreak. However, many current VP1 sequencing protocols are lengthy and cumbersome procedures including two amplification steps, in addition to the need of expensive equipment and reagents (Dill et al., 2017; Knowles et al., 2016; Longjam et al., 2011; Soltan et al., 2017).

In this study, we established for the first time a rapid sequencing protocol for FMDV serotyping using a nanopore sequencing method operated in a mobile suitcase laboratory.

2. Material and methods

2.1. Viruses

The FMDV isolates A_{22} IRQ 24/64, O_1 Manisa TUR/69, C_1 Noville SWI/65, Asia-1 Shamir ISR/89, SAT1 ZIM 25/89, SAT2 ZIM 11/91 and

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SAT3 ZIM 4/81 were selected from archival stocks at the Friedrich-Loeffler-Institut (FLI), Germany.

2.2. Sample preparation and RNA extraction

Fresh cultures of all isolates were grown separately in monolayers of BHK-21 cells (RIE 164, Collection of Cell Lines in Veterinary Medicine, FLI). Infected cells were incubated in a closed system at 37 °C until full cytopathic effect was visible. After freezing and thawing, the supernatant of the seven samples was clarified of cell debris by centrifugation for 10 min at 3200 × *g* at 4 °C. Virus was pelleted through a 30% (wt/vol) sucrose cushion in 40 mM sodium phosphate buffer (pH 7.6) with 100 mM sodium chloride (buffer P as in (Gullberg et al., 2013)) by centrifugation at 125,755 × g in a SW32Ti rotor (Beckman Coulter, Optima 170 LE-70) for 2 h 50 min at 7 °C. Pellets were resuspended in 140 µL buffer P. FMDV RNA of the individual serotypes was extracted using the RNeasy® Mini Kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions. RNA content was determined with a spectrophotometer (NanoDrop 2000c, Thermo Fisher Scientific) by repeated measurements of absorption at 260 nm.

Under field conditions, where no centrifuge is available, the RNA extraction can be performed directly from vesicular material with the Dynabeads SILANE Viral Nucleic Acid kit (Invitrogen, Darmstadt, Germany) as described previously (Abd El Wahed et al., 2013).

2.3. Library preparation

The library preparation was carried out in the mobile suitcase laboratory using a direct cDNA native barcoding protocol (Fig. 1; the full procedure is shown in additional file 1). Firstly, reverse transcription and strand switching was performed with 250 ng of RNA using the *In vitro* SuperScript IV VILO Master Mix (ThermoFisher Scientific, Waltham, MA, USA) with the VNP primer targeting the poly-A tail of FMDV RNA (5′-phosphate-ACTTG CCTGT CGCTC TATCT TCTTT TTTTT TTTTT TTTTT TTVN-3′) and strand-switching primer (5′-TTTCT GTTGG TGCTG ATATT GCTGC CATTA CGGCC-mGmGmG with 2' O-methyl RNA bases-3′), respectively. These primers were suggested by Oxford Nanopore Technologies; for details about their function, please refer to (Zhu et al., 2001). Secondly, 2nd strand synthesis was conducted using the LongAmp Taq Master Mix (New England Biolabs, Ipswich, MA, USA) and PR2 primer (5′-TTTCT GTTGG TGCTG ATATT GC-3′). Thirdly, end-repairing was performed using the NEBNext Ultra II end-repair/dA-tailing module (New England Biolabs). Barcoding was carried out with the NEB Blunt/TA Ligase Master Mix (New England Biolabs) and Native Barcoding Kit 1D (NBD103, Oxford Nanopore Technologies, Oxford, UK) as follows: Barcodes 1, SAT1; 2, SAT2; 3, SAT3; 4, Asia-1; 5, A; 6, O; 7, C. After barcoding, the samples were pooled together. Fourthly, ligation of sequencing adaptor and tether attachment was done using the SQK-DCS108 sequencing kit (Oxford Nanopore) and NEB Blunt/TA Ligase Master Mix. Finally, the preparation was mixed with running buffer FM1 and Library Loading Beads Buffer (Oxford Nanopore) and loaded into an R9.4 flow cell fixed on the top of the MinION device (Oxford Nanopore).

2.4. Data processing

The sequencing run was performed in a MinION (Oxford Nanopore) within 20 min. The data was automatically saved in FAST5 format on

