

COMBINATION OF RANDOM ISOTHERMAL AMPLIFICATION AND NANOPORE SEQUENCING FOR RAPID IDENTIFICATION OF THE CAUSATIVE AGENT OF AN OUTBREAK

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1 **Abstract**

2 **Introduction:** As an outbreak of fever of unknown origin usually starts with
3 nonspecific symptoms and a case definition is only slowly developed and
4 adapted, therefore, identifying the causative agent is crucial to ensure suitable
5 treatment and/or control measures. Polymerase Chain Reaction (PCR) as a gold
6 standard of the molecular diagnostics depends on the previous knowledge of the
7 pathogen genome sequences. Next generation Sequencing is an alternative method,
8 which can be applied to identify the pathogen responsible for the outbreak through
9 sequencing all nucleic acids present in a sample extract. Sequencing data obtained
10 can potentially identify new agents or new variants of known agents.

11 **Aim:** In this pilot study, we explored a sequencing protocol relying on multiple
12 displacement isothermal amplification and nanopore sequencing in order to allow the
13 identification of the causative agent in a sample. To develop the procedure for use in
14 a suitcase laboratory, a mock sample consisting of supernatant from a Zika virus
15 tissue culture was used.

16 **Results:** The whole procedure took around eight hours including sample preparation
17 and data analysis using BLAST search. In total, 63,678 sequence files covering
18 around 10,000 bases were extracted. BLAST search revealed the presence of Zika
19 virus, which was close to an isolate from Senegal.

20 **Conclusion:** In conclusion, the protocol has potential for point of need sequencing to
21 identify RNA viruses. The whole procedure was operated in a suitcase laboratory
22 powered by solar power batteries. However, the procedure is cooling chain
23 dependent and the cost per sequencing run is still very high. In addition, sequencing
24 and data analysis pipelines for optimized and rapid subtraction of background
25 information and assembly of relevant virus information are required.

26 **Introduction**

27 Identifying the causative agent implicated in an outbreak is crucial for selecting the
28 suitable treatment and/or control measures (1). For example, around 25 pathogens
29 can cause influenza like symptoms in the acute phase and up to 20 pathogens have
30 to be considered for diarrhoea.

31 For direct detection of pathogens, polymerase chain reaction (PCR) is a widely used
32 and well-established test for molecular diagnostics. Since specificity of PCR
33 oligonucleotides depends on known sequences of specific target genes, false
34 negative PCR result might be obtained due to a mismatching sequence of a novel
35 variant of a known pathogen or because of a new emerging infectious agent. An
36 alternative promising technology is next generation Sequencing (NGS), which can be
37 applied to identify the pathogen responsible for the outbreak through sequencing of
38 all nucleic acids in a sample allowing generic detection not limited by specific
39 oligonucleotide design. Additionally, NGS data sets on detected infectious agents
40 can be use for phylogenetic and molecular epidemiological analysis to provide
41 insights on strain and origin of the agent. This information can be crucial for
42 organization and distribution of resources during the outbreak control (2, 3).

43 There are many NGS technologies available such as sequencing by synthesis, using
44 HiSeq and MySeq devices (Illumina, USA). These devices have a high data output,
45 an error rate below 2% and the possibility to sequence several samples in parallel
46 (4). Nevertheless, there is a high logistic demand through weight, size and costs of
47 the equipment. Furthermore, cumbersome and long sample and library preparation
48 protocols are necessary in order to generate results (4, 5). In contrast, nanopore
49 sequencing technology (Oxford Nanopore Technology, UK) uses a pore-protein
50 embedded in a membrane to identify individual nucleotide by the unique change in

51 electrical conductivity as a DNA molecule passes through the nanopore protein.
52 Recently, Oxford Nanopore Technology developed a pocket sized (10.5 + 3.5 + 2.5
53 cm) sequencing device (MinION) which has the potential to be applied in the field or
54 rural areas. A flow cell containing the required nanopores is inserted into the MinION
55 in order to operate the sequencing run. The MinION device operates at a constant
56 sequencing temperature (34°C) and translates the measured changes in current to a
57 real-time nucleotide sequence *via* USB connection to a laptop (5-8).

