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Probe capture enrichment next-generation sequencing of complete foot-and-mouth disease virus genomes in clinical samples

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ABSTRACT

Next-generation sequencing (NGS) techniques offer an unprecedented "step-change" increase in the quantity and quality of sequence data rapidly generated from a sample and can be applied to obtain ultra-deep coverage of viral genomes. This is not possible with the routinely used Sanger sequencing method that gives the consensus reads, or by cloning approaches. In this study, a targeted-enrichment methodology for the simultaneous acquisition of complete foot-and-mouth disease virus (FMDV) genomes directly from clinical samples is presented. Biotinylated oligonucleotide probes (120 nt) were used to capture and enrich viral RNA following library preparation. To create a virus capture panel targeting serotype O and A simultaneously, 18 baits targeting the highly conserved regions of the 8.3 kb FMDV genome were synthesised, with 14 common to both serotypes, 2 specific to serotype O and 2 specific to serotype A. These baits were used to capture and enrich FMDV RNA (as cDNA) from samples collected during one pathogenesis and two vaccine efficacy trials, where pigs were infected with serotype O or A viruses. After enrichment, FMDV-specific sequencing reads increased by almost 3000-fold. The sequence data were used in variant call analysis to identify single nucleotide polymorphisms (SNPs). This methodology was robust in its ability to capture diverse sequences, was shown to be highly sensitive, and can be easily scaled for large-scale epidemiological studies.

1. Introduction

Foot-and-mouth disease (FMD) is a highly contagious viral disease that affects all mammalian species belonging to the order *Artiodactyla*. It is an acute, systemic vesicular disease characterised by lesions developing on areas of friction, such as the tongue, mouth, nostrils, teats, udders and feet. Despite low mortality rates in adult animals, FMD severely decreases the productive capacity of livestock and results in devastating economic loss and trade restrictions. The causative agent, foot-and-mouth disease virus (FMDV), belongs to the genus *Aphthovirus*, family *Picornaviridae*. There are seven FMDV serotypes (A, O, C, Asia-1, SAT 1, SAT 2 and SAT 3). Cross-protection following infection or vaccination is serotype-restricted, and is not always complete if vaccines contain different subtypes or variants of the same serotype (Sobrino et al., 2001).

FMD is endemic in many parts of the world and occurs in most countries in South East Asia (SEA), where regular outbreaks of FMDV serotypes O, A, and Asia-1 are reported (Gleeson, 2002; Knowles et al., 2012; Rweyemamu et al., 2008). In this region, FMDV serotype O viruses belonging to the O/SEA topotype (Mya-98 and Cam-94 strains), O/ME-SA topotype (PanAsia lineage; the derivative Pan-Asia-2 sub-lineage and O/Ind2001d sub-lineage) and O/Cathay topotype, serotype A (ASIA topotype; SEA-97 strain) and serotype Asia-1 have been identified (Abdul-Hamid et al., 2011; Le et al., 2010a, b; Qiu et al., 2018).

Single-stranded RNA viruses, including FMDV, evolve rapidly due to their large population size, high replication rate, and the lack of proof-reading ability of their RNA-dependent RNA polymerase (Domingo and Holland, 1997). These viruses exist as heterogeneous and complex populations comprising similar but non-identical genomes referred to as 'intra-host genetic diversity', but the evolutionary importance of this phenomenon remains unclear (Domingo et al., 2002; Eckerle et al., 2010; Holmes and Moya, 2002). Tracing and monitoring the

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transboundary movements of FMDV have been successfully achieved using consensus sequences of the 1D coding region of the viral genome (Kasambula et al., 2012; Knowles and Samuel, 2003; Samuel and Knowles, 2001). However, over shorter epidemic time scales, where viral populations have not substantially diverged, 1D sequencing alone cannot provide the required resolution to discriminate between viruses in field samples collected from neighbouring farms within outbreak clusters (Cottam et al., 2009). Consensus sequencing using the Sanger method identifies the predominant or major viral sequence in a sample but is uninformative about minority variants that are present. Evidence for population heterogeneity, where individual sequences differ from the consensus sequence, was obtained routinely using cloning approaches (Airaksinen et al., 2003; Cottam et al., 2009), providing insights into the evolutionary processes that shape viral populations. Unfortunately, these cloning processes are laborious and usually provide only a limited resolution of the mutant spectrum within a sample. Whole genome sequencing at the consensus level has proven to be a powerful tool for the reconstruction of virus transmission trees (Valdazo-Gonzalez et al., 2012). Next-generation sequencing (NGS) techniques offer a "step-change" increase in the amount of sequence data that can be rapidly generated from a sample and can be applied to sequence viral genomes to obtain ultra-deep coverage (Wright et al., 2011). Although the importance of minor variants remains unclear in relation to both FMDV transmission and evolution (Holmes and Moya, 2002), it has been hypothesised that NGS may help to assess the mechanism of immune escape and virus evolution in both unvaccinated and vaccinated animals (Hauck et al., 2018).

Conventional sequencing protocols are subject to biases such as those encountered during PCR amplification and propagation in cell culture, are restricted by the need for large quantities of starting material, and do not convey sufficient information on how the virus is evolving within the host before transmitting to other susceptible species (Logan et al., 2014). NGS techniques create massive amounts of sequence data in parallel and can be used to produce a snapshot of genetic diversity of the FMDV polyprotein coding region, at both intra-host and intra-herd levels (Radford et al., 2012). This resolution enables monitoring of the entire sequence swarm that exists within FMDV samples and can be employed to assess the impact of transmission within and between hosts upon sub-consensus polymorphisms (Paton et al., 2018). A new method for deep sequencing of FMDV genomes using an enrichment method with biotinylated oligonucleotide baits targeting the FMDV genome is described.

2. Materials and methods

2.1. Sample collection

Samples were collected from pigs that were recruited for two independent vaccine efficacy studies (Nagendrakumar et al., 2015; Vosloo et al., 2015; Wilna et al., 2015) and one pig pathogenesis trial (unpublished). Oral and nasal fluids, and rectal samples were collected using dry cotton swabs (Supplementary Table 1). The swabs were transferred into vials containing 2 ml of virus transportation medium (Basal Medium Eagles with 10% HEPES, 10% Tryptose Phosphate broth, penicillin, streptomycin, gentamicin and amphotericin B).

In each of the vaccine studies, two groups of pigs were vaccinated and challenged on day 4 or 7 post-vaccination using a pig-adapted serotype O or A virus (Nagendrakumar et al., 2015; Wilna et al., 2015). The samples collected on day 3 or 4 post-challenge were used in this study. Swabs collected from two uninfected naïve pigs were used as negative controls. In the pig pathogenesis study, a group of donor pigs was infected with a serotype O virus and a group of naïve pigs was kept in direct contact for 2, 4, 6, 8, 12, 24 or 48 h (unpublished). The samples collected from the donor pigs on day 3 or 4 post inoculation and from the contact pigs on day 3 or 4 post contact were used in this study (Supplementary Table 1).

2.2. RNA extraction

Total RNA from the saliva, nasal and rectal swab samples (Supplementary Table 1) was extracted in duplicate, using the InviMag Virus RNA Mini kit/KF96 (Stratec Molecular) on an automated nucleic acid extraction system (KingFisher* Flex Magnetic Particle Processor, ThermoFisher Scientific) following the manufacturer's protocol. Briefly, swabs (described in Section 2.1) were placed into 2 ml of virus transportation medium and 120 µl of suspension was used for RNA extraction. To obtain positive RNA controls, total RNA was extracted from 120 µl of a 10% w/v suspension, prepared from 1 g of epithelial sample collected from the bulb of the heels of pigs infected with either a serotype O or serotype A virus as described previously (Nagendrakumar et al., 2015; Vosloo et al., 2015; Wilna et al., 2015). Duplicate RNA samples were pooled before further processing. Positive control RNA was extracted from a 10% virus suspension prepared from tissue lesions containing pig-passaged challenge viruses (Nagendrakumar et al., 2015; Vosloo et al., 2015; Wilna et al., 2015). Reverse transcription and quantitative PCR (RT-qPCR) were performed using the Ambion AgPath-ID MasterMix (Life Technologies), as per standard protocols (Shaw et al., 2007), to quantify RNA (copy numbers/ml of total RNA) based on Ct values. *In vitro* transcribed RNA was prepared using the Megascript T7 kit (Ambion) from a plasmid, pBluescript KS+, containing 550 bp from the 5′ UTR region of the FMDV genome (Boyle et al., 2004) and used to generate a standard curve for each RT-qPCR run to quantify the RNA. 18S ribosomal RNA was used as an internal control (Bas et al., 2004).

