

1 **Identification of Bacterial Pathogens and Antimicrobial Resistance Directly**
2 **from Clinical Urines by Nanopore-Based Metagenomic Sequencing**

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19 **Running head:** MinION sequencing for pathogen profiling

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31 **Abstract**

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33 **Background.** The introduction of metagenomic sequencing to diagnostic microbiology has
34 been hampered by slowness, cost and complexity. We explored whether MinION nanopore
35 sequencing could accelerate diagnosis and resistance profiling, using complicated urinary
36 tract infections (UTIs) as an exemplar. **Methods.** Bacterial DNA was enriched from clinical
37 urines (n=10) and from healthy urines 'spiked' with multi-resistant *Escherichia coli* (n=5), then
38 sequenced by MinION. Sequences were analysed using external databases and bioinformatic
39 pipelines or, ultimately, using integrated real-time analysis applications. Results were
40 compared with Illumina data and resistance phenotypes. **Results.** MinION correctly identified
41 pathogens without culture and, among 55 acquired resistance genes detected in the
42 cultivated bacteria by Illumina sequencing, 51 were found by MinION sequencing directly
43 from the urines; with 3 of the 4 failures in an early run with low genome coverage. Resistance-
44 conferring mutations and allelic variants were not reliably identified. **Conclusions.** MinION
45 sequencing comprehensively identified pathogens and acquired resistance genes from urine
46 in a timeframe similar to PCR (4 h from sample to result). Bioinformatic pipeline optimisation
47 is needed to better detect resistances conferred by point mutations. Metagenomic-
48 sequencing-based diagnosis will enable clinicians to adjust antimicrobial therapy before the
49 second dose of a typical (i.e. q8h) antibiotic.

50

51 **Introduction**

52 The UK Prime Minister's O'Neill Commission, reviewing the threat of antibiotic resistance¹,
53 stresses the potential of rapid diagnostics to improve both treatment and antibiotic
54 stewardship. Reducing the time needed to obtain a microbiological diagnosis shortens the
55 duration of broad empirical therapy and its selective pressures.

56 PCR can detect pathogens and resistance genes in specimens without culture, but
57 cannot cover the diversity of organisms and resistance determinants potentially present.
58 Metagenomic sequencing could deliver this comprehensiveness²⁻⁷ but slow turnaround, cost
59 and complexity have impeded introduction into clinical microbiology.

60 Oxford Nanopore's MinION⁸ is the first technology potentially able to deliver
61 sequencing data from clinical samples in a timeframe allowing early de-escalation and
62 refinement of antimicrobial treatment. We examined its applicability to investigation of
63 urinary tract infection (UTIs). These account for over 8 million physician visits p.a. in the USA⁹.
64 Most are trivial but, in severe cases, infection may ascend to the kidneys, with overspill to the
65 bloodstream precipitating bacteraemia and urosepsis. Complicated UTIs are a growing cause
66 of hospitalization, mostly of elderly patients¹⁰, and 35,676 *Escherichia coli* bloodstream
67 infections were recorded in England in 2014-15¹¹, over 60% with a urinary origin. There is
68 growing resistance, particularly in severe and bacteraemic infections, to fluoroquinolones,
69 cephalosporins and lactamase-inhibitor combinations, driving use of previously-reserved
70 carbapenems, even as 'empirical' therapy. With carbapenemases now proliferating, and few
71 alternative therapies in reserve, escalating empiricism becomes increasingly untenable,
72 underscoring the desirability of moving to early targeted therapy, guided by diagnostics.

73

74 **Materials and Methods**

75 *Urines*

76 Ten heavily-infected ($>10^7$ cfu/mL) clinical urines (CUs 1-10) from patients at the Norfolk and
77 Norwich University Hospital (NNUH) were tested. Additionally, urine from a healthy volunteer
78 was spiked with 10^8 cfu/mL of multi-drug resistant *E. coli* strain H141480453, and with
79 cultivated *E. coli* from CU6. The genome sequence of *E. coli* H141480453 was determined
80 previously (Illumina HiSeq) at Public Health England (PHE).

81

82 *Ethics*

83 Ethical approval was not required for the study as testing was performed, for method
84 development purposes, on excess sample from routine clinical urines submitted to the NNUH
85 clinical microbiology laboratory and no patient information was collected.

86

87 *Sample preparation for MinION Nanopore sequencing*

88 Methodology was refined during the project. In its final iteration, urines (4-10 mL) were
89 centrifuged at 300 *g* for 2 min to deplete human cells. The supernatant was collected and re-
90 centrifuged at 12,300 *g* for 5 min, with the resulting bacterial pellet resuspended in 1 mL of
91 phosphate-buffered saline and processed with a MoLYsis Basic 5 Kit (MoLYsis Life Science,
92 Bremen, Germany) to lyse residual human cells and to remove their DNA. Bacterial Lysis
93 Buffer (Roche, Basel, Switzerland) and proteinase K (14-22 mg/mL) (Roche) were added and,
94 after incubation for 10 min at 65°C, DNA was purified using the MagNA Pure Compact Nucleic
95 Acid Isolation Kit (Roche) and DNA Bacteria v3_2 protocol. Variations, in early iterations,

96 were: (i) the initial centrifugation was omitted and no human DNA depletion performed
97 (CU1), and (ii) a NEBNext® Microbiome DNA Enrichment kit (New England BioLabs, Hitchin,
98 UK) was used to remove human DNA instead of MoLYsis (CUs 2-4).

99 To spike urines, 1 mL of overnight broth culture (10^9 cfu/mL) was added to 9 mL donor
100 urine, which was then processed as above, always using the final iteration of the method.

101 The quality and concentration of DNA was assessed using a Qubit® 2.0 Fluorometer
102 (Life Technologies, Paisley, UK), and 2200 TapeStation (Agilent Technologies, Santa Clara, CA);
103 concentrations >15 mg/L were considered acceptable.

104

105 *MinION library preparation and sequencing*

106 To generate a library with an average fragment size of c.8 kb, up to 2 µg of DNA was
107 fragmented by centrifugation at 7,200 rpm (3600 g) in a G-tube (Covaris, Brighton, UK), used
108 according to manufacturer's instructions, then end-repaired (New England BioLabs, Hitchin,
109 UK), cleaned with Agencourt AMPure XP Beads (Beckman Coulter, High Wycombe, UK), and
110 dA-tailed (New England BioLabs, Hitchin, UK). The product was re-cleaned, and eluted in 31
111 µL TrisHCl pH 8.5.

