

1 **Simultaneous detection of DNA and RNA virus species**
2 **involved in bovine respiratory disease by PCR-free rapid**
3 **tagmentation-based library preparation and MinION**
4 **nanopore sequencing**

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19

20 **Abstract**

21 The Oxford Nanopore MinION Mk1B is a portable 90 g device that sequences DNA directly
22 at 450 bases/second generating sequence reads in excess of 400 kb. Recent improvements in
23 error rate and speed of library preparation mean that this device has considerable potential for
24 rapid molecular bovine pathogen diagnostics. We tested the MinION for rapid untargeted
25 detection of viral pathogens associated with bovine respiratory disease (BRD), an
26 economically important disease often involving primary infection of the lung by one or more
27 of a number of DNA and/or RNA viruses. We combined three foetal lung cell cultures which
28 were infected with either Bovine Respiratory Syncytial Virus (BRSV), Bovine Herpes Virus
29 1 (BoHV1) or Bovine Parainfluenza Virus 3 (BPI-3). BoHV1 is a DNA virus and BPI-3 and
30 BRSV are RNA viruses. The cell cultures were treated with DNase and RNase to deplete
31 bovine nucleic acid prior to viral nucleic acid extraction and double-stranded cDNA
32 synthesis. Sequencing libraries were generated by PCR-free tagmentation in under 10
33 minutes and loaded onto a MinION sequencer. Approximately 7,000 sequencing reads were
34 generated and analysed using high-throughput local BLAST against the NCBI nr/nt database.
35 The top BLAST hit for 2,937 of these reads was identified as a virus. Of these, 2,926 (99.6%)
36 were correctly identified either as BoHV1, BRSV or BPI-3.

37

38 **Introduction**

39 Bovine respiratory disease (BRD) is the most costly disease of beef cattle in North
40 America and causes significant losses in most other cattle producing regions including
41 Ireland [1-3]. It is thought that the majority of BRD cases involve primary infection of the
42 lower respiratory tract (LRT) with one or more viruses which predisposes the LRT to
43 secondary infection with a single or multiple bacterial species [4,5].

44 As viruses are the most common primary pathogen in BRD, vaccines containing BRD
45 associated modified live viruses (MLVs) are widely used in the cattle industry. The MLVs
46 that are included in the most recent BRD vaccines (e.g. Rispoval[®]4 (Merck) and Bovi-Shield
47 Gold[®]5 (Zoetis)) are Bovine Respiratory Syncytial Virus (BRSV), Bovine Herpes Virus 1
48 (BoHV1), Bovine Parainfluenza Virus 3 (BPI3). These MLVs have been selected based on
49 viruses that have been most commonly isolated and cultured or detected by qPCR from nasal
50 swabs, lung washes and lung lesions from BRD cases in recent decades.

51 The primers and probes used in the qPCR assays are designed against small fragments
52 (approximately 200 bp) of genomes of known (i.e. cultured and isolated) BRD-associated
53 viral genomes and individual assays have to be performed for each BRD virus. In the
54 majority of BRD cases, no virus is detected either by culturing or by qPCR. For example, in a
55 recent report³ qPCR assays for BoHV1, BVDV, BPI3, BRSV and Bovine Corona Virus
56 detected virus in only 8.7% (391 out of 4444) of nasal swab samples from live animals
57 displaying BRD symptoms. The viruses used in vaccines (i.e. BoHV1, BVDV, BPI3, BRSV)
58 were detected in only 5.4% (241 of the 4444) samples. In addition, despite the wide use of
59 MLV BRD vaccines, BRD remains the leading cause of natural death in the cattle industry.
60 This raises the possibility that other viruses or viral strains that are not easily cultured are
61 involved in BRD and consequently targeted qPCR assays are an inadequate diagnostic
62 method for BRD-associated viruses [6,7].

63 Recently viral metagenomics using next generation sequencing (NGS) platforms have
64 been employed to survey viruses associated with BRD. This untargeted approach detects
65 known and unknown viruses in a single universal assay. Ng *et al.* (2015) conducted viral
66 metagenomic analysis of nasopharyngeal and pharyngeal recess swabs from animals with
67 severe BRD symptoms and detected Bovine Adenovirus 3, Bovine Adeno-associated Virus,
68 Bovine Rhinitis A Virus BSR14, Bovine Rhinitis B Virus, Bovine Influenza D Virus, Bovine
69 Astrovirus, Picobirnavirus, Bovine Parvovirus 2, and Bovine Herpesvirus 6. Bovine
70 Adenovirus 3, Bovine Rhinitis A Virus BSR14 and Bovine Influenza D Virus BRSI1 were
71 the only viruses that were significantly associated with BRD. None of the viruses that are
72 included in current BRD vaccines were detected [7].