2.3. Preparation of Illumina DNA libraries from viral RNA

Illumina libraries were constructed from total RNA using the NEBNext Ultra Directional RNA Library Prep Kit for Illumina (New England Biolabs) in conjunction with NEBNext Multiplex Oligos for Illumina (New England Biolabs) according to the manufacturer's instructions. Briefly, 5 µl of total RNA was added to first strand synthesis buffer and random primers before incubating at 94°C for 2 min to generate RNA fragments larger than 500 nucleotides (nt). Following first and second strand cDNA synthesis, double-stranded cDNA was purified using Mag-Bind RxnPure Plus beads (Omega Bio-Tek) and eluted in 60 µl nuclease-free water. To obtain a library size between 400 and 600 nt, size selection of the libraries was performed using Mag-Bind RxnPure Plus beads (Omega Bio-Tek) in a two-step selection, by adding 35 µl, then subsequently 15 µl of beads to the reaction. The library was eluted in 20 µl nuclease-free water and amplified by PCR using the following conditions: initial denaturation at 98°C for 30 s; 20 cycles of denaturation for 10 s at 98°C followed by annealing for 30 s at 60°C and extension for 30 s at 72°C and a final extension step for 5 min at 72°C and a stop reaction at 4°C to hold. Libraries were purified using the MinElute PCR Purification Kit (Qiagen), eluted in 25 µl nuclease-free water, visualised on a 1.5% agarose gel and quantified using a Bioanalyzer High Sensitivity DNA Assay (Agilent).

2.4. Enrichment of viral library

Biotinylated DNA baits that were complementary to the FMDV genome, and 120 nt in length, were designed (Table 1) such that they were located at least 500 nt apart on the FMDV genome, assuming an average deep sequencing library size of the sample would be approximately 300 nt, following protocols described earlier (Kamaraj et al., 2019). Following this approach, the FMDV genomic RNA (after its conversion to cDNA) was captured with fewer baits, unlike some similar methodologies where placements of baits were tiled across the target genome (Bonsall et al., 2005; Depledge et al., 2011; Houldcroft et al., 2016; Miyazato et al., 2016; Vinner et al., 2015). Targeted FMDV genome enrichment was achieved using custom designed biotinylated, 120-mer xGen Lockdown baits (Integrated DNA Technologies) using the

Table 1
Oligonucleotide baits used for lock-down probe capture and enrichment of FMDV RNA from clinical samples. The start and end numbers represent the genomic position of the sequences based on O/MAY/7/2004. The bait sequences were based on O/MAY/7/2004 (GenBank HQ632772 and A/VIT/4/2004 (GenBank HQ632773).

S No	Start	End	RC bait sequence	GC%	Tm (°C)
1	309	190	GGCCCCATCACCGTAGTCTGCTGCGGGGGGAGACCGTAGAGCGTCGAACGGCCCCCAACAGGAGTGCCCAGGACACAGCGCTAACGACCAAGTAGGCGGAGTGTTAAGAGTACTAGTCGGT	62.5	82.9
2	810	691	TTGTTACCAAGGAGGGAGTCCCGCGGCCACCATGCTCCGCTACGAAGCAACAATACCACTACTAACACTCGCCGGTGGAGCCGAGCGTGGAGTCAATCACAGTACAAAGTGTTACCCCTCT	55.0	80.1
3	1311	1192	CAGGCGATTCATAGACCCAGTCGAAGAAAGGTTCATCGACATACCTAAACAACTGCAGGATGGCATTCAGCCAACAGTTGTCATTGTTGTTGGGCCTAGAGTAAAAGGTCTTCTTCTCAC	45.8	75.7
4	1812	1693	CGTTGTCACCAAGTTGTGTGTCCATAGAGTTCTGGTACTGCTGCATGTATGTTGTTGATGATGCTCCCGGTGTTGCCTCGTTCTGCGACCCAGTAGCCGGACTGGATTGTCCGG	53.3	79.0
5[b]	2313	2194	CCACCAATAGGCAGCCCCCGTTGAACTGGTTCCCCACAGCGGTAACTTCGACGTCCCAGCGCTTCCTCATGTAGGCATATGAGTCGGTCAAGCCGCCGTAGACACCTTTGTGGTCAGTCCG	58.3	81.3
6[b]	2814	2695	CCCTGTCCGAGTCCGTCTTCGGTGCAGTATGGCACGTCACCTTCGAAGTGCAGAAACGTGGGACACGCCTCAGCCACATCCAAGAAGTTGGTGAACCGCCCGGCAACATGTTGCGGG	60.0	82.4
7	3315	3196	CACCGTAGTTCTCAACGGTAGCAGTCACGGGGTCGCGCTGACTCGCCCGTCGAAGTGGTCTGCTGGGGGCGTCCACAGGCAAGCGCAGCTCAAAGTCTTTGCCAGCGCTGGCCAGCACGA	64.2	84.6
8	3816	3697	AGTAGGTCTCGGCCCTCTTCATGCGGTACAGCAGTTCTGTCACCCGGGTGGCTTTGATGGCACCGTAGTTGAAAGAGGTAGGCAGCGGCCTCGCCGCCTTCTGAACCAGCACCTGGAGAT	59.20	82.2
9	4317	4198	CGAAACTGAAGACAGGGGCCGGCACGTGAAAGAGACTGGAGAGTCGGAGATCTTCTTCACGACAAAGGTTGCTGTCCAGAATCTCAAGGCCGGTGTCAGCCAGCATGATGGCCACAA	55.0	80.1
10	4818	4699	CTGAATCGGTTCTGCCAGTGAAGTGGGTGGAAATTGCTTGCGCAAGCACGTTCGCAAGGAAACTCTTGCCCTGGCCGGATTTGCCTCGGAGGCAAACGACCACGGGCTCGGGTCTCGACT	58.3	82.3
11	5319	5200	CGACCGGTCAATCACCTCTTGAAGAGTTGGTACACATTCTGAAGAGGCGGTTGGGGCTTGAACATGTCTTGCTGCATTCTCTTCATTTCGACTGCCATGCCGTTGAGAAGGGCGCAGT	52.5	79.4
12	5820	5701	CTTCAGCTTGTGGTTGCTCTTCCACAGATTTGGCGGGCTCGGAGTTCACGTCATCGCTCGTCTTGTGTCCTGGGAGAGTTCTCTCTGTCGAAACCGACGGTGCTGGCACCGCTAGTCTCCA	56.7	80.7
13	6321	6202	TCACGCGGTTCCCACGGTGCAGCACCATGAGCGCGGCGTCTGAGAGCATGTCCTGTCTTTACTTTAATCTCAAACTCAAACACTCTGTATGTCACTGTCTGTCATGGCTCTCGCGTCCA	53.3	79.7
14	6822	6703	CAACCCCTTCGTCCAGTCGCGGATCCTTGTTGGACAAGGCAGCGGGACCGTATTCGGGGTTGAACACACCGTCGGCCACGGTGGGTGCGGAGCTTGGTTTTGCGCATGCAGTGCACGCGCT	62.5	84.4
15	7323	7204	TACAGCCAACCGCAGATCCAATTTGCGGGCCGTTGTTTGAGTGCATTTGTGCACAAAACCTGCCAATCATCATCCTGGTGTAAAGAATATGTTCAACAGGCAGGACATCGACAATGCGGGG	48.3	78.1
16	7824	7705	AAGTAACATCAGTAATGGAATGACCAAGAACAAAACCTTTGTCACTTTTGTCAGCTGGAGTAATGGTTTGACCAAGAGATTTGAAGTGAGGCTTGAGAGCCTCAAAGTCCAGATCATAAT	39.2	73.3
17[a]	2828	2768	CCAATAGGCGCTGCGCGTTCGTTCTGTCTCAACATACGGTTTCCCGCCGTGGAAACAGAGAAAGGTGGGGCACGCCTCTGCTACATCCAACAAGTTTGTAAACCGCCCAGGGTAGTTGG	55.8	73.1
18[a]	2130	2070	GGGGCAGTAGAGCTCGGCGCGCTTCATGGCACGAGGAGTTCTCGGATCTCCGTGGCTCGAACCGCCGCCGTAGTTGAAGGAGGCAGGGAGCTGTGCGGGAGCCTCGCGCGCGAGAGACCC	68.3	74.3