112 The library was then prepared according to the SQK-MAP-006 Genomic Sequencing
113 Kit protocol provided by Oxford Nanopore Technologies (ONT)¹². Variations, in earlier library
114 preparation experiments, were: (i) Kit SQK-MAP-002 was used for CUs 1-4¹³; (ii) Kit SQK-MAP-
115 003 was used with CUs 5-6 and for spiked urine run 1¹³; (iii) Kit SQK-MAP-004 was used¹⁴ for
116 CU7 and urine spiked with *E. coli* recovered from CU6, (iv) Kit SQK-MAP-005⁸ was used with
117 CUs 8-10 and Spiked Urine Run 2, (v) ONT's Rapid Sequencing Kit, with a-15 minute library

118 preparation procedure, was used, in accordance with the manufacturer's instructions, for
119 Spiked Urine Run 4.

120 MinION sequencing was performed using R7.3 flow cells, except for CUs 1-4, where
121 R7.0 cells were used. Sequencing was run for 7.5 - 48 h (see Results Table 1). ONT's MinKNOW
122 software (versions 0.45.2.6 - 2.34.3) was used to collect raw electronic signal data, which were
123 base-called using Metrichor™ software.

124 *BLAST and CARD identification of pathogens and resistance genes using MinION data*

125 Identification of species and resistance genes routinely utilised BLAST search and the CARD
126 (Comprehensive Antibiotic Resistance Database) database¹⁵. MinION data were extracted, in
127 fasta format, from raw HDF5 files using Poretools¹⁶. BLAST database aliases were built for
128 proteobacteria, firmicutes and human sequences. Top hits from each of these separate
129 database aliases were identified. Taxa were distinguished using the in-house script
130 blast_separate_taxa.pl, and taxonomy was assigned using blast_taxonomy_report.pl¹⁷ with
131 some modifications¹⁸.

132 Resistance genes were identified by aligning MinION reads to the CARD database using
133 LAST, with parameters optimised for low-accuracy long matches¹⁹⁻²¹. Some sequences in
134 CARD contain resistance-gene-flanking regions, leading to false positive results, therefore
135 putative matches were verified by visualisation in Artemis (Sanger)^{22, 23} and by examination
136 of the coordinates. Consensus sequences were built upon the CARD database reference
137 sequences using the MinION read alignments by Samtools 0.1.19, Samtools mpileup, bcftools,
138 vcfutils.pl and vcf2fq, ultimately generating indexed Bam files^{24, 25}. BLASTn (BLAST v 2.2.30+)
139 top hits were identified, using consensus sequences, against the CARD database, seeking
140 >80% identity over the length of a gene. In addition, reciprocal BLAST best-hits were identified

141 between the consensus sequences and the CARD database. The resulting output data were
142 parsed and sorted with a final report generated by a Python script¹⁸.

143

144 *WIMP and ARMA alignment for pathogen identification and resistance gene detection*

145 ONT's 'What's In My Pot?' (WIMP)²⁶ Metrichor application identifies the uropathogen in real-
146 time, using a reference database and Kraken 11; along with Metrichor's Antimicrobial
147 Resistance Mapping Application (ARMA)²⁷ for real-time detection of antibiotic resistance
148 genes. Both applications only became available toward the end of the study, and were used
149 for Spiked Urine Run 3 only.

150

151 *Illumina library preparation*

152 Two methods were used to sequence DNA from the bacteria cultivated from the urines. At
153 PHE's Genomic Services Unit, genomic DNA was prepared using a GeneJET Genomic DNA
154 Purification Kit (ThermoFisher, Cambridge, UK) and sequenced on a HiSeq instrument
155 (Illumina, Cambridge, UK) in Rapid Run mode²⁸. The library was prepared using the Nextera
156 XT DNA Sample Preparation kits (Illumina), following the manufacturer's protocol. At Brunel
157 University, bacterial DNA was quantified using the Quant-iT™ PicoGreen® dsDNA Kits (Life
158 Technology, Paisley, UK) and a FLUOstar OPTIMA plate scanner (BMG Labtech, Ortenberg,
159 Germany) according to manufacturers' specifications. DNA (300 ng) was fragmented using an
160 Episonic system (Epigentek, New York, USA). Libraries were constructed using the NEBNext
161 Ultra DNA Sample Prep Master Mix Kit (NEB) using an automated protocol on a Biomek FX
162 instrument (Beckman Coulter, High Wycombe, UK). Ligation was performed with Illumina
163 Adapters (Multiplexing Sample Preparation Oligonucleotide Kit) and ligated libraries were

164 size-selected using Agencourt AMPure XP Beads (Beckman Coulter). Samples were sequenced
165 on the 150-base paired-end Illumina HiSeq 2000 platform.

166

167 *CARD alignment for resistance gene detection from cultivated bacteria*

168 The presence of resistance genes in Illumina sequence reads was determined with
169 'Genefinder', an in-house PHE algorithm that uses bowtie2²⁹ to map the reads to a local
170 database of antimicrobial resistance genes, and Samtools 0.1.18^{24, 25} to generate an mpileup
171 file. The script then parses the mpileup file to match to reference sequences, based on read
172 coverage and > 90% nucleotide identity over full length of sequence (the lower threshold
173 adopted for MinION was because of higher expected error rates).

174

175 *Phenotypic characterisation of uropathogens*

176 Bacteria were grown by standard methodology³⁰ and identified by MALDI-TOF mass
177 spectroscopy (Bruker, Bremen, Germany). Minimum inhibitory concentrations (MICs) were
178 determined at PHE by British Society for Antimicrobial Chemotherapy agar dilution, with
179 results categorised on EUCAST criteria³¹.

180

181 **Results**

182 *MinION results and performance improvement*

183 Fifteen MinION runs were performed: 10 with CUs, four using urine spiked with
184 *E. coli* H141480453 and one spiked with *E. coli* from CU6 (Table 1). Early attempts failed
185 because human DNA was insufficiently depleted (CU1), flow cells were poor quality (CU2 and

186 CU4) or DNA was degraded (CU3). Improved sample and library preparation, along with R7.3
187 flow cells, resolved these issues (Fig. 1). From CU5 onwards, MinION produced 6536-34330
188 2-D reads/run, with 2518-22405 "pass 2-D reads," and a mean read-length of 3452-6076 bp.
189 The longest single read was 46213 bp, and single-read identity to reference sequences
190 improved from 70% to 85%. Successive runs for urine spiked with *E. coli* H141480453 illustrate
191 the gains (Fig 2); sequence yield and depth improved from Run 1 to 2; WIMP/ARMA software
192 reduced processing to 7.5h in Run 3; this fell to 4h in Run 4 using the Rapid Library Preparation
193 Kit, despite having to revert to BLAST/CARD analysis since WIMP/ARMA could not analyse the
194 kits 1-D read data.

