Optimisation of rapid untargeted nanopore DNA virus metagenomics using cell cultures and calves experimentally infected with bovine herpes virus-1

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Research Article

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Abstract

Bovine respiratory disease (BRD) is the leading cause of morbidity and mortality in cattle in Ireland, and internationally. This disease is caused by many well-known, and an ever-increasing number of newly associated viruses and bacteria. Consequently, diagnosis of BRD pathogens by targeted real-time polymerase chain reaction (qPCR) diagnostics is too expensive and slow to enable a same-day response that is targeted at the causative pathogen(s). To address this, we developed a same-day, sample to result, untargeted metagenomic MinION sequencing protocol for the identification of DNA viruses associated with BRD from nasal swabs. The procedure comprises non-viral nucleic acid depletion, nucleic acid extraction, rapid transposase-based tagmentation with barcoded adapters, non-biased PCR amplification of tagmented nucleic acid, sequencing on a MinION device, then rapid analysis of resulting sequences on cloud-based software EPI2ME WIMP. The protocol was developed using BoHV1-infected foetal lung cell cultures where we achieved 96% enrichment of the BoHV-1 sequence. Subsequently, the protocol was successfully applied to untargeted detection of BoHV-1 in nasal swabs from calves experimentally challenged with BoHV-1.
Introduction

Bovine respiratory disease (BRD) is the leading cause of morbidity, mortality and economic loss in cattle of all ages both in Ireland, and internationally. The extensive use of vaccines against BRD-associated viral and bacterial pathogens has not reduced the incidence or severity of BRD in cattle. Consequently, large quantities of antimicrobials are still used for therapeutic treatment of BRD in Europe and the US. Easy and economical methodologies that enable the rapid and reliable on-farm detection of viral and bacterial pathogens has the potential to increase effective use of vaccines by informing production and appropriate use of relevant up to date vaccines.

BRD is a multifactorial disease that is associated with an ever-increasing number of species and strains of virus and bacteria. Viruses that are commonly associated with BRD cases include bovine herpesvirus type 1 (BoHV-1), bovine parainfluenza 3 virus (BPI-3), bovine respiratory syncytial virus (BRSV), bovine viral diarrhoea virus (BVDV), bovine coronavirus (BCoV) and bovine adenovirus (BAdV). BRD-associated bacteria commonly include Pasteurella multocida, Mannheimia haemolytica, Histophilus somni, and Mycoplasma spp.

New pathogens e.g. influenza D virus (IDV) and Sneathia amnii are continually added to the list of BRD aetiology. Viruses are known to initiate the disease by weakening the animal’s defences which commonly leads to secondary bacterial infection.

A diagnosis of BRD is generally based on clinical signs which are assessed by visual inspection of the animal, measurement of the rectal temperature and pulmonary auscultation. If identification of the causative pathogen/s is attempted (which is often not the case), a nasal swab from the affected animal is sent to a centralised laboratory where targeted qPCR diagnostics of only four or five of the most likely previously mentioned bacterial or viral pathogens is conducted. As there are at least 40 possible bacterial and
viral pathogens associated with BRD, it is too expensive and time consuming to test for all of these by targeted real-time PCR (qPCR) diagnostics. Consequently, there are considerable delays in receiving the results of aetiological diagnosis of BRD cases which are often inconclusive\textsuperscript{18}. For pathogen identification to be of direct practical use in preventing a BRD outbreak, results would need to be available to a vet within 24 h. Therein, current methods used for BRD-associated pathogen identification fall far short of what is required.

The Oxford Nanopore Technologies (ONT) MinION DNA/RNA sequencer is a portable sequencing device that allows real-time data analyses\textsuperscript{19} and has the potential to enable untargeted same-day (sample to result) viral diagnostics. Sequencing all of the nucleic acid in a sample potentially allows detection of all organisms, including pathogens that are present in a single assay. The MinION sequencer uses a flowcell comprising a membrane containing approximately 1200-1600 active biological nanopores. Less expensive flowcells are also available that contain approximately 80-160 active nanopores. The membrane is flanked on either side by opposing electrical charges which drive negatively charged individual single chain strands of DNA or RNA through the nanopores towards the positive charge. A sensor registers the unique change of current produced by each individual base as it passes through the nanopore. These changes in current are translated into nucleotide sequence information by neural network basecallers\textsuperscript{20} and approximately 4000 FASTQ files are generated every minute as the sequence run progresses\textsuperscript{21}. As soon as these FASTQ files are generated they can be uploaded to a cloud-based software platform called Epi2ME which contains a number of intuitive point and click sequence analysis applications called ‘work flows’. In the present work, the WIMP workflow was used as it identifies viral, bacterial, fungal and yeast sequence using ‘Centrifuge’ software. This means that a pathogen can, depending on its abundance in the samples, be identified within approximately 15 to 30 min of loading a sequencing library on a flowcell.
The size of most viral genomes is several orders of magnitude lower than those of bacteria and eukaryotes. In nasal swabs taken from cattle infected with a BRD-associated virus the vast majority of nucleic acid will therefore be prokaryotic or eukaryotic and just a fraction will be viral. As such, the current depth of nucleotide sequence achievable using next generation sequencing approaches is still insufficient to economically run multiple samples at the same time and reliably detect virus without prior enrichment of viral genomes.