[a] Baits specific for serotype A.
[b] Baits specific for serotype O.

complete genomic sequence of FMDV O/MAY/7/2004 (GenBank HQ632772) targeting the O-Myanmar 98 lineage and FMDV A/VIT/4/2004 (GenBank HQ632773.1) targeting the A SEA-97 lineage, respectively (Table 1). To ensure that this minimalistic targeting did not compromise our ability to obtain full genome coverage, all publicly available full genome sequences for serotype O and A viruses were analysed to assess the efficacy of each bait. Prior to capture of viral sequences, 1 µl each of xGen universal blocking oligo TS-p5 and TS-p7 (Integrated DNA Technologies), matched accordingly to the library index, was added to 20 µl of library DNA and 0.5 µl of 5 µg Cot-1 DNA (Invitrogen) to block binding of baits to non-viral regions of library fragments. Blocked libraries were ethanol precipitated and resuspended in 2.5 µl of nuclease-free water, 3 µl Nimblegen hybridisation solution and 7.5 µl Nimblegen 2X hybridisation buffer (Roche). Following a 10 min incubation at room temperature, resuspended libraries were denatured at 95°C for 10 min and cooled on ice before the addition of the FMDV bait pool. A total of 3 pmol of baits was added and hybridised to the libraries for 4 h at 65°C. To capture virus-specific library fragments, 100 µl of magnetic M-270 streptavidin Dynabeads (Life Technologies) were added to the hybridisation reaction and the mix was incubated for a further 45 min at 65°C, with shaking at 2000 rpm in a ThermoMixer C (Eppendorf). Streptavidin beads were washed to remove unbound DNA using the SeqCap EZ hybridisation and wash kit (Roche) according to the manufacturer's instructions. A post-capture PCR amplification of with P1 and P2 primers (Illumina) was performed using the following conditions: initial denaturation at 95°C for 2 min; 20 cycles of denaturation for 20 s at 95°C followed by annealing for 20 s at 65°C and extension for 15 s at 72°C and a final extension step for 3 min at 72°C and a stop reaction at 4°C to hold. The enriched library was purified using the MinElute PCR Purification Kit (Qiagen) and eluted in 25 µl nuclease-free water, visualised on a 1.5% agarose gel and quantified using a Bioanalyzer High Sensitivity DNA Assay (Agilent). All samples were subjected to the same library preparation and enrichment treatment.

2.5. Analysis of sequencing data

For genome assembly and variant calling, sequencing reads were subject to processing in the VIPR4 pipeline (https://github.com/nf-core/vipr/). Briefly, adapters were trimmed and quality was assessed by Skewer (Jiang et al., 2014) and FASTQC (https://www.bioinformatics.babraham.ac.uk/projects/fastqc/), respectively. Host reads were removed by the decont.py script (https://github.com/CSB5/decont/blob/master/decont.py). The remaining non-host reads were assembled into contigs using the BBTools Tadpole module (https://jgi.doe.gov/data-and-tools/bbtools/). Contigs were expanded by incorporating unassembled viral reads through an iterative mapping process until no further viral reads were incorporated (https://github.com/nf-core/vipr/). A final mapping against this newly created assembly was performed with BWA-MEM (Li and Durbin, 2009) and low-frequency variants were identified by LoFreq2 (Wilm et al., 2012).

Exploratory data analysis was performed using Python (www.python.org/). The minimum frequency of reads mismatched to the consensus at a given position was initially set to 0.5% (1 in 200 reads). Location and frequency of single nucleotide polymorphisms (SNPs) along the FMDV genome were determined and, to ensure the positions were correctly aligned, the coordinates were adjusted relative to a multiple sequence alignment (MSA) for that serotype. Venn diagrams showing the correspondence of SNP locations between different groups of samples were constructed for comparison. Only the positions of SNPs and no indel variants were considered for the data analysis.

3. Results

3.1. Viral genome enrichment using baits

To create a virus capture panel targeting serotype O and A simultaneously, 18 baits targeting the highly conserved regions of the 8.3 Kb FMDV genome were designed using the full genome sequences of FMDV O/MAY/7/2004 (GenBank HQ632772) and FMDV A/VIT/4/2004 (GenBank HQ632773.1). Fourteen baits common to both serotypes O and A, 2 baits specific to serotype O and 2 baits specific to serotype A were pooled for the enrichment of FMDV RNA (as cDNA) in the samples. To test the sensitivity of the baits, total RNA from tissue suspensions containing pig-adapted serotype O or A challenge viruses were used.

Illumina libraries were constructed for the RNA derived from the pig-adapted inoculum (O/VIT/2010 and A/VIT/5/2015) and either sequenced directly, or enriched for FMDV genome with the bait panel and then sequenced. The resulting reads from both unenriched and enriched libraries were mapped against FMDV O/MAY/7/2004 (GenBank HQ632772), targeting the FMDV/O/SEA/Mya98 lineage, or FMDV A/VIT/4/2004 (GenBank HQ632773.1), targeting the FMDV/A/ASIA/SEA-97 lineage. The consensus genomes for the serotype O and A viruses generated under the enriched conditions were matched with genome sequences available in GenBank. Post enrichment mapping achieved 93.70–96.25% genome coverage compared to < 10% without prior enrichment. The sequence data of serotype O matched all the serotype O isolates belonging to FMDV/O/SEA/Mya-98 lineage, and that of serotype A matched with A/Asia/SEA97 lineage (results not shown).

To test the specificity of the baits, total RNA isolated from oral and nasal swabs collected from two uninfected naïve pigs were used for the construction of Illumina libraries and were sequenced either directly or following enrichment with the bait panel. The resulting reads from each condition were mapped against the consensus genome generated previously from the enriched condition. None of the oral or nasal swabs collected from the uninfected naïve pigs showed any FMDV-specific reads. However, when oral and nasal swabs collected from infected pigs were used for library construction, FMDV-specific reads were obtained in samples without (Fig. 1A), and with (Fig. 1B) enrichment. FMDV-specific reads were obtained from the pig-adapted virus inoculum of A/VIT/2005 virus without (Fig. 1A), and with (Fig. 1B) enrichment. In the unenriched and enriched libraries, a maximum of 10.96% and 99.34% of the total reads were mapped as FMDV-specific, respectively. Use of the capture probes therefore resulted in an 11-fold enrichment of viral genomic material, compared to the unenriched library, and resulted in a marked improvement in the depth and coverage of the FMDV genome sequence obtained.

3.2. NGS on clinical samples (oral, nasal and rectal swabs)

To measure the efficacy of the bait panel in clinical samples, the enrichment protocol on thirty oral and nasal swabs was tested (Supplementary Table 1). The FMDV genome composition to the host genome for different samples varied from 0.10% to 95% before enrichment. After enrichment, the FMDV component was between 94% and 99.6% for both serotypes tested, and the number of FMDV-specific reads increased almost 3000-fold to 95% (Supplementary Table 1). In the first round of sequencing, poor reads were obtained from four serotype O samples and eight serotype A samples. The C_T values of the FMDV genome in these samples were > 30 (30.9–45). The library preparation and sequencing protocols were repeated for these twelve samples and the complete genomic sequence was obtained from two serotype O and five serotype A samples.