195

196 *Bacterial identification*

197 Analysis using BLAST and the CARD database was performed on 2-D "Pass" reads for CUs 5- 7
198 and for all spiked urines (except Run 3, using WIMP/AMRA). For CUs 8-10 we combined 2-D
199 reads from "Pass" and "Fail" folders. In all cases, MinION correctly identified the pathogen
200 (Table 2); WIMP achieved this within 15 min. Human DNA accounted for only 1.6-12.3% of
201 reads, confirming that depletion was effective. Breadth of coverage was from 82.6-100%;
202 depth was least for CU5 (2.71x) and greatest - 21.55-22.84x - for spiked urine Run 2 and CU8
203 (Table 2).

204

205 *Resistance gene profiles*

206 Acquired resistance genes were readily identified in MinION outputs, as illustrated in Tables
207 3 (Clinical Urines) and 4 (Spiked Urines). Among 55 acquired resistance genes detected by
208 Illumina sequencing of the cultivated bacteria, 51 were found by MinION directly from urines;

209 3 of 4 exceptions were with CU5, where coverage was poorest. Limitations were: (i) MinION
210 often flagged multiple gene variants whereas Illumina definitively identified alleles, (ii)
211 resistance-conferring mutations were not detected and (iii) plasmid and chromosomal *ampC*
212 were not discriminated and nor could the mode of *ampC* expression be inferred.

213

214 *Clinical Urine 5 (K. pneumoniae)*

215 MinION and Illumina detected *bla*_{CTX-M-15} corresponding to the isolate's ESBL phenotype. Both
216 also found *bla*_{OXA-1}, congruent with amoxicillin-clavulanate resistance. Also in agreement,
217 both found *dfrA14*, explaining trimethoprim resistance. Illumina identified *bla*_{LEN-12}, and
218 *bla*_{SHV-27}, whereas MinION indicated *bla*_{SHV-32}; these probably all correspond to the
219 chromosomal *bla*_{LEN}/*bla*_{SHV} of *K. pneumoniae*. Illumina also detected *bla*_{TEM-1}, which should
220 not expand resistance in the presence of CTX-M-15. The strain was resistant to gentamicin
221 and tobramycin, according with detection, by both MinION and Illumina, of *aacC2*; both
222 methods also found *aac6-1b-cr*, encoding a tobramycin- and amikacin-modifying enzyme. The
223 low amikacin MIC (2 mg/L) does not conflict with this: EUCAST advocates reporting all isolates
224 with AAC(6')-1b as amikacin non-susceptible irrespective of MIC. Streptomycin resistance
225 agreed with the presence of *strA* (detected by both methods) and *strB* (found only by
226 Illumina). Both approaches found *qnrB* and *aac(6')-1b-cr*, according with low-level
227 ciprofloxacin resistance (MIC, 2 mg/L).

228

229 *Clinical Urine 6 (E. coli)*

230 MinION and Illumina both found *bla*_{CTX-M-15} and *bla*_{OXA-1}, again congruent with an ESBL
231 phenotype and amoxicillin-clavulanate resistance. Both also indicated *bla*_{TEM-1}. MinION

232 flagged several acquired *ampC* genes whilst Illumina indicated *bla*_{CMY-113} and *bla*_{MIR-14}, albeit
233 below the 90% threshold. It is likely that all these *ampC* calls really corresponded to *E. coli*
234 chromosomal *ampC*, as the ceftazidime MIC for the isolate was only 8 mg/L, whereas ceftazidime
235 MICs for *E. coli* with acquired plasmid AmpC enzymes are mostly >64 mg/L (PHE data on file).
236 MinION and Illumina both found *aacC2* and *aac(6′)-1b-cr*, agreeing with gentamicin and
237 tobramycin resistance and a raised amikacin MIC. Both detected *aadA5*, but the organism
238 was susceptible to streptomycin and this gene may not be expressed. Detection of *dfrA17* by
239 MinION and Illumina agreed with trimethoprim resistance. Double mutations in *gyrA* and
240 *parC*, explaining high-level ciprofloxacin resistance, were detected by Illumina, not MinION.

241

242 *Clinical Urine 7 (E. coli)*

243 MinION and Illumina again detected *bla*_{CTX-M-15}, agreeing with an ESBL phenotype; *bla*_{OXA} was
244 absent and the isolate was more susceptible than those from CUs 5 and 6 to penicillin-
245 inhibitor combinations. Both methods found *bla*_{TEM-1}. Phenotypic resistance to streptomycin
246 agreed with detection, by both methods, of *aadA1/aadA3* and *strA/strB*; resistance to
247 trimethoprim agreed with detection of *dfrA1* by both techniques. An *ampC* gene (*bla*_{ACT-24})
248 was flagged by MinION, not Illumina. As with CU5, however, a low ceftazidime MIC (4 mg/L)
249 contraindicated plasmid *ampC*, and the result probably reflected miscalling chromosomal
250 *ampC*. The ciprofloxacin MIC (0.25 mg/L) was slightly raised, and a single mutation in *gyrA*
251 was detected by Illumina only.

252

253 *Clinical Urines 8 (E. coli) and 9 (E. cloacae)*

254 The *E. coli* from CU8 was resistant to ampicillin, amoxicillin-clavulanate and ceftazidime (MICs,
255 16-64 mg/L), with diminished susceptibility to cefotaxime (MIC 1 mg/L). Cefotaxime-
256 cloxacillin synergy implied AmpC, as did the raised ceftazidime MIC (>64 mg/L). MinION flagged
257 several acquired *ampC* genes but these were not confirmed by Illumina sequencing, meaning
258 that upregulation of chromosomal *ampC* is the likeliest explanation. CU9 contained *E. cloacae*
259 with a cefotaxime MIC of 2 mg/L, reduced to 0.125 mg/L by cloxacillin, implying partial
260 derepression of *ampC*, the commonest mode of oxyimino-cephalosporin resistance in this
261 species. MinION flagged multiple acquired *ampC* genes and Illumina flagged *bla_{ACT-24}*, all
262 probably reflecting mis-calling of chromosomal Enterobacter *ampC*. No other acquired genes
263 were found in either CU8 or 9 in the isolates, agreeing with their general susceptibility.