73 So far, BRD viral metagenomic studies have used Illumina sequencing by synthesis
74 (SBS). This requires amplification of cDNA using techniques such as multiple displacement
75 amplification (MDA), and library preparation that can take several hours. The read lengths on
76 the Illumina MiSeq are short with a maximum of just 300 bp paired end on a 600 cycle
77 reagent cartridge. SBS is relatively slow with a 600 cycle MiSeq run taking approximately 56
78 hours and no sequence data is available from a MiSeq until the run has completed. However,
79 Illumina SBS platforms remain unsurpassed in terms of number and quality of sequence
80 reads.

81 The MiSeq, which is one of the smaller Illumina NGS platforms (686 mm × 523 mm
82 × 565 mm, 572 grams), is not designed for rapid diagnostics in the field. In contrast, the
83 Oxford Nanopore Technologies MinION Mk1B is a pocket-sized (105 mm × 23 mm × 33
84 mm, 87 grams) field-deployable sequencing device that is based on nanopore sequencing.
85 DNA is sequenced directly when it passes through recombinant *E.coli* CcsG nanopores that
86 are embedded in a membrane and each base causes a characteristic change in the membrane
87 current. This allows extremely rapid direct sequencing of individual DNA molecules. In

88 October 2016 the R9.4 MinION flowcell was released which can run at 450 bases/second per
89 nanopore and generate 10 gigabases of data per MinION flow cell. The library preparation
90 with the ‘Rapid Sequencing Kit’ takes approximately 10 minutes. Loading the library takes
91 approximately 20 minutes and thousands of long sequence reads (maximum length is
92 typically >65 kb) are available for analysis within minutes of loading the library. Unlike the
93 Illumina SBS platforms, the quality of nanopore reads does not decline with length. However,
94 the error rate of reads from the MinION is still far higher than those of Illumina SBS
95 platforms such as the MiSeq.

96 Several labs have used the MinION to detect viruses. Greninger *et al* (2015) reported
97 untargeted metagenomic detection of high titre Chikungunya Virus (CHIKV), Ebola Virus
98 (EBOV), and hepatitis C virus (HCV) from four human blood samples by MinION nanopore
99 sequencing [8]. However, to obtain ≥ 1 μg of metagenomic complementary DNA (cDNA) for
100 the library required for the nanopore sequencing protocol, randomly amplified cDNA was
101 generated using a primer-extension pre-amplification method (Round A/B). Briefly, in Round
102 A, RNA was reverse-transcribed with SuperScript III reverse transcriptase using Sol-
103 PrimerA, followed by second strand synthesis with DNA polymerase with Sol-PrimerB and
104 PCR amplification (25 cycles). Libraries were then prepared by end repair, adenylation and
105 adapter ligation. After nanopore sequencing on the MinION they obtained sequences with an
106 individual error rate of 24%. Despite the high error rate this allowed identification of the
107 correct viral strain in all four isolates, and 90% of the genome of CHIKV was recovered with
108 97–99% accuracy. Quick *et al.* (2016) identified high titre Ebola Virus in samples submitted
109 less than 24 h after collection, with a targeted sequencing approach based on Ebola specific
110 PCR primers that took 15-60 min [9]. For this they used targeted reverse transcriptase PCR
111 (RT–PCR) to isolate sufficient DNA for sequencing using a panel of 38 primer pairs that
112 spanned the Ebola Virus genome. Recently, Kilianski *et al.* (2016) generated unamplified

113 RNA/cDNA hybrids from nucleic acid extracted from either Venezuelan Equine Encephalitis
114 Virus or Ebola Virus cell culture (both RNA viruses) and sequenced them individually on
115 separate sequencing runs on a MinION. They were able to correctly identify each of the RNA
116 viruses following alignment to the respective viral genomes [10].