As a result, full-length consensus genome sequences were obtained from 13 out of 15 oral/nasal swabs from the serotype O vaccine study samples (Fig. 2, A–D). Near full coverage (˜98% and ˜80%, respectively) was obtained for the two rectal swabs (samples 24 and 25, Supplementary Table 1; Fig. 2, D–E), which were also the samples that contained the least amount of viral RNA as determined by RT-qPCR (C_T values of 32.12 and > 40.0, respectively). From the serotype A vaccine study samples, full-length genome sequences were obtained from 12 out of 15 oral/nasal swabs (Fig. 3, A–C). Near full coverage was obtained for the remaining three nasal swabs (Samples 34, 39 and 40, Supplementary Table 1; Fig. 3, D–F), RNA from two of which (Samples 39 and 40) was not detectable when subjected to amplification by RT-qPCR. Complete FMDV genome sequences were obtained from all the oral and nasal swabs from the pig pathogenesis studies (Supplementary Table 1).

3.3. FMDV variant analysis

The minimum frequency of reads mismatched to the consensus at a given position was initially set to 0.5% (1 in 200 reads). Changing the threshold for SNP inclusion greatly influenced the number of variable positions (Table 2). By plotting SNPs relative to their coordinate location in the FMDV genome, the density of variable positions was examined (Fig. 4). Three positions (3833, 5261, 7599) stood out as being

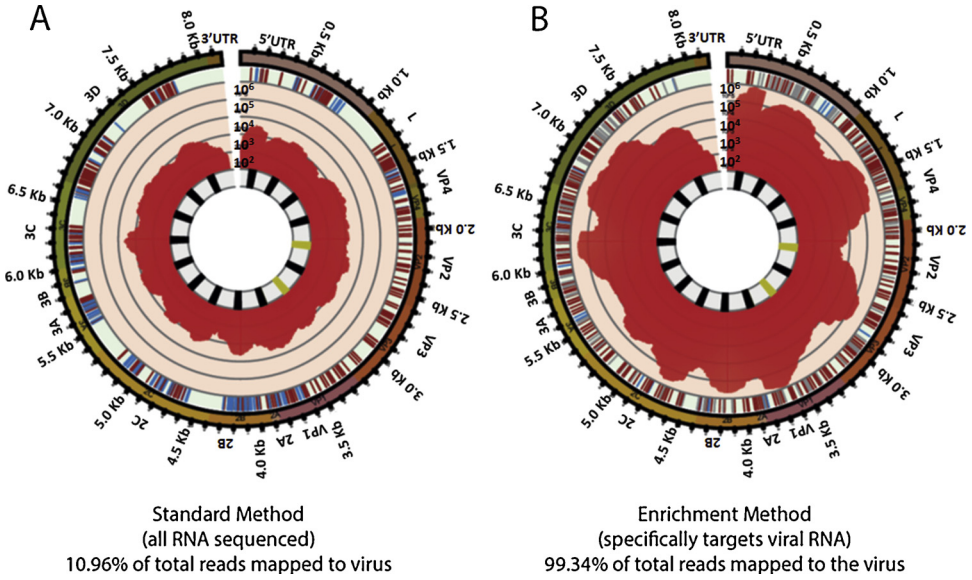

Fig. 1. Sequence coverage maps of unenriched (A) and enriched (B) library samples of pig adapted A/VIT/5/2005 inoculum, generated during optimisation of the enrichment protocol. Each map depicts the viral genome in 5′ to 3′ orientation starting at 12 o'clock and moving in a clockwise direction. The depth of coverage at each position in the genome is indicated by red shading and is shown on a $\log_{10}$ scale. **(For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)**

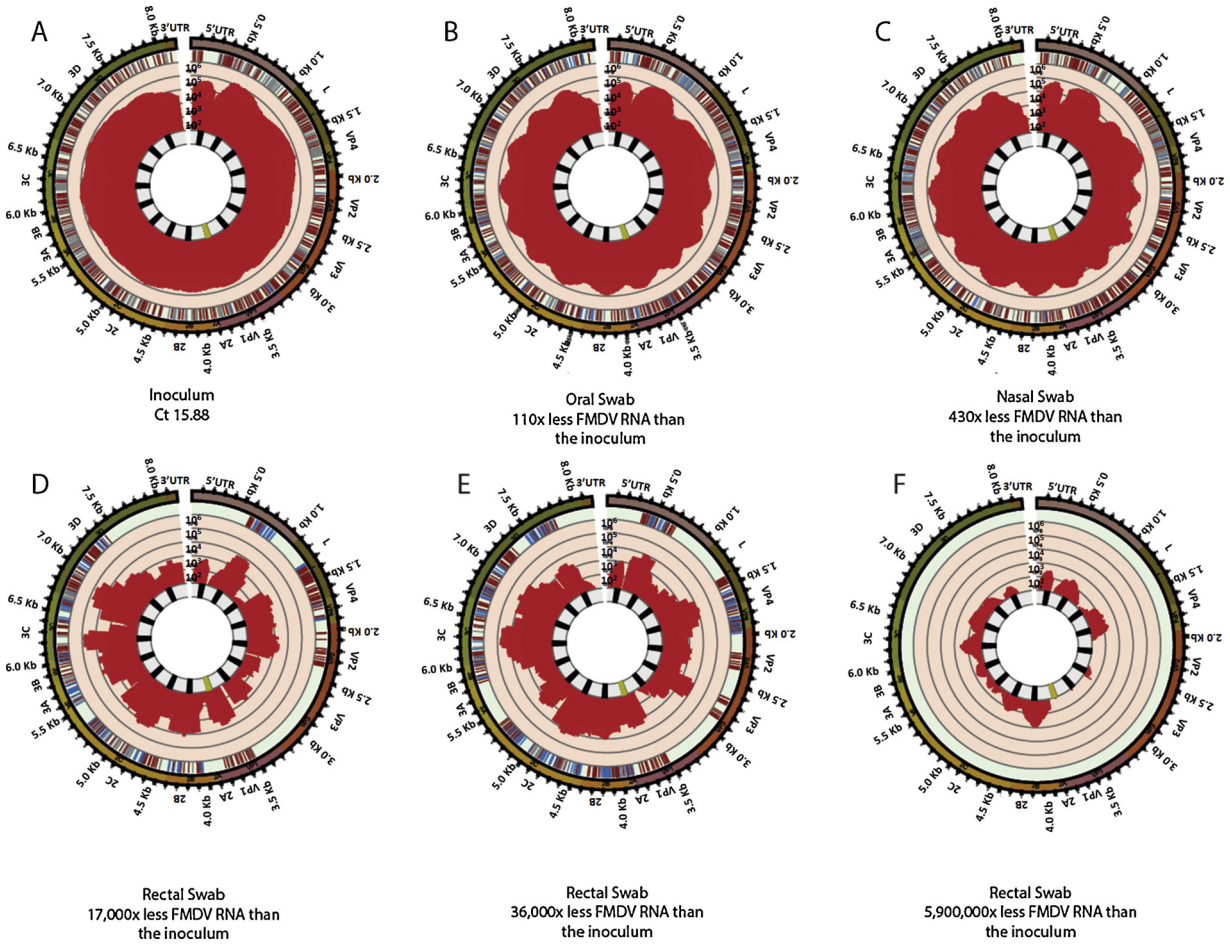

Fig. 2. Direct sequencing of clinical samples (serotype O). Schematic representations of the sequences obtained from FMDV-enriched NGS libraries derived from a pig-adapted O/VIT/2010 (Mya-98 lineage) inoculum (A), as well as from representative oral (B), nasal (C) and rectal (D–F) swabs that were collected from pigs that had been challenged 2–3 days previously. Each map depicts the viral genome in 5′ to 3′ orientation starting at 12 o'clock and moving in a clockwise direction. The depth of coverage at each position in the genome is indicated by red shading and is shown on a $\log_{10}$ scale. The fold enrichment compared to the sequences in the inoculum was calculated as the ratio of the percentage of FMDV sequences mapped in the enriched samples to the unenriched sample. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

more frequently variable in type A viruses (Fig. 4, B2), while clusters of SNPs were apparent for both type A and type O viruses. The majority of observed variants were unique to single samples. However, a small number were identified in multiple samples. Of these, a subset was shared between the different treatment groups (Fig. 5).