264

265 *Clinical Urine 10 (K. pneumoniae)*

266 MinION and Illumina detected *bla_{CTX-M-15}* and *bla_{OXA-1}*, agreeing with an ESBL phenotype and
267 amoxicillin-clavulanate resistance. Both also found *bla_{TEM}*. MinION additionally flagged
268 multiple *bla_{SHV/LEN}* variants whilst Illumina indicated *bla_{SHV-28}*. High gentamicin, tobramycin
269 and amikacin MICs (8-32 mg/L) accorded with detection of *aacC2* and *aac(6')-1b-cr* by both
270 methods, with *aacA4* additionally flagged by MinION. Resistance to streptomycin agreed with
271 detection of *strA* and *strB* by both methods and *aadA3* by MinION only. Trimethoprim
272 resistance accorded with detection of *dfrA14* by both techniques. *qnrB* and *aac(6')-1b-cr* were
273 found by both methods, but high-level ciprofloxacin resistance (>8 mg/L) more likely reflected
274 *gyrA* and *parC* mutations, found only by Illumina.

275

276 *Spiked urines*

277 *E.coli* H141480453 had NDM and OXA-181 carbapenemases and was susceptible only to
278 colistin and tigecycline. Synergy arose between EDTA and imipenem, reflecting metallo- β -
279 lactamase inhibition, but not between cephalosporins and clavulanate or cloxacillin. The four
280 sets of MinION data, directly from urine, closely matched Illumina sequencing. Thus, *bla*_{TEM},
281 *bla*_{CTX-M-group-1}, *bla*_{OXA-48/181}, *bla*_{NDM} and *bla*_{CMY} β -lactamase were consistently identified, though
282 with MinION flagging multiple matches within families whereas Illumina identified single
283 alleles. Among aminoglycoside determinants, *rmtB* was consistently found by both methods,
284 as were *aacC2*, *aac(6')-1b-cr* and *strA/B*; Illumina found *aadA2*, *aadA3* and *aadA5* as did
285 MinION run 3; MinION runs 1 and 2 flagged only one or two of these (*aadA2* and *aadA3* are
286 closely related; *aadA5* differs considerably). *rmtB* alone would confer pan-resistance to
287 aminoglycosides, as observed. Trimethoprim resistance accorded with *dfrA-12* and *dfrA-17*,
288 found by Illumina and 3 of 4 MinION runs. *aac(6')-1b-cr* and *qnrS* variants were consistently
289 flagged by MinION and Illumina but mutations in chromosomal *gyrA* and *parC* –reliably
290 detected by Illumina only- are more likely to explain observed high-level fluoroquinolone
291 resistance. The organism was sulphonamide resistant, and Illumina detected *sul1*, while all
292 MinION runs found both *sul1* and *sul2*; tetracycline resistance agreed with detection of *tet(A)*
293 by Illumina and in 3 of 4 MinION runs. *catB3*, congruent with observed chloramphenicol
294 resistance, was consistently flagged by MinION; Illumina detected a related gene, but with
295 only 69% identity to *catB3*, and a novel variant may be present.

296 One hour of MinION sequencing delivered 0.2x, 3.75x, and 6.96x coverage depth for
297 spiked Runs 1, 2 and 3, respectively (Fig. 3). To assess whether this detected resistance genes
298 adequately, we reanalysed 1h reads from Runs 1-3 on ARMA software, and those of Run 4
299 with BLAST/CARD (its 1-D reads were unsuitable for ARMA). All the acquired resistance genes
300 identified in runs 2, 3 and 4 were recognisable in the 1h data, except for *bla*_{CMY} in run 2 and

301 *strB* in run 4. An *ampC* gene (*bla_{LAT-1}*), was additionally identified in run 4. Lower coverage in
302 Run 1 precluded 1-h detection of several genes.

303 MinION sequencing of healthy urine spiked with *E. coli* from CU6 detected the same
304 acquired genes as: (i) Illumina sequencing of the isolate, and (ii) MinION sequencing direct
305 from CU6, confirming that any bacteria and resistance genes in the urinary tract of the healthy
306 urine-donor did not distort results.

307

308 **Discussion**

309 Rapid pathogen profiling from clinical specimens, without culture, could facilitate better
310 treatment and antibiotic stewardship. PCR diagnostics are under trial for this purpose but can
311 only seek limited numbers of targets. Sequencing could deliver a more comprehensive
312 picture, and we investigated if this was achievable with the MinION. Urine was taken as an
313 exemplar, with a heavy load of infecting bacteria, thereby: (i) yielding sufficient DNA for
314 MinION sequencing, (ii) minimising the confounding effects of commensal bacteria and
315 laboratory/reagent contamination on results and (iii) ensuring a high bacterial cell : human
316 cell ratio. Hasman *et al*⁷ previously applied Ion Torrent sequencing to urine, finding identical
317 resistance genes as in the cultivated pathogens but, with a 24-h turnaround, their method
318 only modestly accelerated conventional workflows.

319 MinION can identify microorganisms^{32, 33} and MinION sequences can predict
320 resistances in cultivated bacteria^{34, 35}. Advantages over other sequencing platforms are: (i)
321 rapid turnaround, (ii) low capital cost and (iii) small size. The technology remained under
322 active development whilst the present studies were undertaken. The manufacturer's
323 improvements, together with refinements in our sample preparation, delivered the stepwise

324 gains illustrated in Fig. 1. Initial experiments, without human cell depletion (CU1), led to a
325 large proportion of human reads, and correspondingly low bacterial sequence yield. We
326 therefore sought to enrich bacterial DNA, initially by NEBNext® Microbiome DNA Enrichment
327 (CUs 2-4), which proved unsatisfactory. From CU5, we combined differential centrifugation,
328 removing most human cells, with MoLYsis technology to lyse residual human cells and remove
329 their DNA. This allowed us to identify pathogens, and the same families of acquired resistance
330 genes as found in pure cultures by Illumina, with good agreement to resistance phenotypes.