58 Here we describe the establishment of a protocol for rapid identification of RNA
59 viruses combining. random isothermal amplification and nanopore sequencing using
60 Zika virus (ZIKV) as model virus. The protocol was performed in a mobile suitcase
61 laboratory (figure 1) in order to allow implementation in outbreak situation (9).

62

63 **Materials and Methods**

64 **Sample origin**

65 ZIKV strains were provided by WHO collaborating Center at the Institute Pasteur of
66 Dakar in Senegal. The monkey strain MR766 and the human strain HD78788 were
67 isolated in 1947 (in Uganda) and 1991 (in Senegal) in Africa, respectively, during
68 surveillance. Viral stocks were prepared by inoculating viral strains into *Aedes*
69 *pseudoscutellaris* clone 61 (AP61) monolayer. Cells were grown in cell culture flasks
70 (25 cm²) until they reached a confluence of approximately 80%. The medium was
71 discarded, and 150 µl virus solution was added to the cells. The flasks were gently
72 agitated every 15 min during incubation to enhance viral infection. After 1 h, 5 ml of
73 Leibovitz 15 (L-15) growth medium (GibcoBRL, Grand Island, NY, USA)
74 supplemented with 5% heat-inactivated fetal bovine serum (FBS) (GibcoBRL, Grand

75 Island, NY, USA), 10% Tryptose Phosphate 1% glutamine, 1% penicillin-
76 streptomycin, 0.05% amphotericin B [Fungizone] (Sigma, GmbH, Germany) was
77 added and the infected cells were incubated at 28°C without CO₂ until a cytopathic
78 effect was observable.. Viral infection was confirmed by an indirect
79 immunofluorescence assay (IFA) using specific hyper-immune mouse ascitic fluid, as
80 described previously (Digoutte *et al.*, 1992).

81 **Sample preparation**

82 Zika virus (ZIKV) RNA was extracted from cell culture supernatant using the QIAamp
83 Viral RNA Mini Kit (QIAGEN Hilden, Germany) following the manufacturer`s
84 instructions. The RNA quantity was measured by NanoDrop ND-1000 spectrometer
85 (Thermo Scientific, Waltham, MA, USA). For elimination of genomic DNA and reverse
86 transcription, the QuantiTect Reverse Transcription Kit (QIAGEN Hilden, Germany)
87 was employed using a prolonged incubation time (25 min) for the reverse
88 transcription step. Second strand cDNA Synthesis was performed with the NEBNext
89 mRNA Second Strand Synthesis Module (New England Biolabs, Ipswich, MA, USA).
90 The double-stranded cDNA (ds-cDNA) was purified with the 1.8X Agencourt AMPure
91 XP Beads Kit (Beckman Coulter, Brea, CA, USA), eluted in 55 µl nuclease-free water
92 and quantified (NanoDrop ND-1000). To fragment and increase the amount of DNA,
93 random amplification was done using the REPLI-g UltraFast Mini Kit (QIAGEN
94 Hilden, Germany), Briefly, 1 µl of ds-cDNA, containing at least 10 ng, was incubated
95 with 1 µl denaturation buffer at room temperature. To terminate the denaturation, 2 µl
96 neutralization buffer was added after 3 min. The denatured ds-cDNA was mixed with
97 16 µl of the master mix containing 15 µl REPLI-g UltraFast reaction buffer and 1 µl
98 REPLI-g UltraFast DNA polymerase and incubated at 30°C for 90 min. The reaction
99 mix was heated to 65°C for 3 min to inactivate the reaction enzymes. Then, the DNA

100 was purified with the 1.8X Agencourt AMPure XP Beads Kit, eluted in 30 µl nuclease
101 free water and quantified (NanoDrop ND-1000).