Non-viral nucleic acid removal can be achieved by separation of eukaryote and prokaryote cells from the much smaller viral capsids by ultracentrifugation\textsuperscript{22}. More recently it has been found that some giant viruses such as mimiviruses, which are associated with pneumonia in humans, are larger in size than some bacteria and pellet at low centrifugation speeds\textsuperscript{23}. As intact viral capsids are nuclease resistant, RNaseA and DNaseI can be used to selectively digest non-viral nucleic acids. DNaseI and RNaseA are applied following cell disruption so that the eukaryotic and prokaryotic nucleic acids are exposed to the nuclease\textsuperscript{24}. However, in a cell infected with virus (i.e. virocell), much of the virus nucleic acid is not protected by a capsid and this unprotected viral sequence can also be lost if cell disruption and nuclease treatment are applied.

Following depletion of non-viral nucleic acid there is often insufficient total nucleic acid to generate enough sequencing library for NGS and TGS platforms, so following double-stranded cDNA synthesis, whole genome amplification (WGA) approaches are usually applied to amplify all of the remaining total nucleic acid in a depleted nucleic acid preparation. These approaches include Sequence-Independent, Single-Primer Amplification (SISPA) and Linker Amplified Shotgun Library (LASL)\textsuperscript{25,26}, which both employ PCR, and isothermal multiple displacement amplification (MDA) using podovirus φ29 polymerase\textsuperscript{26}. Not surprisingly, each WGA method has been shown to preferentially amplify different families of viruses and MDA is prone to generation of chimeric sequence\textsuperscript{26}. 

The protocol we developed employs the LASL WGA approach. Compared to MDA and SISPA, LASL sequencing requires fewer reagents, thus lower cost, and fewer steps, so less time from sample to loading the flowcell. LASL simply comprises tagmentation of nucleic acid with a sequencing adapter, followed by a 70 minute, 30 cycle PCR amplification with barcoded primers.

BoHV-1 is an enveloped DNA virus and was selected for this study as it is of economic importance both to Ireland and internationally. In addition, this virus has a well characterised and predictable pattern of infection. Firstly, non-viral nucleic acid depletion and subsequent non-biased LASL WGA of viral nucleic acids were optimised using bovine foetal lung cells (bFLC) infected with BoHV-1. These optimised procedures were then applied to nasal swabs collected from Holstein-Friesian calves that were experimentally challenged with BoHV-1.

The objective of the present study was to develop and validate, using Holstein-Friesian calves experimentally infected with a known virus, BoHV-1, a protocol for same-day, sample to result, untargeted identification of DNA viruses in nasal swabs from cattle using the portable MinION sequencer and Epi2ME cloud based software. Nasal swabs are the most common sample collected for the identification of pathogens associated with BRD outbreaks.
Results and Discussion

Optimisation of untargeted MinION sequencing of DNA viruses using \textit{in-vitro} bovine foetal lung cells infected with BoHV-1

Non-viral nucleic acid depletion

Initially, optimisation of the non-viral nucleic acid depletion step was conducted using aliquots of the same batch of BoHV-1-infected bFLC culture. These aliquots were subjected to five different combinations of bead beating and nuclease treatment (A-E) (Fig. 1). Nucleic acids were then extracted and qPCR of the $bACTB$ gene and $UL27$ gene was used to calculate the copy number of both the bovine and the BoHV-1 genomes respectively (Fig. 1). The most effective method identified for the depletion of non-viral nucleic acid comprised of an initial bead beating treatment, followed by a single treatment with RNaseA, then two 30 min incubation with Turbo DNase.
Figure 1. Effect of different non-viral nucleic acid depletion treatments on recovery of BoHV-1 and bovine genomic DNA from bovine lung cell cultures. Mean genome copy numbers are shown which were estimated by quantification by qPCR analysis of the BoHV-1 UL27 gene and the bovine bACTB gene. All treatments were performed in triplicate. Error bars represent the SD for each treatment. (A) No depletion treatment. (B) Bead beating only. (C) 1× RNase, 1× DNase. (D) 1× RNase, 2× DNase. (E) Bead beating, 1× RNase, 2× DNase. However, whilst treatment E (bead-beating combined with single RNaseA and double DNase treatment) depletion resulted in the greatest reduction of bovine DNA, it also resulted in the greatest loss of viral nucleic acid. This could be due the exposure of non-capsid viral nucleic acid from infected bovine cells (i.e. virocells) to nucleases following disruption of the cells by bead beating. The number of BoHV-1 genome copies detected were also reduced approximately 20 fold for treatment B (bead beating, no nuclease) compared to treatment A (no bead beating, no nuclease) (Figure 1). This suggests that bead beating alone leads to a considerable reduction in the amount of BoHV-1 DNA that can be subsequently be detected by qPCR. Bead beating may fragment some of the exposed viral DNA to sizes too small to be recovered by the QIAamp UltraSens Virus Kit or amplified by the UL27 primers. In order to
achieve high sensitivity of BoHV-1 detection on the MinION it was necessary to increase the ratio of viral to non-viral nucleic acid even if that meant losing BoHV1 DNA.