331 Most sequence analysis was post-run, using BLAST search and CARD database.
332 However the Metrichor WIMP and ARMA software, adopted late in these studies, allowed
333 real-time analysis. With this approach, adding together times for analysis (1h), sequencing
334 (1h), library preparation (3h), DNA extraction and sample transport (2.5h) suggests a total 7-
335 8h turnaround, equating to one dosage interval for a 'typical' q8h antibiotic. Further
336 acceleration is feasible using the 15-min library preparation kit (as with spiked urine Run 4)
337 reducing turnaround to c. 4h (Fig. 3). This is similar to PCR methodology, and would inform
338 much earlier de-escalation and refinement of therapy than now. During the WIMP/ARMA-
339 based analysis c. 32 MB of 2-D sequencing data were generated, with almost 7x depth of
340 coverage after 1h (57 MB of 2-D data with 11.37x depth were available after 2h). Based on
341 Lander and Waterman's³⁶ equation, we calculate that 7x depth covers 99.905% of the *E. coli*
342 genome (4.6 MB), leaving little risk of missing an acquired resistance gene. Moreover, MinION
343 sequencing error rates are diminishing rapidly (>90% identity with recent R9 pore
344 chemistry)³⁷.

345 Although the approach has great potential, challenges remain:

346 Firstly, we used heavily-infected urines ($>10^7$ cfu/mL) to deliver the c.1 μ g of DNA
347 required for sequencing, whereas significant bacteriuria is defined as $>10^5$ cfu/mL. Low-input
348 procedures have been described and should address this issue³⁸; these reduce the DNA
349 requirement for nanopore sequencing to 20 ng.

350 Secondly, we tested one urine sample per flow cell. While this offers flexibility, it is
351 expensive, with cells costing US\$500-900 each. This is balanced: (i) if a day's hospitalisation
352 is saved, or (ii) if expensive antibiotics can be avoided. Alternatively, Oxford Nanopore have
353 introduced a PCR-free barcoding kit, allowing multiplexing of 12 samples; this would reduce
354 the cost per sample but would necessitate batching, extending turnaround.

355 Thirdly, allelic variants were poorly distinguished. In particular, the MinION-based
356 pipeline (i) failed to detect mutations associated with fluoroquinolone resistance or *ampC* up-
357 regulation (likely to have been present, e.g. in the *E. coli* from CU8 and the *E. cloacae* from
358 CU9); (ii) flagged multiple alleles (e.g. of *bla*_{TEM}, *bla*_{SHV}, *bla*_{CTX-M}, *bla*_{NDM}) whereas Illumina
359 indicated single types, and (iii) confused acquired (plasmid-mediated) and endogenous
360 chromosomal *ampC* and *bla*_{SHV/LEN}. Distinguishing *bla*_{NDM} or *bla*_{CTX-M} variants is unimportant,
361 as all alleles have similar resistance implications; however, SNPs determine hydrolytic
362 spectrum and inhibitor vulnerability within the TEM, SHV and GES families, thereby
363 determining whether a therapy is appropriate or not. In the case of AmpC, plasmid types are
364 copiously expressed and have clear resistance association, whereas the implications of
365 chromosomal types depends on their level of expression, which is determined by the
366 promoter sequence in *E. coli* or by mutation of regulatory genes (principally *ampD*) in species
367 with inducible *ampC* expression, e.g. *E. cloacae*. To further complicate matters, the plasmid-
368 mediated types, which occur across species, are chromosomal escapes from other species –

369 CMY-2, the commonest, is from *Citrobacter freundii* and DHA-1 from *Morganella morganii*.
370 There are potential ways to address the challenges of distinguishing closely-related variants
371 and predicting AmpC expression. SNPs and sequence variants can be called using MinION
372 data,³⁹ though this slow. In future, reads aligning to CARD could be isolated and polished to
373 improve consensus accuracy, facilitating precise identification. What is more, long MinION
374 reads can give context to the position of resistance genes, potentially enabling differentiation
375 between plasmid-borne and chromosomal *ampC* genes. Optimally, MinION reads will enable
376 the assembly of complete plasmids and in some cases, single reads will cover the full length
377 of a plasmid.

378 Fourthly, a gene may be present but fail to cause resistance, owing to poor expression,
379 silencing or inactivation. MinION and Illumina found *aadA5* in CU6 but the *E. coli* isolate was
380 streptomycin susceptible. Tyson *et al.*⁴⁰ previously noted poorer genotype-phenotype
381 concordance for streptomycin than for other resistances (81.3% versus 100%).

382 Lastly, optimising the cut-off to only call 'true positive' results for resistance genes is
383 challenging. We used 90% identity for Illumina and (owing to lower base-calling accuracy)
384 80% identity for MinION. This lower cut-off probably explains the larger number of
385 misidentifications of plasmid *ampC* by MinION and the calling of *catB3* in *E. coli* H141480453
386 by MinION but not Illumina. A technical aspect, independent of MinION, was occasional
387 misdetection of resistance genes due to inclusion of flanking regions of integrons in CARD
388 (not shown). This might be resolved by adjusting cut-offs, but is better addressed by stricter
389 database curation.

390 All the clinical urine samples tested in this study were infected with single pathogens.
391 Polymicrobial UTIs were not sought, however MinION data can identify and differentiate

392 multiple species in metagenomic samples⁴¹. Multiple strains of the same species would be
393 harder to distinguish, but all their resistance genes would be represented in the sequence,
394 whereas conventional culture would be liable to randomly select and test one of the strains
395 present.

396 Given the improvements achieved already we believe that the technology can be
397 enhanced to overcome these challenges. If so, MinION profiling from urosepsis patients could
398 allow beneficial refinement of antibiotic regimens within the first dosage interval after clinical
399 diagnosis.

400

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406 DML. Drafting of manuscript: KS, DML, JOG, LCC, MD, AMK. Critical revision: DML, JOG, JW,
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408

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502

503

Table 1. Clinical and spiked urines subjected to MinION sequencing in chronological order