102 **Library preparation and sequencing**

103 For library preparation, the protocol for amplicon sequencing, SQK-NSK007, was
104 used as recommended by Oxford Nanopore Technology. Briefly, 45 µl containing at
105 least 1µg ds-cDNA were used for end-repairing and dA-tailing using the NEBNext
106 Ultra II end-repair / dA-tailing module. The end-prepped DNA was purified with the
107 1.8X Agencourt AMPure XP Beads Kit and eluted in 31 µl nuclease free water. DNA
108 recovery aim was at least 700 ng/µl. Adapter ligation and tethering was carried out
109 with the NEB Blunt/TA Ligase Master Mix. The DNA was purified using the
110 Dynabeads® MyOne™ Streptavidin C1 Kit (Thermo Fisher Scientific, Waltham, MA,
111 USA) and solved in 25 µl of Oxford Nanopores` Elution Buffer. Six microliter of the
112 adapted and tethered DNA was mixed with 31.5 µl nuclease free water and 37.5 µl of
113 Oxford Nanopores` Running Buffer FM1 and then loaded into the flow cells in the
114 MinION device.

115 **Data processing**

116 The MinION device generates data in fast5 format. These reads were processed with
117 the METRICHORE AGENT (Oxford Nanopore Technology, Oxford, UK). Afterwards,
118 the files were transformed to fastq format with PORETOOLS (10). Duplicate reads
119 were deleted and the remaining sequences were loaded in BLAST search using
120 GENEIOUS 9.1.6 (Biomatters Ltd., Auckland, New Zealand). Contigs were aligned to
121 Zika strain KF383115 *via* Map to Reference option in GENEIOUS.

122

123 **Results**

124 The described procedure took around eight hours as shown in table 1. In total, 63678
 125 sequences were extracted and transformed to fastq format. After uploading the
 126 sequences to BLAST, ZIKV sequences were identified in approximately 4% of the
 127 reads. The complete original ZIKV sequence (GenBank accession number:
 128 KF383115) was recovered with 2454 reads with an average read length of XXX
 129 (Max.: 585, Min.: 36, Std. Dev.: 122.6) (figure 2 and 3). The average coverage was x
 130 fold the minimum coverage was x-fold. Pairwise identity in BLAST analysis was
 131 67.4%.

132 Additionally a total of 411 correct ZIKV reads were found in the FAST5 fail sequence
 133 file. If included in the assembly they matched correctly to the respective ZIKV
 134 sequence (figure 3).

135

136 **Table 1: Sequencing Workflow**

Procedure	Reagents/Software	Time (min)
RNA extraction	QIAamp Viral RNA Mini Kit	30
DNA digestion and reverse transcription	QuantiTect Reverse Transcription Kit	35
second strand cDNA synthesis	NEBNext mRNA Second Strand Synthesis Module	90
random isothermal amplification	REPLI-g UltraFast Mini Kit	120
library preparation	Nanopore sequencing kits: SQK-NSK007	70
sequencing	MinION device and R9 flow cell	20
data analysis and BLAST search	PORETOOLS and Geneious 9.1.6	120
Total		485

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138

139 **Discussion**

140 Identifying the causative agent of an outbreak using sequencing instead of molecular
141 techniques like PCR could have a high impact on selecting and implementing the
142 right patient management and control measures.

143 The most widespread sequencing device is the MiSeq, as Illumina`s smallest device,
144 which has still a size of 68.6 + 52.3 + 56.5 cm and a weight of approximately 57 kg.
145 Moreover, it has a higher data output (15 Giga bases) in comparison to the MinION
146 (10 Giga bases). Nevertheless, read length by MiSeq is limited to around 300 bp and
147 a maximum of 22-25 million reads can be produced in a run time between 4h and
148 56h (11, 12). In contrast, the MinION has through its nanopore technology no limit in
149 read length and number. Moreover, reads are generated in 20-120 min and data are
150 easily accessible on laptop or PC.