117 In the present study, we tested the potential of untargeted nanopore sequencing on the
118 MinION Mk1B for rapid simultaneous identification of a mixture of DNA and RNA viruses
119 that are associated with BRD. Often more than one virus (with either a DNA or RNA
120 genome) causes infection in the respiratory tract of an animal. We sequenced nucleic acid
121 that we extracted from a mixture of three foetal lung cell cultures which were infected either
122 with BRSV, BPI-3 or BoHV1 [11]. BoHV1 is a member of the family Alphaherpesviridae
123 with a 150 kb linear double-stranded DNA monopartite genome. BRSV and BPI-3 are both
124 members of the family Paramyxoviridae each with a 15 kb negative sense single-stranded
125 RNA genome. BoHV1, BRSV and BPI-3 genomes are all packaged in a protein capsid which
126 is surrounded by an outer lipid membrane envelope. BoHV1 also has a protein tegument
127 between the capsid and the envelope.

128 We report correct simultaneous identification of combined DNA and RNA virus
129 species involved in BRD by PCR-free rapid (10 min) tagmentation-based library preparation
130 and nanopore sequencing on the portable Oxford Nanopore Technologies MinION Mk1B
131 sequencer.

132

133 **Methods**

134 **Viral cultures**

135 Foetal lung cell cultures, infected with either BoHV1, BPI-3 or BRSV, were stored at -80°C.

136 The cultures were sourced from (Agri-Food and Biosciences Institute (AFBI), Veterinary

137 Science Division, Stormont, Belfast, N. Ireland). BPI-3 (TCID₅₀ = 10^{6.5}/100 µL in FCL) was
138 cultured from a diagnostic lung sample. BHV-1 (TCID₅₀ = 10^{6.75}/100 µL in FCL) was
139 cultured from a diagnostic lung sample. BRSV RISP_RS SP.C 11/03/15) (TCID₅₀ =
140 10^{3.75}/100 µL in FCL) is a vaccine strain of BRSV cultured in the FCL cell line.

141

142 Nuclease treatment

143 The three frozen viral cultures were crushed to a fine powder with a sterile pestle and mortar
144 under liquid nitrogen. Crushed frozen powder for each virus culture was weighed (BoHV1
145 (480 mg), BRSV (370 mg), BPI-3 (150 mg)) and combined in a 1.5 mL Eppendorf DNA low
146 bind tube (Eppendorf, Hamburg, Germany). The volume was adjusted to 1 mL with
147 DNA/RNA/DNase/RNase-free PBS (Sigma Aldrich Ltd., Arklow, Ireland) and 2.5 µL
148 RNaseA (4 mg/mL) (Promega, Madison, WI, USA), 100 µL of 10× Turbo DNase buffer and
149 10 µL of Turbo DNaseTM (Life Technologies Ltd, Paisley, UK) were added. The solution was
150 inverted slowly six times and incubated for 30 min at 37°C. Then another 10 µL of Turbo
151 DNase was added, the mixture was inverted slowly again 6 times and incubated for a further
152 30 min.

153

154 Nucleic acid extraction

155 Immediately following nuclease treatment, the remaining nucleic acids were extracted with
156 the QIAamp Ultra Sens Virus Kit DNA extraction kit (Qiagen, UK) according to
157 manufacturer's instructions except that carrier RNA was substituted with 5.6 µL of a solution
158 of 5 mg/mL linear acrylamide (Thermo Fisher Scientific, MA, USA). The final elution was
159 performed with 2× 30 µL of buffer AVE (total 60 µL) which was supplied in the kit.