Sample and date	Flow cell chemistry	Sequencing time (h)	Total number of reads	Mean readlength (bp)	Number of 2-D reads	Number of 2-D 'pass' reads	Mean readlength of 2-D 'pass' (bp)	Total number of 2-D 'fail' reads	Mean readlength of 2-D 'fail' (bp)
CU1 09-07-2014	R7.0	24	12295	3647	1645	0	0	0	0
CU2 12-07-2014	R7.0	24	8299	2859	621	0	0	0	0
CU3 04-09-2014	R7.0				No results				
CU4 09-09-2014	R7.0	21	3829	1728	184	0	0	0	0
Urine spiked with E. coli H141480453 run 1; 06-11-2014	R7.3	30	45652	2827	15216	10109(66%)	4103	5107	3880
CU5 16-01-2015	R7.3	25.5	22968	3292	8191	2518(26.5%)	3980	5673	3491
CU6 24-01-2015	R7.3	23	57289	4700	15932	12183(48%)	5510	3749	4848
CU7 05-02-2015	R7.3	17.5	76499	4473	17050	10137(18.8%)	5414	9776	4447
Urine spiked with E. coli from CU6 09-03-2015	R7.3	14	56394	5419	13206	7678(27.9%)	6076	5528	5421
CU8 02-03-2015	R7.3	33	86294	4664	20799	13798(36%)	5324	7001	4221
CU9 30-03-2015	R7.3	26	28 767	4 926	6536	4376(29%)	5741	2160	4572
CU10 16-05-2015	R7.3	35	141 511	3 107	34330	15074(23%)	3452	19256	2908
Urine spiked with E. coli H141480453 run 2; 04-05-2015	R7.3	48	138 720	4 424	33589	17123(27.7%)	5013	16466	4040
Urine spiked with E. coli H141480453 run 3; 23-10-2015	R7.3	7.5	97961	4308	28787	22405(77%)	4416	6382	2467
Urine spiked with E. coli	R7.3	29	21441	2043	-	-	-	-	-

Table 2. Pathogen identification using MinION sequencing for 6 clinical and spiked urines

	Clinical Urine 5	Clinical Urine 6	Urine spiked with <i>E. coli</i> from CU6	Clinical Urine 7	Clinical Urine 8	Clinical Urine 9	Clinical Urine 10	Urine spiked with <i>E. coli</i> H141480453 Run 1	Urine spiked with <i>E. coli</i> H141480453 Run 2	Urine spiked with <i>E. coli</i> H141480453 Run 3
Reads used	2-D pass only	2-D pass only	2-D pass only	2-D pass only	2-D pass and fail	2-D pass and fail	2-D pass and fail	2-D pass only	2-D pass only	2-D pass only
% non-human DNA reads matching Gram-negative bacteria	76%	84%	83%	84%	81%	95%	85%	98%	89%	-
% DNA reads matching human	6.6%	8.5%	8.5%	8.1%	12.3%	1.7%	9.7%	1.6%	4.2%	-
Best species match to MinION sequence data	<i>K. pneumoniae</i> CG43	<i>E. coli</i> JJ1886	<i>E. coli</i> JJ1886	<i>E. coli</i> PMV-1	<i>E. coli</i> 536	<i>E. cloacae</i> NCTC 9394	<i>K. pneumoniae</i> CG43	<i>E. coli</i> APEC O78	<i>E. coli</i> APEC O78	<i>E. coli</i> APEC O78
Best species match to Illumina sequence data	<i>K. pneumoniae</i> MGH 78578	<i>E. coli</i> JJ1886	<i>E. coli</i> JJ1886	<i>E. coli</i> IHE3034	<i>E. coli</i> 536	<i>E. cloacae</i> NCTC 9394	<i>K. pneumoniae</i>	<i>E. coli</i> ST410	<i>E. coli</i> ST410	<i>E. coli</i> ST410
% Breadth of coverage to best match organism	82.57%	99.59%	100%	92.19%	99.9%	86.25%	96.70%	95.13%	96.13%	-
Average depth of coverage versus best match organism	2.71 x	15.65 x	10.58 x	10.77 x	22.84 x	9.16 x	17.61 x	7.25 x	21.55 x	21.51 x
Run time (h)	25.5	23	14	17.5	36	26	35	30	48	7.5

Table 3. Genes found by MinION sequencing for 6 clinical urines compared with antibiotic MICs and Illumina sequencing for cultured isolates

Urine and species	Method ^a	Penicillins and inhibitor combinations			Cephalosporins, monobactams and inhibitor combinations								Fluoro-quinolone	Aminoglycosides				Antifolate	
		Amp	Aug	Ptz	Ctx	Ctx-clox	Ctx-clav	Caz	Caz-clav	Cpm	Cpm-clav	Fox	Azt	Cip	Amk	Tob	Gen	Str	Tmp
CU5 <i>K. pneumoniae</i>	MICs	>64	16	8	128	64	≤0.06	16	0.25	8	≤0.06	4	16	2	2	16	32	R	R
	MinION	<i>bla_{OXA-1}</i>			<i>bla_{CTX-M-15}</i> , <i>bla_{SHV-32}</i>								<i>qnrB</i> , <i>aac(6')-lb-cr</i>	<i>aac(6')-lb-cr</i> , <i>aacC2</i> , <i>strA</i>				<i>dfrA14</i>	
	Illumina	<i>bla_{TEM-1}</i> , <i>bla_{OXA-1}</i>			<i>bla_{CTX-M-15}</i> , <i>bla_{SHV-27}</i> , <i>bla_{LEN-12}</i>								<i>qnrB</i> , <i>aac(6')-lb-cr</i>	<i>aac(6')-lb-cr</i> , <i>aacC2</i> , <i>strA</i> , <i>strB</i>				<i>dfrA14</i>	
CU6 <i>E. coli</i>	MICs	>64	16	4	128	32	≤0.06	16	0.25	8	≤0.06	8	32	>8	4	16	16	S	R
	MinION	<i>bla_{TEM(mv*)}</i> , <i>bla_{OXA-1}</i>			<i>bla_{CTX-M gp1 (15)}</i> , <i>ampC</i> (<i>bla_{CMY mv*}</i> , <i>bla_{ACC-4}</i> , <i>bla_{MIR-9}</i> , <i>bla_{DHA-22}</i>)								<i>aac(6')-lb-cr</i>	<i>aac(6')-lb-cr</i> , <i>aacC2</i> , <i>aadA5</i>				<i>dfrA17</i>	
	Illumina	<i>bla_{TEM-1}</i> , <i>bla_{OXA-1}</i>			<i>bla_{CTX-M-15}</i>								<i>aac(6')-lb-cr</i> ; <i>gyrA</i> (83:SL;87:D-N); <i>parC</i> (80:S-I; 84:E-V)	<i>aac(6')-lb-cr</i> , <i>aacC2</i> , <i>aadA5</i>				<i>dfrA17</i>	
CU7 <i>E. coli</i>	MICs	>64	8	2	128	32	≤0.06	8	0.12	4	≤0.06	4	16	0.25	2	1	0.5	R	R
	MinION	<i>bla_{TEM(mv*)}</i>			<i>bla_{CTX-M gr1}</i> , <i>ampC</i> (<i>bla_{ACT-24}</i>)								<i>aadA1</i> , <i>aadA3</i> , <i>strA</i> , <i>strB</i>				<i>dfrA1</i>		
	Illumina	<i>bla_{TEM-1}</i>			<i>bla_{CTX-M-15}</i>								<i>gyrA</i> (83:S-L)	<i>aadA1</i> , <i>strA</i> , <i>strB</i>				<i>dfrA1</i>	
CU8 <i>E. coli</i>	MICs	64	32	4	1	≤0.12	0.25	0.5	0.5	≤0.12	0.12	>64	0.25	≤0.12	1	0.5	0.5	S	S
	MinION Illumina	<i>ampC</i> (<i>bla_{CMY mv*}</i> , <i>bla_{ACC-4}</i> , <i>bla_{MIR-4}</i> , <i>bla_{DHA-6}</i> , <i>bla_{FOX4}</i>)																	
CU9 <i>E. cloacae</i>	MICs	>64	64	4	2	≤0.12	2	1	1	≤0.12	0.12	>64	0.25	≤0.12	1	0.5	0.5	S	S
	MinION Illumina	<i>ampC</i> (<i>bla_{CMY mv*}</i> , <i>bla_{ACT-18, 24}</i>) <i>ampC</i> (<i>bla_{ACT-24}</i>)																	
CU10 <i>K. pneumoniae</i>	MICs	>64	32	>64	>256	256	0.125	128	1	64	≤0.06	16	>64	>8	8	>32	>32	R	R
	MinION	<i>bla_{TEM(mv*)}</i> , <i>bla_{OXA-1}</i>			<i>bla_{CTX-M gr1}</i> , <i>bla_{SHV(mv*)}</i> ,								<i>aac(6')-lb-cr</i> , <i>qnrB</i>	<i>aac(6')-lb-cr</i> , <i>aacA4</i> , <i>aacC2</i> , <i>aadA3</i> , <i>strA</i> , <i>strB</i>				<i>dfrA14</i>	
	Illumina	<i>bla_{TEM-1}</i> , <i>bla_{OXA-1}</i>			<i>bla_{CTX-M-15}</i> , <i>bla_{SHV-28}</i> , <i>bla_{LEN-12}</i>								<i>gyrA</i> (83:S-I), <i>parC</i> (80:S-I), <i>aac(6')-lb-cr</i> , <i>qnrB</i>	<i>aac(6')-lb-cr</i> , <i>aacC2</i> , <i>strA</i> , <i>strB</i>				<i>dfrA14</i>	