**Effect of non-viral nucleic acid depletion on nanopore sequencing**

The effect of non-viral nucleic acid depletion treatment E (Fig. 1) (bead beating and nuclease treatment) on nucleotide sequencing was then assessed. For this, non-barcoded PCR-free libraries were generated using the field sequencing library preparation kit (LRK001) from nucleic acid extracted from non-depleted samples (no bead beating or nuclease treatment) and depleted samples (bead beating and nuclease treatment) prior to extraction. The samples were either BoHV-1-infected bFLC *in vitro* cultures or a nasal swab from a BoHV-1-infected animal. These libraries were sequenced on a MinION (one library per R9 flowcell) and generated nucleotide sequences were analysed using the Epi2ME WIMP workflow. As expected, the percentage of reads that were assigned to viruses was significantly increased in the depleted cell culture libraries (mean = 96.94%, SD ±0.12, n = 3) compared to non-depleted cell culture libraries (mean = 45.61%, SD ±0.82, n = 3) (Table 1). The percentage of viral reads was also dramatically increased in the depleted nasal swab library (12.91%) compared to the non-depleted (0.42%) nasal swab library (Table 1). However, the non-depleted cell culture libraries had higher viral read counts (mean = 22,626, SD ±8,888, n = 3) than the depleted cell culture libraries (mean = 4,164, SD ±1108, n = 3). Whereas the non-depleted library prepared from the swab had lower viral read counts (657) than the depleted library made from the same swab (1,903).
| Taxonomic assignment | Sequence Read Counts | Non-depleted | Depleted |
|----------------------|----------------------|--------------|----------|
|                      | bFLC 1 | bFLC 2 | bFLC 3 | Swab | bFLC 1 | bFLC 2 | bFLC 3 | Swab |
| Eukaryota            | 31,850 | 14,427 | 33,459 | 154,002 | 75 | 54 | 51 | 12,541 |
| Viruses              | 26,163 | 12,514 | 29,202 | 657 | 5,433 | 3,677 | 3,384 | 1,903 |
| Bacteria             | 528 | 252 | 563 | 2,057 | 90 | 62 | 60 | 279 |
| Archaea              | 31 | 11 | 31 | 126 | 0 | 0 | 0 | 13 |
| Eukaryota %          | 54.38 | 53.03 | 52.90 | 98.19 | 1.34 | 1.42 | 1.46 | 85.10 |
| Viruses %            | 44.67 | 46.00 | 46.17 | 0.42 | 97.05 | 96.94 | 96.82 | 12.91 |
| Bacteria %           | 0.90 | 0.93 | 0.89 | 1.31 | 1.61 | 1.63 | 1.72 | 1.89 |
| Archaea %            | 0.05 | 0.04 | 0.05 | 0.08 | 0.00 | 0.00 | 0.00 | 0.09 |

Table 1. Total and % sequence read counts for the four phylogenetic superkingdoms in non-depleted and depleted (bead beating and nuclease treatment) libraries prior to nucleic acid extraction from three aliquots from a bFLC in vitro culture and a nasal swab from a calf infected with BoHV1. PCR-free tagmented libraries were generated with the ONT Field Sequencing Kit and sequenced on a MinION using rapid base calling. FASTQ files were subjected to EPI2ME WIMP analysis.