160

161 Double-stranded cDNA synthesis

162 Double-stranded cDNA was generated with the Maxima H Minus Double-Stranded cDNA
163 Synthesis Kit (Thermo Fisher Scientific, Waltham, MA, USA) according to the
164 manufacturer's instructions. Briefly, to generate the first cDNA strand (i.e. reverse
165 transcription), 13 μ L of extracted nucleic acid was added to 1 μ L of random hexamer and
166 mixed by gentle pipetting six times then incubated for 5 min at 65°C. The reaction was placed
167 on ice for 1 minute, then centrifuged in a minifuge for 30 sec and placed back on ice. Then 5
168 μ L of 4 \times First Strand Reaction Mix and 1 μ L of First Strand Enzyme Mix were added, mixed
169 by slowly pipetting the entire volume up and down six times, then incubated on an Eppendorf
170 Master Cycler (Eppendorf, Hamburg, Germany) at 25°C (10 min), 50°C (30 min) and 85°C (5
171 min). The tube was removed from the Master Cycler and centrifuged for 10 seconds in a
172 minifuge and placed on ice. To generate the second cDNA strand, the entire first strand
173 cDNA reaction mixture (20 μ L) was combined with molecular grade water (55 μ L) (Sigma-
174 Aldrich, Arklow, Ireland), 5 \times second strand reaction mix (20 μ L) and second strand enzyme
175 mix (5 μ L). The entire volume (100 μ L) was mixed by slowly pipetting up and down six
176 times and was then incubated at 16°C (60 min). The reaction was stopped by adding 6 μ L of
177 0.5 M EDTA (pH 8.0) (Sigma-Aldrich, Arklow, Ireland) and pipetting the entire volume up
178 and down six times. To remove residual RNA, 10 μ L (100 U) of RNase1 (supplied in the
179 Maxima H Minus Double-Stranded cDNA Synthesis Kit) was added to the second strand
180 reaction mixture which was mixed by slowly pipetting up and down six times and incubated
181 at room temperature (22-25°C) for 5 min. The double-stranded cDNA reaction was purified
182 using a Qiagen MinElute PCR clean up kit (Qiagen, Manchester, UK). This removed
183 enzymes and primers and retained purified double-stranded cDNA and gDNA.

184

185 Double-stranded cDNA/gDNA library preparation

186 The purified double-stranded cDNA/gDNA was tagged using a Rapid Sequencing Kit
187 SQK-RAD001 (Oxford Nanopore Technologies, Oxford, UK). Briefly, 7.5 μ L of double-
188 stranded cDNA/DNA was added to 7.5 μ L of FRM (Oxford Nanopore Technologies, Oxford,
189 UK), then mixed by slowly pipetting up and down six times. The reaction was incubated in a
190 thermal cycler at 30°C for 1 min then at 75°C for 1 min. 1 μ L of RAD (Oxford Nanopore
191 Technologies, Oxford, UK) was added to the 15 μ L tagmentation reaction and mixed by slow
192 pipetting, then 0.2 μ L of blunt/TA ligase master mix (New England BioLabs, Ipswich, MA,
193 USA) was added to each tube and mixed again by slow pipetting. The reaction (designated
194 pre-sequencing mix) was then incubated for 5 min at room temperature.

195

196 Running the library

197 RAD (37.5 μ L), H₂O (31.5 μ L) and pre-sequencing mix (6 μ L) were combined in an
198 Eppendorf LoBind tube (Eppendorf, Hamburg, Germany) and mixed by pipetting. The
199 resulting 75 μ L was loaded onto a Spot-on flowcell (FLO-MIN106) (Oxford Nanopore
200 Technologies, Oxford, UK) on a MinION Mk1B (Oxford Nanopore Technologies, Oxford,
201 UK) according to the manufacturer's instructions. The flowcell was run for 16 h on
202 MinKNOW software (Oxford Nanopore Technologies, Oxford, UK) using the Protocol
203 'NC_48hr_Sequencing run_FLO-MIN105_plus_1D_Basecaller.py'.

204

205 High throughput local BLAST search

206 The MinION generated 17,138 FAST5 sequence files which we converted to FASTA files
207 using pore tools [12]. This resulted in 7,057 FASTA files that contained sequence reads.
208 These sequence reads were then subjected to a local high throughput BLAST search against

209 the NCBI nr/nt database using a 24 core processor. The top hit BLAST result for each read
210 was used for identification of the 7,057 sequences.

211

212 **Results**

213 **Viral identification**

214 Results of the high throughput BLAST search of the 7,057 MinION sequence reads are
215 summarised in Table 1 and full details are included in S1 Table. A large number of these
216 sequence reads (41.6%) were identified as viruses. The vast majority of the virus-identified
217 sequence reads (99.6%) were identified as one of the three expected viruses BoHV1, BPI-3
218 and BRSV (Table 1). Only 11.6% of the sequence reads were identified as bovine and 46% of
219 the sequence reads were identified as non-bovine/non-viral sequences.