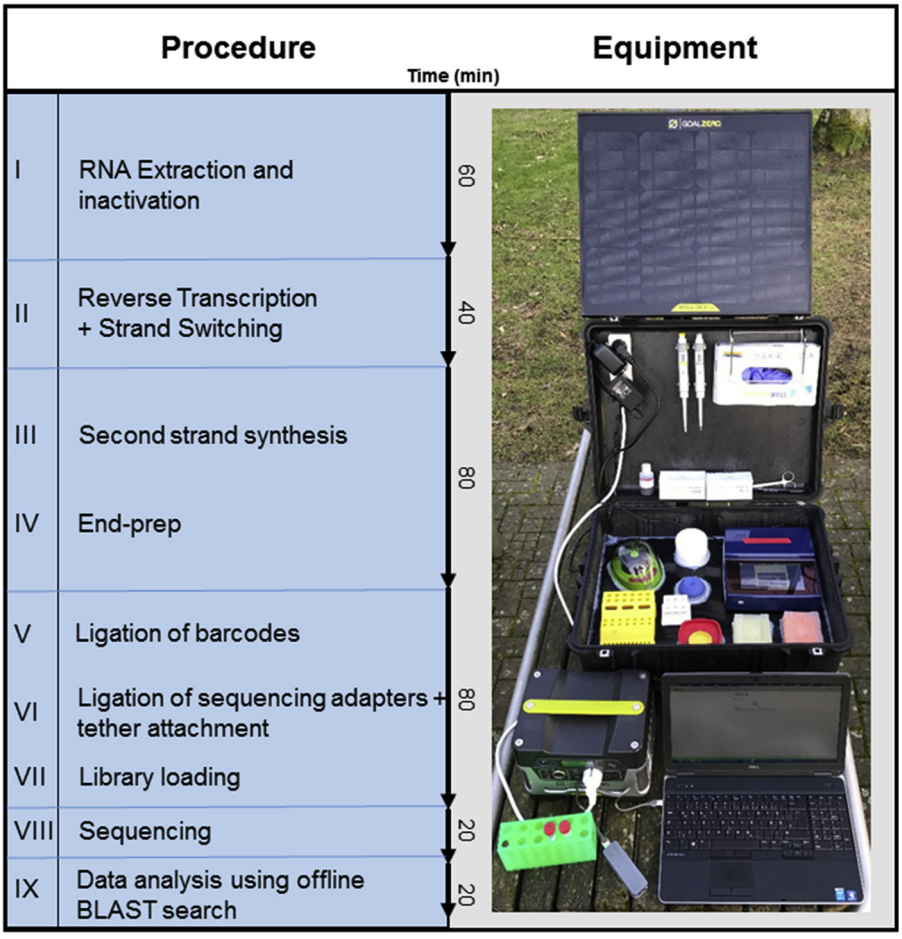

Fig. 1. Work flow and the required equipment for the serotyping of FMDV.

the laptop *via* the MinKnow Software (Oxford Nanopore). The sequence files were automatically transferred to FASTQ format and categorized into barcodes using ALBACORE 2.0.1 (Oxford Nanopore). Duplicated reads and sequences less than 300 and more than 9000 nucleotides were deleted using GENEIOUS v9.1.6 (Biomatters Ltd., Auckland, New Zealand). Thereafter, sequences were aligned to offline local BLAST databases encompassing either P1, P2, P3 or the whole FMDV genome (for full sequence and accession numbers, please see additional file 2). The offline database was created ahead of time according to the procedure in the GENEIOUS manual. Local BLAST search was also performed using GENEIOUS applying the MEGABLAST algorithm and showing results as Query-centric alignment only. A maximum of 1000 hits per read was allowed with an E-value of 1e-100. Decision on the serotype was made based on the total number of possible GenBank data hits aligned to the nanopore sequence data. In order to determine the specificity of using the offline database of either the whole FMDV genome or particular gene region for serotyping, the number of hits from a particular serotype aligned correctly to sequence reads was divided by the total number of hits of all serotypes placed under the same barcode. A hit is one FMDV sequence with a distinct GenBank accession number.

3. Results

In total, 12,193 sequence files were generated and processed to FASTQ, from which 7372 could pass our sequence filtration. Categorization of the reads into the various barcodes is shown in Fig. 2. BLAST search revealed between 14.3 and 32.5 percent of the reads as FMDV sequences. Serotyping using BLAST searches to the whole genome as well as to sequences of the P2 and P3 region of FMDV revealed a very poor specificity, 24.8, 23.6 and 21.4%, respectively. In contrast, applying the P1 database for alignment to the reads *via* MEGABLAST yielded the highest specificity (98.3%).

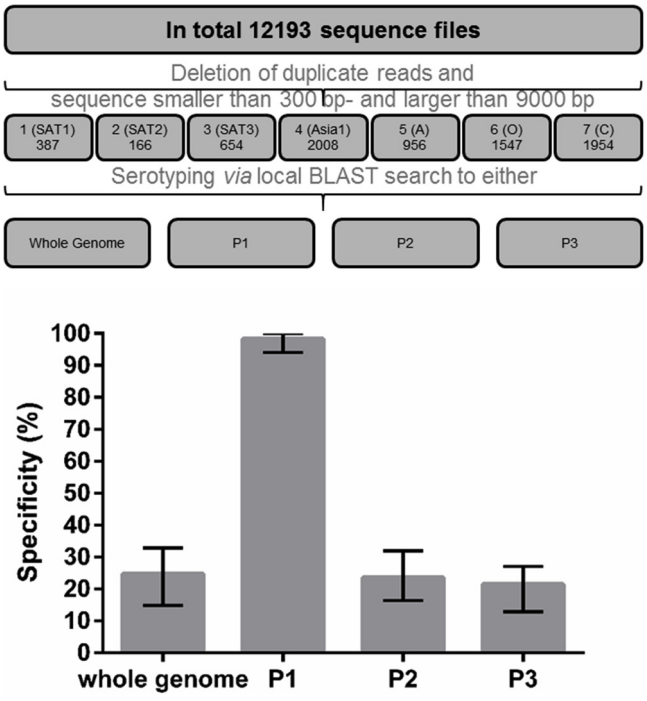

Fig. 2. Flow chart of analysing the sequence data using offline BLAST search. The lower graph shows the success rate of BLASTing the reads to the right FMDV serotype.