Optimisation of bias-free PCR amplification of tagmented libraries

Due to the high cost of R9 flowcells at the time of writing, we aimed to develop a protocol whereby an optimum number of samples could be processed on a single R9 flowcell. In order to achieve this, the rapid PCR barcoding kit provided the simplest, most rapid option for library preparation of the depleted libraries. This kit involves random insertion of partial adapters into ds DNA by tagmentation with a Tn5 transposase complex, followed by PCR amplification with barcoded primers that target the inserted adaptors. During pilot experiments we observed that tagmented libraries generated with the Rapid PCR Barcoding Kit from nucleic acid extraction from high titre BoHV1-infected lung cell cultures showed unexpectedly low read counts for BoHV1 after 30 cycles of PCR using the Log Amp Taq that was recommended by ONT. These samples showed high read counts for BoHV-1 in PCR-
free libraries prepared with the PCR-free Field Sequencing Kit so we suspected that low BoHV1 read counts with the Rapid PCR Barcoding Kit was due to PCR bias as the BoHV1 genome has a very high GC content (72%). This prompted us to compare Long Amp Taq with Ultra II Q5 to assess their PCR bias during library preparation and consequent differences in BoHV-1 read counts. Long Amp Taq is recommended by ONT for use with their Rapid PCR Barcoding Library Preparation Kit as it can amplify long templates and generate long reads. However, it is not optimised for amplifying templates with extreme GC content such as the BoHV-1 genome. Ultra II Q5 polymerase is optimised for amplification of templates with a wide range of GC content but not for long templates. Many viral genomes have unusually high or low GC content, so GC bias of polymerases could lead to certain viruses being underrepresented or missed altogether following bias metagenomic library amplification\textsuperscript{30}. Libraries were generated with either Long Amp Taq or Ultra II Q5, using either 10, 20 or 30 PCR cycles, after which they were sequenced on the MinION. Libraries generated with Ultra II Q5 polymerase showed a consistently high percentage (≥ 96%) of BoHV-1 sequence reads for 10, 20, and 30 PCR amplification cycles whereas libraries generated with Long Amp Taq showed a dramatic reduction in the percentage of BoHV-1 sequence reads with increasing PCR cycle number, and a dramatic increase in the percentage of bacterial sequence reads that were detected after 20 and 30 PCR cycles (Fig. 2). This is most likely due to positive amplification bias of the Long Amp Taq towards the lower GC content of the bacterial genomes.
Figure 2. Comparison of PCR polymerase bias on percentage of BoHV-1 reads in MinION sequencing libraries. Percentage read counts for taxonomic assignments following EPI2ME WIMP analysis of FASTQ files are shown. Libraries were generated from the same nucleic extract (bead beating + nuclease) from BoHV1 infected bFLCs using the ONT rapid PCR barcoding kit. Either Long Amp Taq polymerase or Ultra II Q5 polymerase were used for the PCR step. For each polymerase, libraries were generated using either 10, 20 or 30 PCR cycles (indicated on X-axis) and a 300 second extension time. Three libraries were generated for each of the 3 PCR cycle numbers.

In terms of sequence read counts, the Q5 polymerase dramatically increased the numbers of BoHV-1 reads after 20 PCR cycles (109 fold increase) and 30 cycles (169 fold increase) (Table 2). After 30 PCR cycles with Q5, tens of thousands of BoHV-1 reads were identified. With LongAmp Taq there was actually a 0.3 fold decrease in the BoHV-1 read
count between 10 and 20 cycles and only a 3.7 fold increase between 10 and 30 cycles with just a few hundred BoHV-1 reads after 30 cycles (Table 2). The LongAmpTaq showed much higher amplification of bacterial sequence than Q5. Amplification of eukaryotic nucleotide sequence was similar using both polymerases.

| Taxonomic assignment       | Sequence read counts |
|----------------------------|----------------------|
|                            | 10 PCR cycles | 20 PCR cycles | 30 PCR cycles |
| BoHV-1 (LongAmp)           | 81           | 115          | 65           |
| BoHV-1 (Q5)                | 231          | 36           | 92           |
| Bacteria (LongAmp)         | 21           | 18           | 7            |
| Bacteria (Q5)              | 0            | 0            | 0            |
| Eukaryote (LongAmp)        | 7            | 4            | 0            |
| Eukaryote (Q5)             | 2            | 1            | 3            |
| Other viruses (LongAmp)    | 0            | 0            | 0            |
| Other viruses (Q5)         | 0            | 0            | 0            |

Table 2. Comparison of PCR polymerase bias on BoHV-1 detection sensitivity in MinION sequencing libraries. Libraries were generated from the same nucleic extract (bead beating + nuclease) from BoHV1 infected bFLCs using the ONT rapid PCR barcoding kit. Either Ultra II Q5 polymerase or Long Amp Taq polymerase were used for the PCR step. For each polymerase, libraries were generated using either 10, 20 or 30 PCR cycles. Three libraries were generated for each of these 3 PCR cycle numbers. Read counts for taxonomic assignments following EPI2ME WIMP analysis of FASTQ files are shown.

We also tested if it was possible to reduce the time of the PCR extension step for each PCR cycle with the Q5 polymerase. The 5 minute extension step recommended by ONT for LongAmp Taq resulted in a PCR amplification step that took 4 hours and 10 min. Extension times tested for Q5 were 5 min (as recommended by ONT), 3 min, 2 min, and 40 s. Unexpectedly, the longest reads were obtained with 40 s of extension, with an average read
length of 1,400 bp. The reduction of the PCR extension step to 40 s led to a reduction of the overall 30 cycle PCR amplification to 80 min.

**MinION sequencing of nasal swabs collected from calves challenged with BoHV-1 or PBSBoHV-1 and PBS challenged calves**

From the day prior to challenge to the sixth day post-infection, rectal temperatures and clinical signs were recorded for each calf. Rectal temperatures increased from day 2 post-challenge in calves challenged with BoHV-1 whereas rectal temperatures did not increase in the PBS challenged control calves (Fig. 3).