220

221

222 **Table 1. Summary of all top virus hits (species level) following high throughput local**
223 **BLAST search of MinION sequence reads of rapid sequence library prepared from**
224 **combined BPI-3, BRSV and BoHV1 calf foetal lung cell cultures against NCBI nr/nt**
225 **database.**

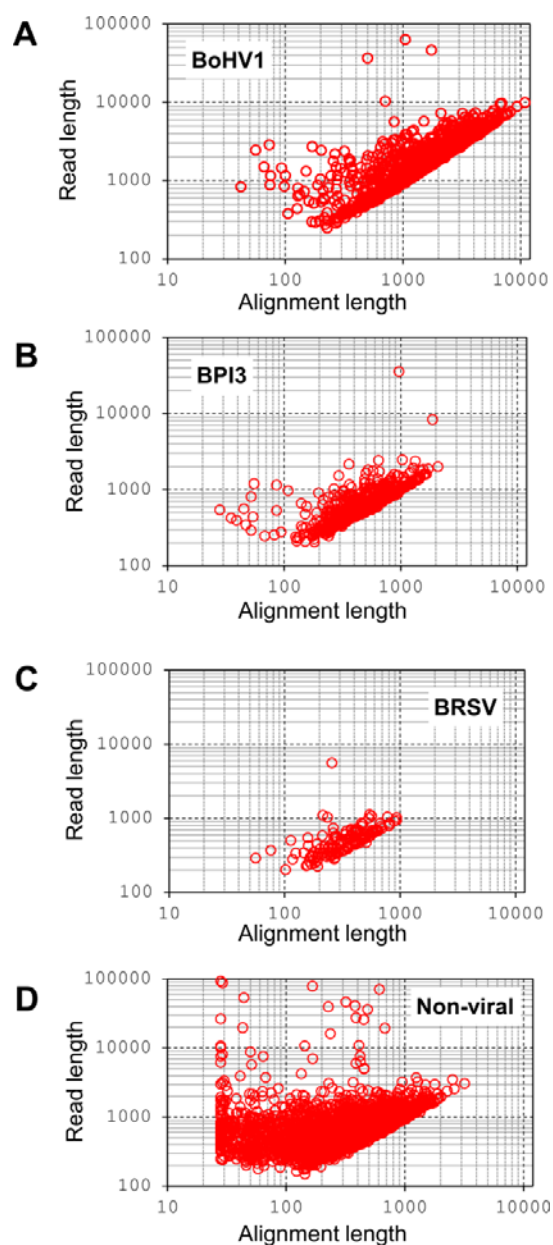
Organism identified	Number of reads	% of total reads	% of virus reads
BPI-3 (RNA virus, 15 kb genome)	746	10.57	25.40
BRSV (RNA virus, 15 kb genome)	139	1.97	4.73
BoHV1 (DNA virus, 150 kb genome)	2,041	28.93	69.5
BoHV (no type designated)	4	0.06	0.14
BoHV5	4	0.06	0.14
Herpesvirus type 1	1	0.01	0.03
<i>Bos taurus</i> BCL2/adenovirus	1	0.01	0.03
Nile crocodilepox virus	1	0.01	0.03
Total number of sequence reads	7,057	-	-
Total number of virus sequence reads	2,937	42.2	-

226

227 Read lengths and alignments

228 Twenty five of the reads were >10 kb and the longest read was 93,542 bp. However,
229 these very long reads only had very short alignments to sequences on the NCBI nr/nt
230 data base (Fig 1). The read length of sequences for which the top BLAST hit was virus,
231 were longer for BoHV1 than for BRSV and BPI-3 (Fig 1). BoHV1 also had the longest
232 alignments out of all viral and non-viral reads (Fig 1). There were three very long viral
233 reads (36,880 bp, 46,607 bp, and 63,234 bp) for which the top hit was either the
234 BoHV1 complete genome or the BoHV1.2 complete genome. However, the alignment

235 length of these long reads to these genomes was short (499 bp, 1, 739 bp, 1, 046 bp
236 respectively) (Fig 1).