4. Discussion

Reconstruction of viral transmission pathways relies on phylogenetic analysis of viral sequences recovered from field samples. Molecular sequence data are an important component of FMD control strategies, allowing the generation of phylogenetic trees (Knowles and Samuel, 2003). Whole genome sequencing is a powerful tool for reconstructing fine-scale transmission pathways due to the increased resolution of genomic information (Cottam et al., 2008; Wright et al., 2013). Logan et al. (2014) described a methodology for generating whole genome sequences on the Illumina MiSeq. Using this method, 94% of an FMDV genome that had previously been identified as cell culture negative was recovered. King et al. (2016) reported a method of PCR amplification of a 7.6 kb fragment of the FMDV O_1 BFS 1860 genome (starting within the 5′ UTR and ending after the stop codon of the polyprotein encoding region) and subsequent deep sequencing to characterise the diversity of FMDV populations in six infected cattle present on a single farm during the series of outbreaks in the UK in 2007. The potential problem with this method is biased amplification of a few target RNA molecules in subsequent PCR cycles, thereby less common sequences will not be represented or detectable following amplification and therefore will be missed, even with NGS.

This paper demonstrated proof of concept for obtaining high-quality full-length FMDV genome sequence data directly from clinical samples, including those containing very low concentrations of FMDV RNA, without isolation and propagation of the virus in cell culture. All RNA samples that had C_T values ≤ 31.10 (approximately 10^3–10^4 copy numbers/ml) consistently yielded full-genome coverage. In addition, a number of samples (such as samples 36, 38 and 41, Supplementary Table 1) gave full-genome sequence even when the RNA concentration was at or below the limit of detection using RT-qPCR. In fact, genome coverage of > 80% was obtained from every sample tested, demonstrating the power of the enrichment technique for obtaining viral genome sequence directly from clinical samples. However, no correlation was found between the C_T values obtained in real-time RT-PCR and the results of the sequencing with enrichment. In addition, the impact of the integrity of genomic RNA, especially in degraded samples was not assessed. The baits used were designed to specifically target the FMDV-A and FMDV-O viruses used in this study. At 80–85% identity, baits begin to bind at lower efficiency (Kamaraj et al., 2019). Although these baits still contribute to the capture of the target genomes, baits with sequence identities falling below 80% are not expected to effectively pull down RNA. Therefore, it is recommended that if the target genome does not have ≥ 85% identity with the existing bait, a new bait

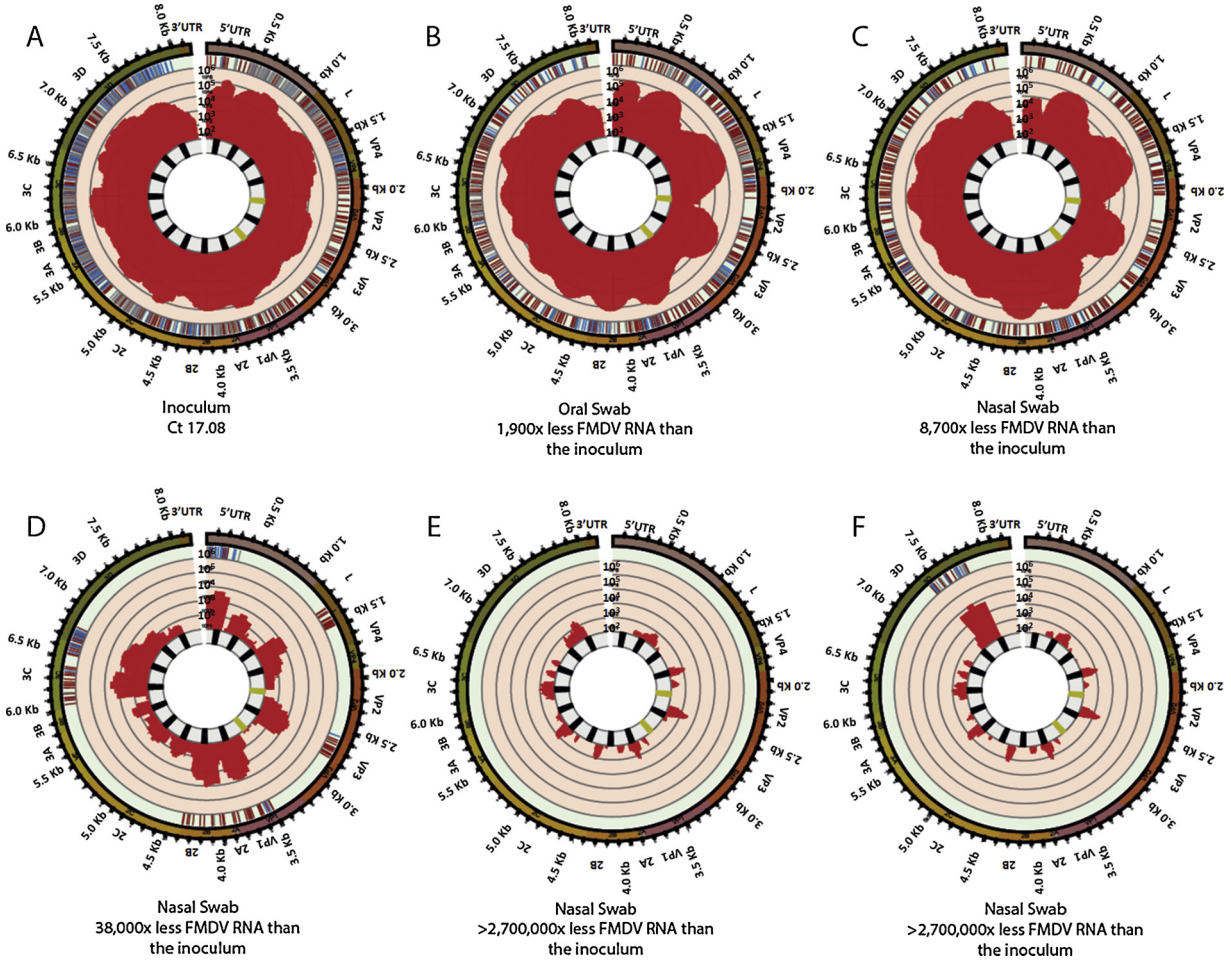

Fig. 3. Direct sequencing of clinical samples (serotype A). Schematic representations of the sequences obtained from FMDV-enriched NGS libraries derived from a pig-adapted A/VIT/2005 (SEA-97 lineage) inoculum (A), as well as from oral (B) and nasal (C–F) swabs that were collected from pigs that had been challenged 2–3 days previously. Each map depicts the viral genome in 5′ to 3′ orientation starting at 12 o'clock and moving in a clockwise direction. The depth of coverage at each position in the genome is indicated by red shading and is shown on a $\log_{10}$ scale. The fold enrichment compared to the sequences in the inoculum was calculated as the ratio of the percentage of FMDV sequences mapped in the enriched samples to the unenriched sample. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

Table 2
The number of variable positions based on the percentage threshold for SNP inclusion. With an increase in the percent threshold, the number of SNPs and the number of samples containing a SNP at a given position decrease.

Threshold (%)	Number of SNP positions		Number of clinical samples with overlapping SNP positions[a]	
	Serotype A	Serotype O	Serotype A	Serotype O
0.5	2200	1861	17	16
1	1762	1317	16	12
2	1256	975	15	12
3	998	834	14	11
4	845	743	13	10
5	725	671	13	8
10	330	475	13	7
20	104	339	10	6
30	49	233	4	5
40	21	118	2	3
50	2	17	1	1

[a] Samples were considered to have overlapping SNP positions if they shared one or more genomic locations containing a SNP, but does not imply that those SNPs are identical.

(s) be designed using the BaitMaker algorithm; available at GitHub (https://umasangumathi.github.io/BaitMaker/).