![Figure 3. Rectal temperatures of BoHV-1 experimentally infected calves and PBS challenged (control) calves from day -1 to day 6 relative to the challenge on day 0.](image)
MinION sequencing of nasal swabs calves challenged with BoHV-1

Nasal swabs were collected from the six BoHV-1 challenged calves and six control (PBS challenged) calves from day -1 to day 6 relative to the challenge. Each swab was diluted in 3 mL of PBS and a 1 mL aliquot of this was used for depleted nucleic acid extraction for sequencing and library preparation. Nucleic acid extraction and library preparation were performed in batches comprising the 8 nasal swabs that were collected from each animal (one swab per day) plus a clean swab as negative control. Each batch of libraries was run on a separate flowcell. The libraries were sequenced on the MinION device attached to a MinIT for 24 h using rapid base calling and FASTQ files were uploaded to Epi2ME WIMP for taxonomic assignment.

BoHV-1 reads were identified by Epi2ME WIMP in all sequence libraries that had been generated from nasal swabs taken from the BoHV-1 challenge group from day 1 to day 6 post infection. BoHV-1 was not detected in any of the nasal swabs from calves challenged with PBS (Table 3). BoHV-1 was not detected on day -1 and 0 in four of the six calves challenged BoHV-1. One or two reads were identified as BoHV-1 for day -1 for one calf from the BoHV-1 challenge group, and for day 0 for two calves from the BoHV-1 challenge group. One or two reads were also identified as BoHV-1 in the negative extraction control that was included in the batch of extractions from those same two BoHV-1 challenged animals (BoHV1_1 and BoHV1_2). This could have resulted from either leaky barcodes during sequencing or cross contamination during sample processing.
| Calf no. | Sequence read counts | Day (d) of study |
|---------|----------------------|------------------|
|         | d -1    | d 0 | d 1 | d 2 | d 3 | d 4 | d 5 | d 6 | -ve |
| PBS_1   | 0      | 0   | 0   | 0   | 0   | 0   | 0   | 0   | 0   |
| PBS_2   | 0      | 0   | 0   | 0   | 0   | 0   | 0   | 0   | 0   |
| PBS_3   | 0      | 0   | 0   | 0   | 0   | 0   | 0   | 0   | 0   |
| PBS_4   | 0      | 0   | 0   | 0   | 0   | 0   | 0   | 0   | 0   |
| PBS_5   | 0      | 0   | 0   | 0   | 0   | 0   | 0   | 0   | 0   |
| PBS_6   | 0      | 0   | 0   | 0   | 0   | 0   | 0   | 0   | 0   |
| BoHV1_1 | 0      | 1   | 635 | 44,864 | 1779 | 314 | 356 | 1,186 | 1 |
| BoHV1_2 | 1      | 2   | 69,864 | 3024 | 204,042 | 26,519 | 7   | 884  | 2 |
| BoHV1_3 | 0      | 0   | 14   | 1,607 | 1  | 220  | 103  | 2,218 | 0 |
| BoHV1_4 | 0      | 0   | 174  | 46,381 | 478 | 259  | 284  | 47   | 0 |
| BoHV1_5 | 0      | 0   | 134  | 1,405 | 385 | 939  | 86   | 110  | 0 |
| BoHV1_6 | 0      | 0   | 7    | 554   | 751 | 163  | 355  | 2,300 | 0 |

**Table 3.** Numbers of BoHV-1 sequence reads identified in swabs by DNA virus MinION sequence protocol. Epi2ME WIMP analysis of rapid base-called FASTQ files generated by MinION sequencing of nucleic acid extracted from nasal swabs collected from the 6 PBS control calves and 6 BoHV-1 experimentally infected calves on the day prior to challenge (d - 1), the day of challenge (d 0) with BoHV-1 or PBS to d 6 post-challenge. -ve represents the extraction that was performed on a clean swab which was included as a negative extraction control in each batch of swab extractions.

**Detection of viruses other than BoHV-1 in nasal swabs from BoHV-1 calf challenge model**

For each swab, many single reads were assigned to viruses and phages other than BoHV-1 including bacteriophages and eukaryotic viruses (Supplementary Table S1). Where one or two reads were assigned to each of these eukaryotic viral taxa they were possibly a result of incorrect assignment of bovine, fungal or yeast genomes which were present in large amounts in these nasal swabs or inaccurate submissions to the RefSeq data base employed by WIMP. Incorrect assignment in WIMP due to inaccurate submissions to RefSeq have been reported.
previously\textsuperscript{31}. However, this was not checked in the current work. Bacteriophage taxonomic assignments were not surprising given the large numbers of bacteria in these nasal swab samples. More than 100 reads were assigned to Proteus phage VB\textsubscript{PmiS-Isfahan}, Acinetobacter phage YMC13/03/R2096, Bubaline alphaherpesvirus and Bovine Alphaherpes Virus5 in some swabs. Proteus phage VB\textsubscript{PmiS-Isfahan} seemed to be common in the nasal passages of these animals. Reads were also assigned to alphaherpes virus taxa other than BoHV-1 such as BoHV-5. These only occurred in animals that were challenged with BoHV-1 indicating that they were missassigned BoHV-1 sequences. We used rapid FASTQ basecalling in MinKnow software as our aim was to go from sample to result in a single day. Accurate FASTQ base calling may have reduced the number of non-BoHV-1 viral reads but it was too slow for the same day protocol so we did not test it.