Legend: AMP, ampicillin; AUG, amoxicillin-clavulanic acid; AZT, aztreonam; PTZ, piperacillin-tazobactam; CTX, cefotaxime; CTX-Clav, cefotaxime-clavulanic acid, CAZ, ceftazidime; CAZ-Clav, ceftazidime-clavulanic acid; CPM, cefepime; CPM-Clav, cefepime-clavulanic acid; FOX, ceftazidime; CIP, ciprofloxacin; AMK, amikacin, TOB- tobramycin; GEN, gentamicin; STREP, streptomycin; TRIM, trimethoprim. All β -lactamase inhibitors were used at 4 mg/L. **(R)**: resistant; **(I)**: intermediate; **(S)**: susceptible based on EUCAST criteria; **(A)**: acquired genes found only by Illumina; **(M)**: acquired gene families detected only by MinION; ***mv**: multiple (>5) different gene variants of this family flagged.

^a MICs are expressed as mg/L; MinION results are for the urine, tested directly; Illumina results are for the cultivated bacteria. Only relevant genes are listed.

^b *gyrA* and *parC* were found in all clinical samples by both sequencing methods. They are only detailed when mutations were detected.

Table 4. Acquired resistance genes identified during four MinION runs for urine spiked with *E. coli* H141480453, compared with Illumina sequencing of the cultivated organism

Genes	Illumina	MinION run 1 (run time= 30 h)	MinION run 2 (run time= 48 h)	MinION run 3 ARMA (run time= 1 h)	MinION run 4 (run time= 1 h)
β-Lactamase genes					
<i>bla</i> _{TEM}	1 ^a	1, mv*	1, mv*	1, mv*	1, mv*
<i>bla</i> _{CTX-M}	group-1 (15)	group-1 (1, 3, 15, 52, 114)	group-1 (15, mv*)	mv* not including <i>bla</i> _{CTX-M-15}	mv* not including <i>bla</i> _{CTX-M-15}
<i>bla</i> _{OXA}	1, 181	31 (=1,30), 181	2, 7, 30, 232 (=181)	1, 181, mv*	181, mv* not including <i>bla</i> _{OXA-1}
<i>bla</i> _{NDM}	4	4, 6, 7	4, 5, 7, 12, 13	1	mv*
<i>bla</i> _{CMY}	2	34, 45, 111	mv* not including <i>bla</i> _{CMY-2}	mv* not including <i>bla</i> _{CMY-2}	mv* not including <i>bla</i> _{CMY-2}
others	-	-	-	-	<i>bla</i> _{LAT-1}
Aminoglycoside resistance genes					
<i>aacC</i>	<i>aacC2</i>	<i>aacC2</i>	<i>aacC2</i>	<i>aacC2</i>	<i>aacC2</i> , <i>aacC8</i>
<i>aadA2, aadA, aadA5</i>	<i>aadA2, aadA3, aadA5</i>	<i>aadA2, aadA3</i>	<i>aadA5</i>	<i>aadA2, aadA3, aadA5, mv*</i>	mv* not including <i>aadA2, A3, A5</i>
<i>rmtB</i>	<i>rmtB</i>	<i>rmtB</i>	<i>rmtB</i>	<i>rmtB</i>	<i>rmtA</i>
<i>aac6'-1b-cr</i>	<i>aac6'-1b-cr</i>	<i>aac6'-1b-cr</i>	<i>aac6'-1b-cr</i>	<i>aac6'-1b-cr</i>	<i>aac6'-1b</i>
<i>strA/B</i>	<i>strA/B</i>	<i>strA/B</i>	<i>strA/B</i>	<i>strA/B</i>	<i>strA</i>
Quinolone resistance genes					
<i>qnr</i>	<i>qnrS1</i>	<i>qnrS3</i>	<i>qnrS3, qnrS7</i>	<i>qnrS1</i>	<i>qnrS</i>
<i>aac(6')-Ib-cr</i>	<i>aac(6')-Ib-cr</i>	<i>aac(6')-Ib-cr</i>	<i>aac(6')-Ib-cr</i>	<i>aac(6')-Ib-cr</i>	<i>aac(6')-Ib</i>
Trimethoprim resistance genes					
<i>dfrA</i>	<i>dfrA-12, dfrA-17</i>	not detected	<i>dfrA-12, dfrA-17</i>	<i>dfrA-12, dfrA-17</i>	<i>dfrA7 (A17), A12, A21, A22</i>
Others					
<i>cat</i>	not detected	<i>catB3</i>	<i>catB3</i>	<i>catB3</i>	<i>catB3/B6</i>
<i>sul</i>	<i>sul1</i>	<i>sul1, sul2</i>	<i>sul1, sul2</i>	<i>sul1, sul2</i>	<i>sul1, sul2</i>
<i>tet</i>	<i>tetA, tetR</i>	<i>tetA, tetB, tetC</i>	<i>tetE</i>	<i>tetA, tetR</i>	<i>tetA, tetR</i>