The genetic dynamics of FMDV during persistent infections of naturally infected Asian buffalo was investigated using NGS to obtain 21 near complete FMDV genome sequences from 12 sub-clinically infected buffalo over a period of one year (Ramirez-Carvajal et al., 2018). Seven persistently infected animals yielded more than one virus of the same serotype, showing a long-term intra-host viral genetic divergence at the consensus level of less than 2.5%. Quasispecies analysis showed few nucleotide variants and non-synonymous substitutions of progeny virus despite intra-host persistence of up to 152 days. The group employed a SISPA approach (Moser et al., 2016) to multiplex several samples in a single instrument run. The SISPA method produced low sequence coverage at the 3′ and 5′ ends of viral genomes (Djikeng et al., 2008). The study also revealed that the heterogeneous depth of coverage across the genome hindered the ability to detect SNPs or to include samples for pairwise analysis in areas with low coverage. It has been reported that the structural elements of FMDV constrain NGS (Logan et al., 2014) and viral-specific oligonucleotides based on conserved sequences have been suggested to improve sequencing (Djikeng et al., 2008). In this study, the latter method was used by developing biotinylated oligonucleotide baits for the capture of FMDV RNA and subsequent sequencing using the MiSeq platform.

Next-generation sequencing reveals the fine polymorphic

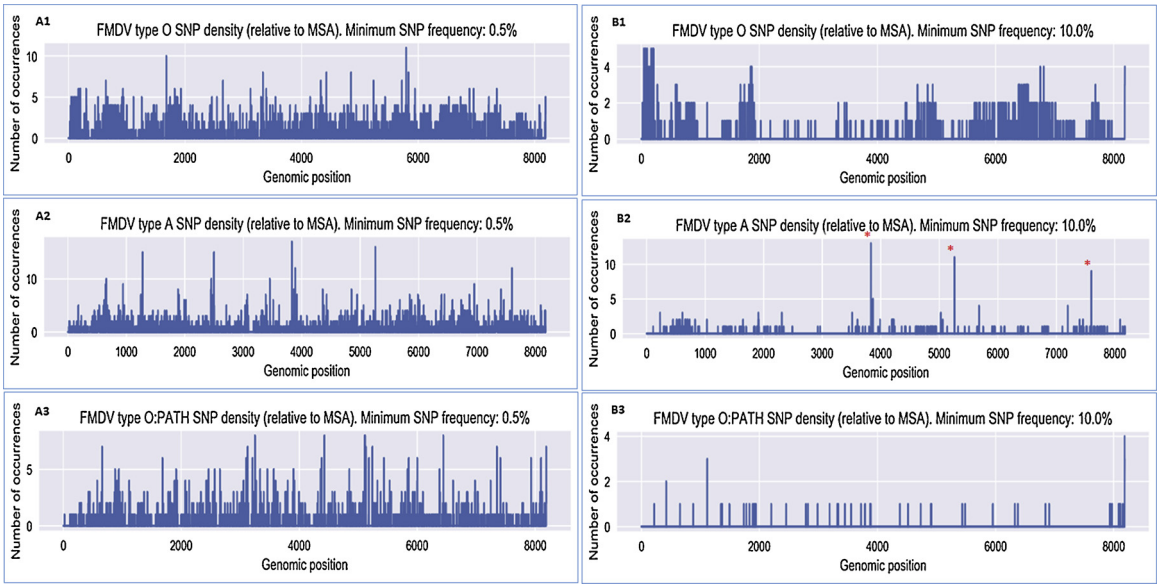

Fig. 4. Location and frequency of SNP positions along the FMDV genome at thresholds of 0.5% (A1-3) and 10% (B1-3), respectively. (A1, B1) Serotype O vaccine studies; (A2, B2) Serotype A vaccine studies; (A3, B3) Pig pathogenesis study. To ensure positions were correctly aligned, the coordinates are relative to a multiple sequence alignment (MSA) for that serotype. Height of the bars indicates the number of samples in which this position was observed to be variable. Red asterisks highlight positions found to be more frequently variable, as described in the text. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

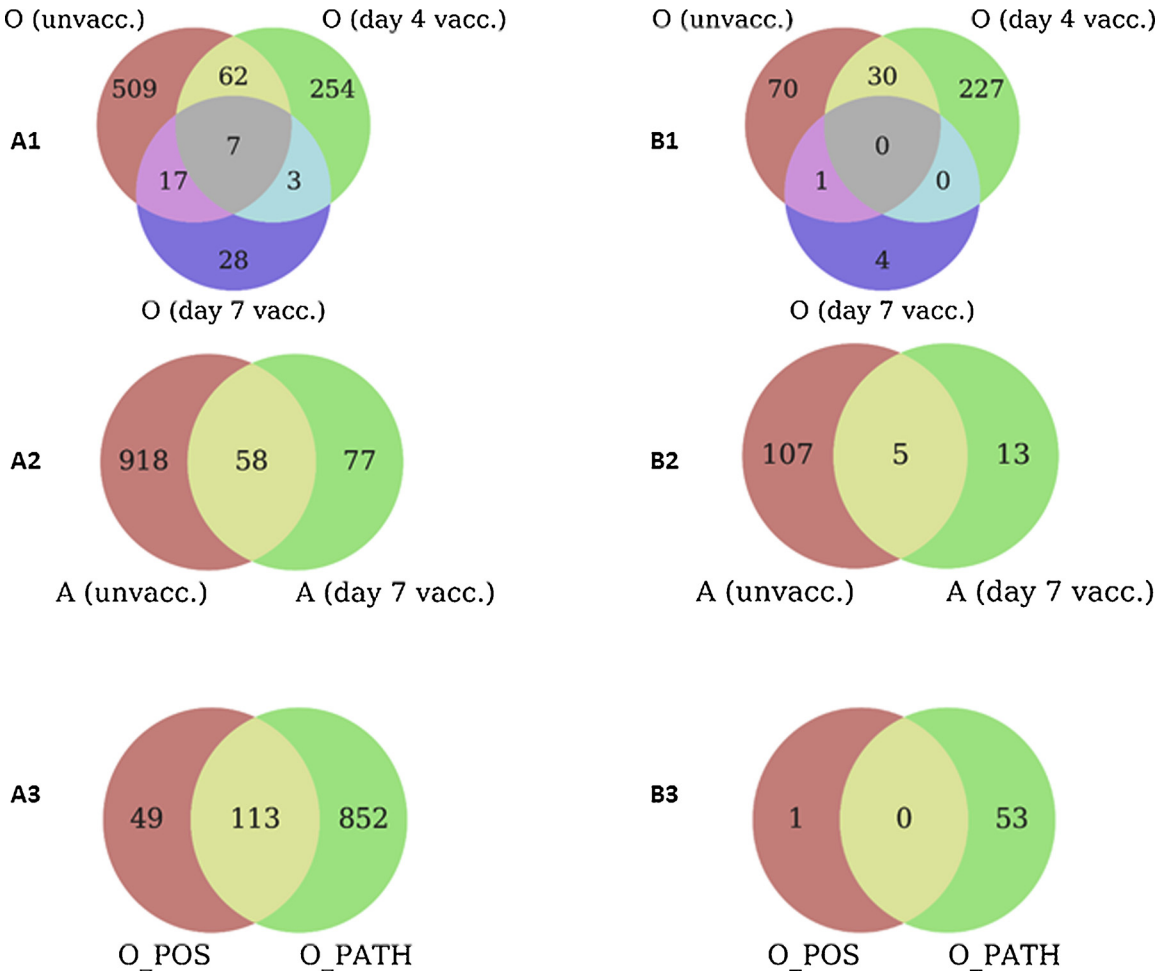

Fig. 5. Correspondence of SNP locations between different groups of samples at thresholds of 0.5% (A1-3) and 10% (B1-3), respectively. (A1, B1) Serotype O vaccine studies; (A2, B2) Serotype A vaccine studies; (A3, B3) Pig pathogenesis study. Overlapping regions correspond to SNP positions that were observed in at least one member of each group.

substructure of the viral population, from nucleotide variants present at just below 50% frequency to those present at fractions of 1%. A total of 2622, 1434, and 1703 polymorphisms were present in the inoculum and the two foot lesions, respectively. Using variant call analysis, 2200 and 1861 SNPs were identified for serotype O and A, respectively, at a set threshold of 0.5% (Table 2). Changing the threshold for SNP inclusion greatly influenced the number of variable positions. As the threshold increased the number of samples that were variant at a given position also changed (Table 2). With an increase in the per cent threshold, the number of SNPs and the number of samples containing a SNP at a given position decreased. Samples were considered to have overlapping SNP positions if they shared one or more genomic locations containing a SNP, but this does not imply that those SNPs are identical. Therefore, the threshold percentage setting is very important in calling SNPs and variants. This analysis, while basic, shows that the enrichment method has great potential for facilitating advanced SNP analysis. While not explored in great depth here, it clearly illustrates the high-resolution SNP data that can be expected due to increasing the depth of coverage via enrichment.