151 We have discovered that 1/5 of the correct ZIKV reads was placed into the "fail" file.
152 The METRICHORE AGENT classifies the reads into pass and fail reads by neuronal
153 network computing assessing definite conductivity readout events at the pore exit for
154 5-6 mers. This complicated sequence definition needs quality scoring to decide on
155 the statistical trustworthiness of the sequencing result. Fails are defined through the
156 following approach. Initially base calling (1D base calling) of template and
157 complement reads is performed separately. If the resulting sequence length ratio is
158 between 0.5-2.0, all sequences are stacked together for base 2D base calling. If
159 resultant 2D sequences are assessed with a Q-score > 9 they are sorted into a
160 FAST5 fail sequence file (13).

161 Short Illumina device reads have a 0.1% non-random error rate, which means an
162 error at one site can still dominate the base calling process. The MinION reads have
163 a 10% error rate but sites are distributed at random throughout the sequence which

164 is compensated for by base calling and which therefore do not dominate at one site
165 reducing the overall error rate in comparison to Illumina reads (14). Our results
166 suggest that the analysis algorithm and the Q-score need to be optimised for viral
167 RNA sequences. At this current development stage therefore a recommended
168 assembly approach would be first to use all pass reads to identify the infectious
169 agent. To improve the result, the fail reads can be included in a 2nd step.

170 In general however the passed sequence assembly result already produce a robust
171 result with a average coverage of 40.

172 The MinION was successfully used in the Ebola virus outbreak in Guinea (7) and
173 during the Zika virus outbreak in Brazil (15). In both cases, specific PCR fragment
174 sequencing strategies were used. RT-PCR assays were applied to reverse transcribe
175 RNA and create multiple fragments to increase the sequencing efficacy (7, 16). This
176 strategy limits sequencing output to targeted agents, which is ideal for molecular
177 epidemiological analysis. The use of PCR leads to logistic issues due to heavy
178 devices and requirement of a cold chain for the reagents. In Brazil, this was solved
179 by transporting the whole laboratory in a caravan. The generic sequencing approach
180 described here is intended for diagnostic identification of unknown infectious agents.
181 It uses only random isothermal steps throughout the procedure and PCR cycling is
182 not required which avoids the use of a thermal cycler.

183 We have already shown that isothermal amplification can be easily implemented in a
184 mobile suitcase laboratory (9, 17, 18) and we successfully adapted this concept for
185 the workflow needed for library preparation for the MinION sequencing procedure
186 (figure 1). The suitcase, contains all materials and reagents needed for sequencing in
187 one box of 56.0 + 45.5 + 26.5 cm in size and less than 23 kg in weight.

188 All steps of data collection and analysis except the BLAST search were performed
189 offline using MINKNOW and METRICHORE AGENT as well as GENEIOUS. This is a
190 major improvement since during the Ebola outbreak base calling for MinION datasets
191 was only possible through cloud computing which needed internet capacity often not
192 available locally (7). The simple structure and clear layout of these analysis
193 programmes makes it easy for users without bioinformatic background to obtain
194 basic information about origin and phylogeny of the sequenced target. Therefore, a
195 bioinformatician is not necessarily needed for analysis of the datasets obtained. To
196 perform BLAST offline a database of infectious agent sequences only located on the
197 laptop needs to be assembled. It could be replenished with new entries to GenBank
198 whenever online.

199 Currently, the following challenges have to be solved. In our hands, the sequencing
200 reagents can be kept at 25°C for one day without any changes in their efficacy
201 (confirmed by Oxford Nanopore Technologies, UK). However for long-term storage a
202 -20°C freezer is still required. Moreover, the price per sequencing run is very high
203 (around \$1500), as one flow cell costs between \$500 and \$900 depending on the
204 amount of ordered flow cells. In addition, the shelf life of the flow cells is around 8
205 weeks at 4°C. One of the biggest drawbacks is that the manufacturer is progressively
206 changing the reagents and flow cells so that it is difficult to match biochemistry to
207 flow cells.

208 The goal of this pilot study was to establish a protocol for pathogen identification
209 during an outbreak field investigation. In principle this seems possible in a suitcase
210 laboratory setup. The next steps will be to assemble an offline solution to compare
211 identified sequences with preloaded database and to identify cold chain independent
212 reagents.

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