237

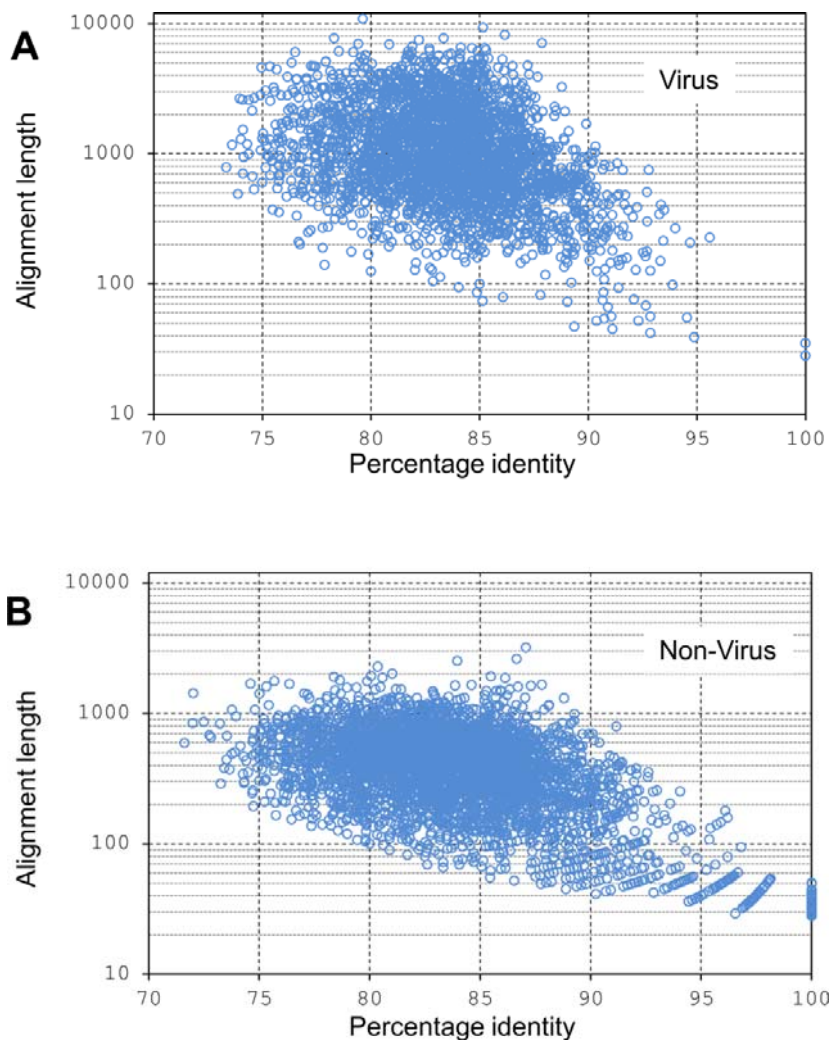
238 **Fig 1. Scatterplot of alignment length (following high-throughput local BLAST search against NCBI nr/nt**
239 **database) against read length of MinION sequence reads.** Sequence reads were generated from a rapid
240 sequencing library prepared from combined BPI-3, BRSV and BoHV1 calf foetal lung cell cultures. A: Reads
241 for which the top BLAST hit was BoHV1. B: Reads for which the top BLAST hit was BPI-3. C: Reads for
242 which the top BLAST hit was BRSV. D: Reads for which the top BLAST hit was not a virus.

243

244 The average percentage identity for alignments was 83.5% for viruses and 84.2% for

245 non-viruses (Fig 2). Reads with >90% identity were <2 kb for both viruses and non-viruses

246 (Fig 2).



247

248 **Fig 2. Scatterplot of alignment length against percentage identity of MinION sequence reads following**

249 **high-throughput local BLAST against NCBI nr/nt database.** Sequence reads were generated from a rapid

250 sequencing library prepared from combined BPI-3, BRSV and BoHV1 calf foetal lung cell cultures A: Reads for

251 which the top BLAST hit was virus B: Reads for which the top BLAST hit was not virus.

252

253 **Discussion**

254 In the present study, combined DNA and RNA viruses were correctly identified following
255 double-stranded cDNA synthesis, library preparation using the Oxford Nanopore
256 Technologies Rapid Sequencing Kit, nanopore sequencing on a MinION Mk1B, and local
257 high throughput BLAST against the entire NCBI nr/nt database. The rapid sequencing library
258 preparation took approximately 10 min and, although the sequencer was run for 16 h, more
259 than 5000 FAST5 files were generated in the first hour of sequencing, which was adequate
260 for identification of the three viruses. This highlights the potential of the MinION for rapid
261 diagnosis of viruses in regional veterinary laboratories and veterinary practices. As the
262 MinION is highly portable, it also has potential for pen-side use by veterinarians and
263 veterinary technicians to characterise viruses in animals that display BRD symptoms and this
264 viral detection method could be applied to DNA extracted from lung washes or
265 nasopharyngeal swabs. However, the numbers of reads generated for the nuclease-treated
266 viral cell cultures was only 7,057 which would be insufficient to detect low titre viruses
267 directly in clinical matrices. With the current depth of sequence available on the MinION, the
268 only way to detect low titre viruses with this device will be through further improvement in
269 depletion of host DNA and unbiased amplification of the library.