**Conclusion**

A ‘same-day-sample-to-result’ untargeted sequencing protocol to identify BRD-associated DNA viruses in nasal swabs was developed using the MinION nanopore sequencer and Epi2ME WIMP software. This protocol, which could be carried out within a six hour time frame, allowed correct identification of BoHV-1 in nasal swabs collected from calves that were experimentally infected with BoHV-1. The portability of the MinION nanopore sequencer means this protocol has potential for point-of-care viral pathogen testing in cattle.

**Methods**

**Bovine foetal lung cells infected with BoHV-1**

Bovine foetal lung cells were isolated from a bovine foetus and used as the *in-vitro* preparation for viral infection. The source, origin, and characteristics of these bFLC are shown in Supplementary Table S2. BoHV-1 strain 2011-415 was isolated from the trachea of
a 9 months old calf that died after showing clinical signs of pneumonia and was diagnosed with IBR. A post-mortem examination showed fibrin and purulent debris on the nares, severe necrotic, fibrinous and purulent tracheitis affecting the whole length of the trachea and bronchi, consolidation with foci of necrosis and purulent material in the airways, fibrinous pleuritis, and interstitial and alveolar emphysema. Furthermore, tissue samples from the trachea were collected and tested for the presence of BoHV-1 by qPCR. The sample tested positive for BoHV-1 and the virus was isolated and stored at -80°C. A dilution of BoHV-1 (TCID50 = 106.75/100 μL in bFLC) was prepared at a concentration of 1:100 in 2% Glasgow minimal essential medium (G-MEM). A T75 flask (Thermo Fisher Scientific, MA, USA) containing bFLC was infected with 5 mL of the dilution. As a negative control, an additional T75 flask containing bovine foetal lung cells and 5 mL of 2% buffer G-MEM was also used. The two flasks were incubated at 37°C for 90 min in a CO₂ incubator (Thermo Fisher Scientific, CA, USA). Afterwards, 15 mL of 2% G-MEM buffer was added. The flasks were replaced in the CO₂ incubator at 37°C. At 48 h post infection, the two flasks were observed via phase-contrast light microscopy at 4 × magnification, with 90% of the cells displaying viral cytopathology. The flasks were then placed in a freezer at -80°C for 2 h, and subsequently thawed and their contents transferred to sterile 50 mL centrifuge tubes (Thermo Fisher Scientific, MA, USA). The tubes were then centrifuged for 5 min at 3,660 × g, and 1 mL aliquots of the supernatant were transferred to sterile 1.5 mL microfuge tubes (Eppendorf, Hamburg, Germany) and frozen at -80°C.

**Experimental calves**

All animal experiments were carried out in accordance with the UK Animals (Scientific Procedures) Act 1986 and with the approval of the Agri-Food and Biosciences Institute
Northern Ireland Ethical Review Committee. The study is reported in accordance with ARRIVE guidelines (https://arriveguidelines.org).

As part of a larger study, 12 Holstein-Friesian bull male calves (mean age 21.3 weeks, SD ±3.4) were selected from a larger group of 43 Holstein-Friesian bull calves. The enrolment of calves for the challenge study was based on two criteria: low level of BoHV-1 MDA, and negative PCR result for BoHV-1 tested two weeks before challenge. The 12 selected calves were assigned to two groups (BoHV-1-challenge and PBS-challenge) with 6 calves per group. For the PBS challenge group the mean age = 21.4 (SD ±3.3) weeks, mean weight = 173.3 (SD ±23.7) kg, mean MDA = 18.0 (SD ±4.5) %. For the BoHV-1-challenge group mean age = 21.0 (SD ±4.5), mean weight = 175.8 (SD±35.6) kg, MDA = 20.6 (SD ±13.2) %. At day 0, the BoHV-1-challenge group was infected by intranasal atomisation with a solution of BoHV-1 (dose = 6.3 × 10⁷/mL × 1.35 mL per animal). The animals in the PBS-challenge group were mock challenged (day 0) with an intranasal atomisation of sterile PBS solution. The two groups were housed in two separate biocontainment level 3 sheds each with a 10 m x 5 m floor covered in straw. Daily clinical assessments, nasal swabs, and blood samples were collected from each animal every day from the day before (day -1) challenge until day 6 when the animals were euthanized.
Nasal swabs from experimental calves

For each nasal swab sample, the exterior of the nasal nares of the calf were sterilised with 70% ethanol then a sterile swab was removed from its sterile tube and inserted approximately 20 cm into the nostril and rolled on the internal nasal membrane for approximately 5 s. The swab tip was cut with scissors (sterilised with 70% ethanol) into a 2 mL sterile tube and immediately frozen on dry ice. Nasal swabs from the BoHV-1 calf challenge model were stored for approximately 6 months at -80°C prior to nucleic acid extraction and sequencing.