This technology has the potential to facilitate an in-depth study of the intra-host genetic diversity during FMDV infection, with or without vaccination, thereby revealing the location and frequency of SNPs within specific viral populations under different selection criteria. Such data, which are currently being evaluated for the genome sequences reported herein, will provide further insights into, and enhance our understanding of, the infection dynamics and evolutionary processes of this highly varied and complex viral pathogen.

Ethical approval

The pig studies were approved by the institutional animal ethics committee of the Australian Animal Health Laboratory, Geelong (AEC1497, AEC 1571).

Declaration of Competing Interest

None to declare.

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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.2019.113703.

References

Abdul-Hamid, N.F., Firat-Sarac, M., Radford, A.D., Knowles, N.J., King, D.P., 2011. Comparative sequence analysis of representative foot-and-mouth disease virus genomes from Southeast Asia. Virus Genes 43, 41–45.

Airaksinen, A., Pariente, N., Menendez-Arias, L., Domingo, E., 2003. Curing of foot-and-mouth disease virus from persistently infected cells by ribavirin involves enhanced mutagenesis. Virology 311, 339–349.

Bas, A., Forsberg, G., Hammarstrom, S., Hammarstrom, M.L., 2004. Utility of the housekeeping genes 18S rRNA, beta-actin and glyceraldehyde-3-phosphate-dehydrogenase for normalization in real-time quantitative reverse transcriptase-polymerase chain reaction analysis of gene expression in human T lymphocytes. Scand. J. Immunol. 59, 566–573.

Bonsall, D., Ansari, M.A., Ip, C., Trebes, A., Brown, A., Klenerman, P., Buck, D., Consortium, S.-H., Piazza, P., Barnes, E., Bowden, R., 2005. ve-SEQ: robust, unbiased enrichment for streamlined detection and whole-genome sequencing of HCV and other highly diverse pathogens. F1000Research 4, 1062.

Boyle, D.B., Taylor, T., Cardoso, M., 2004. Implementation in Australia of molecular diagnostic techniques for the rapid detection of foot and mouth disease virus. Aust. Vet. J. 82, 421–425.

Cottam, E.M., Wadsworth, J., Knowles, N.J., King, D.P., 2009. Full sequencing of viral genomes: practical strategies used for the amplification and characterization of foot-and-mouth disease virus. Methods Mol. Biol. 551, 217–230.

Cottam, E.M., Wadsworth, J., Shaw, A.E., Rowlands, R.J., Goatley, L., Maan, S., Maan, N.S., Mertens, P.P., Ebert, K., Li, Y., Ryan, E.D., Juleff, N., Ferris, N.P., Wilesmith, J.W., Haydon, D.T., King, D.P., Paton, D.J., Knowles, N.J., 2008. Transmission pathways of foot-and-mouth disease virus in the United Kingdom in 2007. PLoS Pathog. 4, e1000050.

Depledge, D.P., Palser, A.L., Watson, S.J., Lai, I.Y., Gray, E.R., Grant, P., Kanda, R.K., Leproust, E., Kellam, P., Breuer, J., 2011. Specific capture and whole-genome sequencing of viruses from clinical samples. PLoS One 6, e27805.

Djikeng, A., Halpin, R., Kuzmickas, R., Depasse, J., Feldblyum, J., Sengamalay, N., Afonso, C., Zhang, X., Anderson, N.G., Ghedin, E., Spiro, D.J., 2008. Viral genome sequencing by random priming methods. BMC Genom. 9, 5.

Domingo, E., Baranowski, E., Escarmis, C., Sobrino, F., 2002. Foot-and-mouth disease virus. Comp. Immunol. Microbiol. Infect. Dis. 25, 297–308.

Domingo, E., Holland, J.J., 1997. RNA virus mutations and fitness for survival. Annu. Rev. Microbiol. 51, 151–178.

Eckerle, L.D., Becker, M.M., Halpin, R.A., Li, K., Venter, E., Lu, X.T., Scherbakova, S., Graham, R.L., Baric, R.S., Stockwell, T.B., Spiro, D.J., Denison, M.R., 2010. Infidelity of SARS-CoV Nsp14-exonuclease mutant virus replication is revealed by complete genome sequencing. PLoS Pathog. 6, e1000896.

Gleeson, L.J., 2002. A review of the status of foot and mouth disease in South-East Asia and approaches to control and eradication. Rev. Sci. Tech. 21, 465–475.

Hauck, N.C., Kirpach, J., Kiefer, C., Farinelle, S., Maucourant, S., Morris, S.A., Rosenberg, W., He, F.Q., Muller, C.P., Lu, I.N., 2018. Applying unique molecular identifiers in next generation sequencing reveals a constrained viral quasispecies evolution under cross-reactive antibody pressure targeting long alpha helix of hemagglutinin. Viruses 10. https://doi.org/10.3390/v10040148.

Holmes, E.C., Moya, A., 2002. Is the quasispecies concept relevant to RNA viruses? J. Virol. 76, 460–465.

Houldcroft, C.J., Bryant, J.M., Depledge, D.P., Margetts, B.K., Simmonds, J., Nicolaou, S., Tutill, H.J., Williams, R., Worth, A.J., Marks, S.D., Veys, P., Whittaker, E., Breuer, J., 2016. Detection of low frequency multi-drug resistance and novel putative maribavir resistance in immunocompromised pediatric patients with cytomegalovirus. Front. Microbiol. 7, 1317.

Jiang, H., Lei, R., Ding, S.W., Zhu, S., 2014. Skewer: a fast and accurate adapter trimmer for next-generation sequencing paired-end reads. BMC Bioinform. 15, 182.

Kamaraj, U.S., Tan, J.H., Mei, O.X., Pan, L., Chawla, T., Uehara, A., Wang, L.-F., Ooi, E.E., Gubler, D.J., Tissera, H., Ng, L.C., Wilder-Smith, A., de Sessions, P.F., Barkham, T., Anderson, D.E., Sessions, O.M., 2019. Application of a targeted-enrichment methodology for full-genome sequencing of Dengue 1-4, Chikungunya and Zika viruses directly from patient samples. PLoS Negl. Trop. Dis. 13, e0007184.

Kasambula, L., Belsham, G.J., Siegismund, H.R., Muwanika, V.B., Ademun-Okurut, A.R., Masembe, C., 2012. Serotype identification and VP1 coding sequence analysis of foot-and-mouth disease viruses from outbreaks in eastern and northern Uganda in 2008/9. Transbound. Emerg. Dis. 59, 323–330.

King, D.J., Freimanis, G.L., Orton, R.J., Waters, R.A., Haydon, D.T., King, D.P., 2016. Investigating intra-host and intra-herd sequence diversity of foot-and-mouth disease virus. Infect. Genet. Evol. 44, 286–292.

Knowles, N.J., He, J., Shang, Y., Wadsworth, J., Valdazo-Gonzalez, B., Onosato, H., Fukai, K., Morioka, K., Yoshida, K., Cho, I.S., Kim, S.M., Park, J.H., Lee, K.N., Luk, G., Borisov, V., Scherbakov, A., Timina, A., Bold, D., Nguyen, T., Paton, D.J., Hammond, J.M., Liu, X., King, D.P., 2012. Southeast Asian foot-and-mouth disease viruses in Eastern Asia. Emerg. Infect. Dis. 18, 499–501.

Knowles, N.J., Samuel, A.R., 2003. Molecular epidemiology of foot-and-mouth disease virus. Virus Res. 91, 65–80.