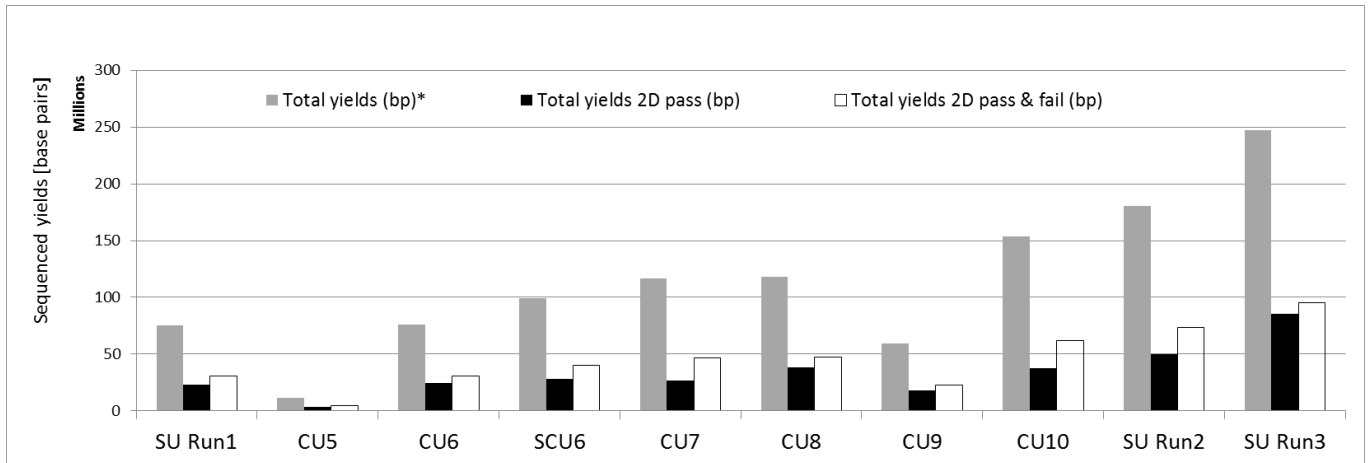
^aβ-Lactamase gene variant detected e.g. here '1' means *bla*_{TEM-1}

*mv- multiple variants (> 5) flagged

Grey: additional acquired genes detected only by MinION

Acquired resistance genes in MinION runs 1, 2 and 4 were sought using BLAST and CARD searches, whereas in run 3 they were sought using ARMA software.

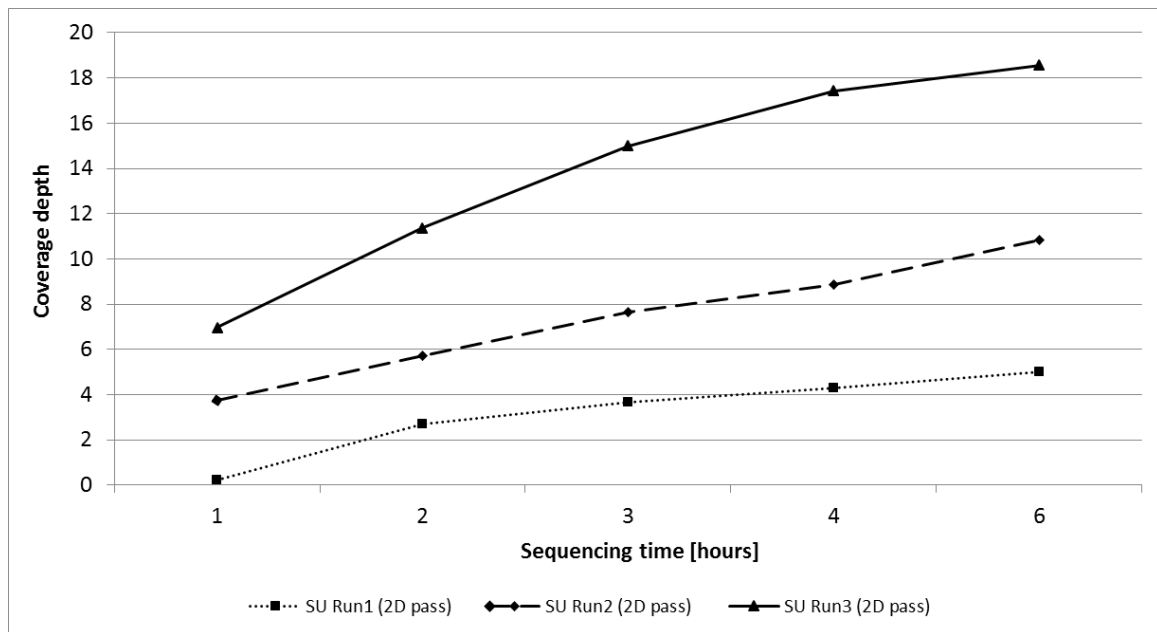
Fig. 1. Improvement of MinION sequencing performance and yields over 6h of sequencing run time



Legend: SU Spiked urine; CU clinical urine. Runs are shown in chronological order, see Table 1.

Grey total yields (1-D template + 1-D complement); **Black** total yields 2-D pass (bp); **White** total yields 2-D pass & fail (bp)

Fig. 2. Timeline of coverage depth for successive runs with urine spiked with *E. coli* strain



Legend: ...■... SU Run1, Spiked urine with multi-drug resistant *E. coli* H141480453 Run1; --- Run2, Spiked urine with multi-drug resistant *E. coli* H141480453 Run2; — SU Run3, Spiked urine with multi-drug resistant *E. coli* H141480453 Run3.

Fig. 3. Timeframe of MinION sequencing with the 15-min library preparation kit used in Spiked Urine Run 4