Immediately prior to nucleic acid extraction, nasal swabs in tubes were removed from storage in a -80°C freezer to a class 2 biological safety cabinet. A volume of 1.5 mL of molecular grade PBS (Sigma, Germany) was added to each tube and the tubes were vortexed for 1 minute to release the nasal material from the swabs. The resulting PBS eluate was then transferred to a sterile 15 mL tube. A further 1.5 mL of PBS was added to each tube containing the swabs, the tubes were vortexed again and the PBS eluate was removed and added to the 1.5 mL in the 15 mL tube resulting in 3 mL of PBS nasal swab eluate for each swab sample.

Non-viral nucleic acid depletion

Either 250 µL of BoHV-1 infected bFLC in vitro culture or 1 mL of nasal swab eluate was transferred to a Pathogen Lysis Tubes L (Qiagen, Manchester, UK). For the negative extraction control, 250 µL of molecular grade phosphate-buffered saline (PBS, pH 7.4) (Sigma, Aldrich, Germany) was added to a Pathogen Lysis Tube L (Qiagen, Manchester, UK).

To prevent escape of aerosols from tubes during bead beating, tube lids were sealed with Parafilm (Sigma Aldrich, Germany). Tubes were placed in a FastPrep-24 disruptor
instrument (MP Biomedicals, CA, USA) and shaken at high speed (4 ms\(^{-1}\)) for 30 s. The tubes were then removed and centrifuged at 500 \(\times\) g for 45 s to collect the contents to the bottom of the tube. The supernatant from each Pathogen Lysis Tube was then carefully transferred to 2 mL DNA LoBind tubes (Eppendorf AG, Hamburg, Germany) and the volume was adjusted to 1 mL with molecular grade PBS. An aliquot of 2.5 µL of RNaseA (4 mg/mL) (Promega, Madison, WI, USA) was added to the tubes which were then incubated for 15 min at 37°C in an Eppendorf Thermostat Plus (Eppendorf AG, Hamburg, Germany). Turbo DNase (10 µL) and 10× Turbo DNase buffer (100 µL) were then added, (Thermo Fisher Scientific, CA, USA)\(^{32}\) and the tubes were gently mixed by pipetting six times and incubated for 30 min at 37°C in an Eppendorf Thermostat Plus. A further 10 µL of Turbo DNase was added and the contents were again mixed by gently pipetting six times and incubated for a further 30 min at 37°C. DNase inactivation reagent (112.5 µL) (Thermo Fisher Scientific, CA, USA) was then added to the samples and mixed by gentle pipetting and incubated for 5 min at 24°C. The mixture was centrifuged at 10,000 \(\times\) g for 90 s then the supernatant containing the RNA was transferred to a fresh tube without disturbing the pellet of DNase Inactivation Reagent. Samples were also processed with the omission of certain treatments to test whether these treatments were necessary. Five such treatment regimes were tested. These were: (A) no bead beating or nuclease treatment, (B) bead beating only, (C) no bead beating, 1× RNase, 1× DNase, (D) no bead beating, 1× RNase, 2× DNase. (E) bead beating, 1× RNase, 2× DNase. A negative extraction control (a tube containing only molecular grade PBS, or a sterile unused swab) was included with each batch of extractions to monitor contamination of reagents and cross contamination of samples during the extraction process.

**Nucleic acid extraction and purification**
Total nucleic acid was immediately extracted and purified from the nuclease treated samples using the QIAamp UltraSens Virus Kit (Qiagen, Manchester, UK) according to the manufacturer’s instructions with the exception of the substitution of 5.6 µL of carrier RNA with 5.6 µL of a solution of 5 mg/mL linear acrylamide.

**Library generation**

MinION libraries were generated with the Rapid PCR Barcoding Kit (SQK-RPB004) (Oxford Nanopore Technologies, Oxford, UK). For each library, 6 µL of nucleic acid extraction plus 2 µL of fragmentation mix (FRM) were added to a thin walled 0.5 mL PCR tube (Eppendorf, Hamburg, Germany). The tubes were incubated in a Master Cycler Gradient PCR machine (Eppendorf, Hamburg, Germany) at 30°C for 1 min, then 80°C for 1 min, after which they were immediately placed on ice. This resulted in tagmentation of the DNA in the nucleic acid extraction with sequencing adapters.