Le, V.P., Nguyen, T., Lee, K.N., Ko, Y.J., Lee, H.S., Nguyen, V.C., Mai, T.D., Do, T.H., Kim, S.M., Cho, I.S., Park, J.H., 2010a. Molecular characterization of serotype A foot-and-mouth disease viruses circulating in Vietnam in 2009. Vet. Microbiol. 144, 58–66.

Le, V.P., Nguyen, T., Park, J.H., Kim, S.M., Ko, Y.J., Lee, H.S., Nguyen, V.C., Mai, T.D., Do, T.H., Cho, I.S., Lee, K.N., 2010b. Heterogeneity and genetic variations of serotypes O and Asia 1 foot-and-mouth disease viruses isolated in Vietnam. Vet. Microbiol. 145, 220–229.

Li, H., Durbin, R., 2009. Fast and accurate short read alignment with Burrows-Wheeler transform. Bioinformatics 25, 1754–1760.

Logan, G., Freimanis, G.L., King, D.J., Valdazo-Gonzalez, B., Bachanek-Bankowska, K., Sanderson, N.D., Knowles, N.J., King, D.P., Cottam, E.M., 2014. A universal protocol to generate consensus level genome sequences for foot-and-mouth disease virus and other positive-sense polyadenylated RNA viruses using the Illumina MiSeq. BMC Genom. 15, 828.

Miyazato, P., Katsuya, H., Fukuda, A., Uchiyama, Y., Matsuo, M., Tokunaga, M., Hino, S., Nakao, M., Satou, Y., 2016. Application of targeted enrichment to next-generation sequencing of retroviruses integrated into the host human genome. Sci. Rep. 6, 2.

Moser, L.A., Ramirez-Carvajal, L., Puri, V., Pauszek, S.J., Matthews, K., Dilley, K.A., Mullan, C., McGraw, J., Khayat, M., Beeri, K., Yee, A., Dugan, V., Heise, M.T., Frieman, M.B., Rodriguez, L.L., Bernard, K.A., Wentworth, D.E., Stockwell, T.B., Shabman, R.S., 2016. A universal next-generation sequencing protocol to generate noninfectious barcoded cDNA libraries from high-containment RNA viruses. mSystems 1, e00039–15.

Nagendrakumar, S.B., Hong, N.T., Geoffrey, F.T., Jacqueline, M.M., Andrew, D., Michelle, G., Van Phuc, K., Ngon, Q.V., Phuong le, T.T., Phuc, N.N., Hanh, T.X., Van Hung, V., Quynhanh le, T., Tan, T.M., Long, N.T., Wilna, V., 2015. A Malaysia 97 monovalent foot-and-mouth disease vaccine (&6PD50/dose) protects pigs against challenge with a variant FMDV A SEA-97 lineage virus, 4 and 7 days post vaccination. Vaccine 33, 4513–4519.

Paton, D.J., Gubbins, S., King, D.P., 2018. Understanding the transmission of foot-and-mouth disease virus at different scales. Curr. Opin. Virol. 28, 85–91.

Qiu, Y., Abila, R., Rodtian, P., King, D.P., Knowles, N.J., Ngo, L.T., Le, V.T., Khounsy, S., Bounma, P., Lwin, S., Verin, B.C., Widders, P., 2018. Emergence of an exotic strain of serotype O foot-and-mouth disease virus O/ME-SA/Ind-2001d in South-East Asia in 2015. Transbound. Emerg. Dis. 65, e104–e112.

Radford, A.D., Chapman, D., Dixon, L., Chantrey, J., Darby, A.C., Hall, N., 2012. Application of next-generation sequencing technologies in virology. J. Gen. Virol. 93, 1853–1868.

Ramirez-Carvajal, L., Pauszek, S.J., Ahmed, Z., Farooq, U., Naeem, K., Shabman, R.S., Stockwell, T.B., Rodriguez, L.L., 2018. Genetic stability of foot-and-mouth disease virus during long-term infections in natural hosts. PLoS One 13, e0190977.

Rweyemamu, M., Roeder, P., Mackay, D., Sumption, K., Brownlie, J., Leforban, Y., Valarcher, J.F., Knowles, N.J., Saraiva, V., 2008. Epidemiological patterns of foot-and-mouth disease worldwide. Transbound. Emerg. Dis. 55, 57–72.

Samuel, A.R., Knowles, N.J., 2001. Foot-and-mouth disease type O viruses exhibit genetically and geographically distinct evolutionary lineages (topotypes). J. Gen. Virol. 82, 609–621.

Shaw, A.E., Reid, S.M., Ebert, K., Hutchings, G.H., Ferris, N.P., King, D.P., 2007. Implementation of a one-step real-time RT-PCR protocol for diagnosis of foot-and-mouth disease. J. Virol. Methods 143, 81–85.

Sobrino, F., Saiz, M., Jimenez-Clavero, M.A., Nunez, J.I., Rosas, M.F., Baranowski, E., Ley, V., 2001. Foot-and-mouth disease virus: a long known virus, but a current threat. Vet. Res. 32, 1–30.

Valdazo-Gonzalez, B., Polihronova, L., Alexandrov, T., Normann, P., Knowles, N.J., Hammond, J.M., Georgiev, G.K., Ozyoruk, F., Sumption, K.J., Belsham, G.J., King, D.P., 2012. Reconstruction of the transmission history of RNA virus outbreaks using full genome sequences: foot-and-mouth disease virus in Bulgaria in 2011. PLoS One 7, e49650.

Vinner, L., Mourier, T., Friis-Nielsen, J., Gniadecki, R., Dybkaer, K., Rosenberg, J., Langhoff, J.L., Cruz, D.F., Fonager, J., Izarzugaza, J.M., Gupta, R., Sicheritz-Ponten, T., Brunak, S., Willerslev, E., Nielsen, L.P., Hansen, A.J., 2015. Investigation of human cancers for retrovirus by low-stringency target enrichment and high-throughput sequencing. Sci. Rep. 5, 13201.

Vosloo, W., Morris, J., Davis, A., Giles, M., Wang, J., Nguyen, H.T., Kim, P.V., Quach, N.V., Le, P.T., Nguyen, P.H., Dang, H., Tran, H.X., Vu, P.P., Hung, V.V., Le, Q.T., Tran, T.M., Mai, T.M., Le, Q.T., Singanallur, N.B., 2015. Collection of oral fluids using cotton ropes as a sampling method to detect foot-and-mouth disease virus infection in pigs. Transbound. Emerg. Dis. 62, e71–5.

Wilm, A., Aw, P.P., Bertrand, D., Yeo, G.H., Ong, S.H., Wong, C.H., Khor, C.C., Petric, R., Hibberd, M.L., Nagarajan, N., 2012. LoFreq: a sequence-quality aware, ultra-sensitive variant caller for uncovering cell-population heterogeneity from high-throughput sequencing datasets. Nucleic Acids Res. 40, 11189–11201.

Wilna, V., Hong, N.T., Geoffrey, F.T., Jacqueline, M.M., Jianning, W., Van Phuc, K., Ngon, Q.V., Phuong le, T.T., Hung, D., Hanh, T.X., Van Hung, V., Anh le, T.Q., Tien, M.T., Quang le, T.V., Long, N.T., Nagendrakumar, S.B., 2015. Efficacy of a high potency O1 Manisa monovalent vaccine against heterologous challenge with a FMDV O Mya98 lineage virus in pigs 4 and 7 days post vaccination. Vaccine 33, 2778–2785.

Wright, C.F., Knowles, N.J., Di Nardo, A., Paton, D.J., Haydon, D.T., King, D.P., 2013. Reconstructing the origin and transmission dynamics of the 1967-68 foot-and-mouth disease epidemic in the United Kingdom. Infect. Genet. Evol. 20, 230–238.

Wright, C.F., Morelli, M.J., Thebaud, G., Knowles, N.J., Herzyk, P., Paton, D.J., Haydon, D.T., King, D.P., 2011. Beyond the consensus: dissecting within-host viral population diversity of foot-and-mouth disease virus by using next-generation genome sequencing. J. Virol. 85, 2266–2275.