4. Discussion

We have developed a rapid sequencing method for serotyping of FMDV. Direct cDNA sequencing using a MinION device was combined with an offline local BLAST search in order to allow point-of-need FMDV serotyping. The whole protocol was carried out within five hours. Moreover, a bioinformatic background is not required due to the user-friendly interface of GENEIOUS as all steps of data processing are easy to execute and results are presented in a clear way as query-centric alignment. In addition, no highly complicated analysis procedure is needed as the nanopore data was used for identifying the serotype, not for whole genome assembly, which is more convoluted.

In this study, we applied a local BLAST search to align the obtained reads to FMDV sequences stored in GenBank. By establishment of an offline local data-calling algorithm, we decreased the time to result and overcame the need of a stable and powerful internet connection, which is usually absent in the areas most affected by FMD. The FMDV open reading frame consists of four functional regions (L, P1-3). P1 encodes the structural proteins of the virus, while L, P2 and P3 contain non-structural proteins that play an important role in virulence, viral replication and virus-host interaction (Gao et al., 2016). It is not surprising that the P1 region worked best for serotyping because i) P2 and P3 are more conserved between the different serotypes ii) the NCBI database did not contain enough sequences of the P2 and P3 regions to cover all variants of the serotypes (Carrillo et al., 2005). Co-infection with different serotypes can be efficiently determined *via* the calculation of the number of hits allocated to each serotype.

Using sequencing overcomes the problem of variability in the genome as viruses from all serotypes can be detected independent of the geographical region or virus pool.

Previously published sequencing protocols rely mainly on sequencing by synthesis using either Sanger or Illumina sequencing (Knowles et al., 2016; Logan et al., 2014). These approaches are cost-effective and yield accurate sequences with high throughput. However, both methods are not field applicable, due to the need of expensive and complex devices, and have time-consuming protocols. Additionally, Illumina sequencing requires a bioinformatic background for data analysis. In contrast, the nanopore sequencing produced results in a very short time and the whole procedure can be performed in a mobile suitcase laboratory (Abd El Wahed et al., 2015), with no need for sophisticated infrastructure or laboratory capacities. This enables serotyping directly at the site of an outbreak in low-resource settings. In FMDV-free countries, native samples from suspect animals must be handled with greater care, but after they have been treated with a lysis buffer, the downstream analysis can be safely performed under BSL-2 conditions.

A −20 °C freezer is still needed for long-term storage of sequencing reagents. Fortunately, the sequencing kits and the flow cells can be kept at room temperature for one day, which is more than enough to perform the experiment. One of the big limitations of the technology is the cost per one sequencing run for seven samples (€2300) and another €8500 is needed as a starting cost for the suitcase laboratory.

Rapid sequencing of RNA viruses was established before by others (Batovska et al., 2017) employing the SuperScript III First-Strand Synthesis System (ThermoFisher Scientific, Waltham, MA, USA) and random hexamer primers for cDNA synthesis along with the SQK-NSK007 kit (Oxford Nanopore Technologies, Oxford, UK) for library preparation. By contrast, in our study we have used the SuperScript IV VILO kit, which produces higher quality cDNA in less time (10 min). In addition, the application of a poly-T primer binding to the poly-A tail of the FMDV RNA has allowed the synthesis of a long cDNA strand instead of multiple cDNA fragments in the case of random hexamer primers. Furthermore, the SQK-NSK007 kit is designed for the sequencing of a single sample, while the SQK-DCS 108 kit allowed us to sequence seven samples simultaneously in the same run.

Generally, various sequencing methods have a limit of detection at 10^4 DNA molecules per tested sample (Liu et al., 2012). In clinical cases

of FMDV, the viral load in the vesicular lesions is high ($\geq 10^{11}$ genome copies/g) and in the preclinical phase it ranges from $10^{5.1}$ to $10^{9.8}$ genome copy/g (Pharo, 2002; Stenfeldt et al., 2015). In carrier animals, the viral load in probang samples is low: $10^{3.5}$-$10^{5.2}$/ml (Stenfeldt et al., 2016). However, the amount of highly purified RNA needed for sequencing can easily be obtained either directly or by a nucleic acid concentration method.

5. Conclusions

In conclusion, we have developed a fast and reliable method for sequence-based serotyping of FMDV. The whole procedure can be easily implemented in a regular laboratory workflow or with a mobile suitcase laboratory in a low-resource setting. The next points to address will be the decrease of pipetting steps and the switch to lyophilized reagents, which are stable at ambient temperature.

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Declarations of interest

None.

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Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:https://doi.org/10.1016/j.jviromet.2018.10.020.

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