For PCR amplification of the tagmented DNA, 8 µL of the tagmentation reaction, 16 µL of nuclease-free water, 1 µL of rapid barcode primer (RLB), and either 25 µL of LongAmp® Taq DNA polymerase master mix (New England BioLabs Inc.) or 25 µL of NEBNext Ultra II Q5 master mix (New England BioLabs Inc.) were added to a 0.5 mL thin walled PCR tube (Eppendorf, Hamburg, Germany). The tube contents were mixed by gently flicking the tube and then centrifuged for 10 s in a minifuge. The tubes were then placed in a Master Cycler Gradient PCR machine (Eppendorf AG, Hamburg, Germany). Cycle conditions for LongAmp® Taq were: 95 °C (3 mins), then 10, 20 or 30 cycles of 95 °C (15 s), 56 °C (15 s), 65 °C (6 mins), then 65 °C (6 mins) followed by a hold step at 4°C. Cycle conditions for NEBNext Ultra™ II Q5® were 98°C (3 min), then 10, 20 or 30 cycles of 98°C (10 s), 65°C (30 s), 72°C (either 40 s, 120 s, 180 s or 300 s), then 72°C (2 min) followed by a hold step at followed by a hold step at 4°C.
Following PCR, the 12 PCR libraries were combined in a single 1.5 mL Eppendorf DNA Lo-Bind (Eppendorf AG, Hamburg, Germany) tube and a total volume of 360 µL of AMPureXP beads (i.e. 30 µL of beads for each individual barcoded library) (Beckman Coulter Inc., Brea, CA, USA) were added to the 12 pooled PCR reactions and mixed gently by pipetting. The library pool and beads were incubated in a rotator mixer for 5 min at room temperature. The tube was then removed from the rotator mixer, centrifuged for approximately 20 s in a minifuge, then placed on a magnet for 5 min and the supernatant was discarded. Being careful not to dislodge the beads, 1 mL of 70% ethanol was added to the beads, then immediately removed. This ethanol wash step was then repeated once. The tube was then centrifuged for 30 s using a minifuge and placed on the magnet until the beads had bound to the side of the tube leaving the ethanol at the bottom. Residual ethanol was removed from the bottom of the tube by pipetting without touching the beads. The tube was then left open for 60 s to allow the pellet to air dry. The tube was then removed from the magnet and the pellet was resuspended in 25 µL of a solution of 10 mM Tris-HCl (pH 8.0), 50 mM NaCl. The solution was mixed by pipetting until the pellet was completely resuspended and the suspension was then incubated for 2 min at room temperature to elute the purified-pooled-barcoded libraries. The tube was placed on the magnet for 5 min and the eluate containing the purified-pooled-barcoded libraries was collected and transferred to a new 1.5 mL DNA LoBind tube (Eppendorf AG, Hamburg, Germany). A volume of 1 µL of the purified-pooled-barcoded libraries was removed to measure its DNA concentration on a Qubit fluorometer using the Qubit 1× dsDNA HS Assay Kit (Invitrogen, Thermo Fisher Scientific), following the manufacturer’s instructions. The volume of library corresponding to 60 ng dsDNA was calculated and removed to a new 1.5 mL DNA LoBind tube. This volume was then adjusted to 11 µL with 10 mM Tris-HCl (pH 8.0), 50 mM NaCl. One microliter of rapid adapter (RAP) was then added to the 11 µL of the pooled barcoded library, mixed gently by flicking
the tube, centrifuged for approximately 10 s in a minifuge to collect the liquid in the bottom 
of the tube, and incubated at room temperature for 5 min. Subsequently, the sample and 
flowcell were prepared for priming and loading following the manufacturer’s instructions. 
The library was loaded into the spot-on port on a spot-on flowcell (FLO-MIN106D R9) 
(Oxford Nanopore Technologies, Oxford, UK) and sequenced for 48 h on a MinION 
sequencer (Min-101B, MinION MK1B) (Oxford Nanopore Technologies, Oxford, UK) 
attached to a MinIT compute module (Oxford Nanopore Technologies, Oxford, UK) using 
MinIT software version (19.05.02) for MinKnow Guppy-based basecalling^{35}. Each flowcell 
was tested with the ONT configuration program in MinKnow software immediately prior to 
each run to ensure that the number of active pores was >1000. The output selected for each 
run was fast basecalling, FASTQ files only.

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**Author contributions**

G.E., M.S.M., BE, BT, KR, PC and SMW conceived and designed the sequencing analysis of animal samples. KR, TB, MSM and GE designed nanopore sequencing approaches. GE and MSM designed and developed the lab sequencing protocol. GE conducted the sequencing. KL, BE and SMW conceived and designed the animal challenge model. K.L. conducted the animal challenge model, nasal swabbing and cell cultures. GE, MSM and PC analysed the data. GE and MSM wrote the manuscript. All authors reviewed and edited the manuscript.

**Competing interests**

The authors declare no competing interests.
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