270 For nucleic acid extraction and cDNA synthesis we used a spin column method with a
271 microcentrifuge and thermocycler. Magnetic bead-based nucleic acid extraction would
272 eliminate the need for a centrifuge in the field but bead-based extractions tend to be
273 inefficient compared to spin columns. At present, to treat the sample with nucleases, extract
274 the viral nucleic acid then prepare and purify double-stranded cDNA takes approximately 5
275 hours but there is scope to optimise and simplify these steps to significantly reduce this time.
276 We used our own high-throughput BLAST pipeline against the entire NCBI nr/nt data base
277 for 7,057 reads. CPU time for this on a 24 core processor was 130 minutes. We also

278 conducted a local BLAST search against only viral sequences (either against 90,000 virus
279 sequences from NCBI or 7,000 sequences downloaded from Virusite) which took
280 approximately 20 seconds. For virus-only BLAST searches all top hits were viral but many of
281 these hits were not one of the three expected viruses. Reads that did not match the correct
282 viruses were generally less than 100 bp, so removing short reads may work for virus-only
283 BLAST. However, BLAST search against the entire NCBI nr/nt data base, though slower,
284 appeared to be more robust as 99.6% of the viral top hits were correct to viral species level.

285 Due to the low ratio of viral nucleic acid to host nucleic acid, other viral
286 metagenomics protocols commonly use whole genome amplification or PCR. However, in
287 our study amplification was not necessary as 41.6% of the MinION sequence reads were
288 identified as viral by BLAST search against the NCBI nr/nt data base. This may be due to the
289 fact that we used stringent nuclease treatment with Turbo DNase which has 50× the activity
290 of wild type DNase and that the titre of the virus was high in the cell cultures compared to
291 clinical samples such as swabs. Other protocols also use spin filters to deplete eliminate host
292 cells but it is not clear from the literature whether this is a benefit or an added complication
293 that might bias the viruses that are detected [13]. Therefore we did not include a filtering step.

294 Interestingly the read lengths and alignment lengths were longer for the DNA virus
295 (BoHV1) than the two RNA viruses (BPI-3 and BRSV). This is possibly due to the efficiency
296 of the reverse transcriptase and the DNA polymerase in the double-stranded cDNA synthesis.
297 As such, there may be bias towards DNA viruses in this protocol which will have to be
298 allowed for if accurate quantification of the viruses is required. Direct RNA sequencing
299 (without prior cDNA synthesis) using nanopore technology was recently announced [14].
300 However, for truly universal virus detection by sequencing we require direct DNA and RNA
301 sequencing on the same flowcell which is not currently available.

302 The average error rate for 1D rapid sequencing libraries is currently 15% which
303 explains the low percentage alignments to viral sequences on the NCBI nr/nt database that we
304 observed. Oxford Nanopore Technologies claim this error rate will decrease to 5% for the
305 rapid 1D library preparation and will be 1% for the slower 2D library preps by the middle of
306 2017. Surprisingly, despite the current high 15% error rate and low alignment percentages,
307 our study showed that 99.6% of the MinION sequencing reads, for which the top BLAST hit
308 was a virus, were correctly identified to species level. Several different viral strains were
309 detected for BoHV1. (e.g. BoHV1 strain Cooper, BoHV1 subtype1 and BoHV subtype2).
310 BoHV1 was a field isolate so it is possible that several strains were present. However, with its
311 current error rate it is unlikely that MinION Mk1B 1D rapid sequencing can distinguish
312 between viral strains or subtypes but as error rates are constantly improving this will likely be
313 resolved in the near future.

314 We conclude that double-stranded cDNA synthesis, PCR-free tagmentation, nanopore
315 sequencing on a MinION Mk1B with a 9.4 flowcell, and high-throughput local BLAST
316 search can be used for rapid simultaneous species level identification of mixed RNA and
317 DNA BRD-associated viruses in high titre cell cultures.

318

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378 **Supporting information**

379 **S1 Table. Results of local BLAST search of MinION Mk1B sequence reads against the**
380 **NCBI nr/nt database.**

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