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

K. Schmidt[1], S. Mwaigwisya[1], L. C. Crossman[2], M. Doumith[3], D. Munroe[4], C. Pires[5], A.M. Khan[5], N. Woodford[3], N.J. Saunders[5], J. Wain[1], J. O'Grady[1*], D. M. Livermore[1, 3]

[1]*Norwich Medical School, University of East Anglia, Norwich, United Kingdom*

[2]*SequenceAnalysis.co.uk, Norwich Research Park, Norwich, United Kingdom*

[3]*AMRHAI Reference Unit, National Infection Service, Public Health England, London, United Kingdom*

[4]*Microbiology Department, Norfolk and Norwich University Hospital, Norwich, United Kingdom*

[5]*Brunel University London, Uxbridge, United Kingdom.*

***Corresponding author:** Justin O'Grady

Telephone: +44 (0) 1603 59 7005

E-mail: (Justin.OGrady@uea.ac.uk)

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Abstract

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

Introduction

The UK Prime Minister's O'Neill Commission, reviewing the threat of antibiotic resistance[1], stresses the potential of rapid diagnostics to improve both treatment and antibiotic stewardship. Reducing the time needed to obtain a microbiological diagnosis shortens the duration of broad empirical therapy and its selective pressures.

PCR can detect pathogens and resistance genes in specimens without culture, but cannot cover the diversity of organisms and resistance determinants potentially present. Metagenomic sequencing could deliver this comprehensiveness[2-7] but slow turnaround, cost and complexity have impeded introduction into clinical microbiology.

Oxford Nanopore's MinION[8] is the first technology potentially able to deliver sequencing data from clinical samples in a timeframe allowing early de-escalation and refinement of antimicrobial treatment. We examined its applicability to investigation of urinary tract infection (UTIs). These account for over 8 million physician visits p.a. in the USA[9]. Most are trivial but, in severe cases, infection may ascend to the kidneys, with overspill to the bloodstream precipitating bacteraemia and urosepsis. Complicated UTIs are a growing cause of hospitalization, mostly of elderly patients[10], and 35,676 *Escherichia coli* bloodstream infections were recorded in England in 2014-15[11], over 60% with a urinary origin. There is growing resistance, particularly in severe and bacteraemic infections, to fluoroquinolones, cephalosporins and lactamase-inhibitor combinations, driving use of previously-reserved carbapenems, even as 'empirical' therapy. With carbapenemases now proliferating, and few alternative therapies in reserve, escalating empiricism becomes increasingly untenable, underscoring the desirability of moving to early targeted therapy, guided by diagnostics.

Materials and Methods

Urines

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

Ethics

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

Sample preparation for MinION Nanopore sequencing

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

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

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

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

MinION library preparation and sequencing

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

The library was then prepared according to the SQK-MAP-006 Genomic Sequencing Kit protocol provided by Oxford Nanopore Technologies (ONT)[12]. Variations, in earlier library preparation experiments, were: (i) Kit SQK-MAP-002 was used for CUs 1-4[13]; (ii) Kit SQK-MAP-003 was used with CUs 5-6 and for spiked urine run 1[13]; (iii) Kit SQK-MAP-004 was used[14] for CU7 and urine spiked with *E. coli* recovered from CU6, (iv) Kit SQK-MAP-005[8] was used with CUs 8-10 and Spiked Urine Run 2, (v) ONT's Rapid Sequencing Kit, with a-15 minute library

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

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

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

Identification of species and resistance genes routinely utilised BLAST search and the CARD (Comprehensive Antibiotic Resistance Database) database [15]. MinION data were extracted, in fasta format, from raw HDF5 files using Poretools[16]. BLAST database aliases were built for proteobacteria, firmicutes and human sequences. Top hits from each of these separate database aliases were identified. Taxa were distinguished using the in-house script blast_separate_taxa.pl, and taxonomy was assigned using blast_taxonomy_report.pl[17] with some modifications[18].

Resistance genes were identified by aligning MinION reads to the CARD database using LAST, with parameters optimised for low-accuracy long matches[19-21]. Some sequences in CARD contain resistance-gene-flanking regions, leading to false positive results, therefore putative matches were verified by visualisation in Artemis (Sanger)[22, 23] and by examination of the coordinates. Consensus sequences were built upon the CARD database reference sequences using the MinION read alignments by Samtools 0.1.19, Samtools mpileup, bcftools, vcfutils.pl and vcf2fq, ultimately generating indexed Bam files [24, 25]. BLASTn (BLAST v 2.2.30+) top hits were identified, using consensus sequences, against the CARD database, seeking >80% identity over the length of a gene. In addition, reciprocal BLAST best-hits were identified

between the consensus sequences and the CARD database. The resulting output data were parsed and sorted with a final report generated by a Python script[18].

WIMP and ARMA alignment for pathogen identification and resistance gene detection

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

Illumina library preparation

Two methods were used to sequence DNA from the bacteria cultivated from the urines. At PHE's Genomic Services Unit, genomic DNA was prepared using a GeneJET Genomic DNA Purification Kit (ThermoFisher, Cambridge, UK) and sequenced on a HiSeq instrument (Illumina, Cambridge, UK) in Rapid Run mode[28]. The library was prepared using the Nextera XT DNA Sample Preparation kits (Illumina), following the manufacturer's protocol. At Brunel University, bacterial DNA was quantified using the Quant-iT™ PicoGreen® dsDNA Kits (Life Technology, Paisley, UK) and a FLUOstar OPTIMA plate scanner (BMG Labtech, Ortenberg, Germany) according to manufacturers' specifications. DNA (300 ng) was fragmented using an Episonic system (Epigentek, New York, USA). Libraries were constructed using the NEBNext Ultra DNA Sample Prep Master Mix Kit (NEB) using an automated protocol on a Biomek FX instrument (Beckman Coulter, High Wycombe, UK). Ligation was performed with Illumina Adapters (Multiplexing Sample Preparation Oliogonucleotide Kit) and ligated libraries were

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

CARD alignment for resistance gene detection from cultivated bacteria

The presence of resistance genes in Illumina sequence reads was determined with 'Genefinder', an in-house PHE algorithm that uses bowtie2[29] to map the reads to a local database of antimicrobial resistance genes, and Samtools 0.1.18[24, 25] to generate an mpileup file. The script then parses the mpileup file to match to reference sequences, based on read coverage and > 90% nucleotide identity over full length of sequence (the lower threshold adopted for MinION was because of higher expected error rates).

Phenotypic characterisation of uropathogens

Bacteria were grown by standard methodology[30] and identified by MALDI-TOF mass spectroscopy (Bruker, Bremen, Germany). Minimum inhibitory concentrations (MICs) were determined at PHE by British Society for Antimicrobial Chemotherapy agar dilution, with results categorised on EUCAST criteria[31].

Results

MinION results and performance improvement

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

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

Bacterial identification

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

Resistance gene profiles

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

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

Clinical Urine 5 (K. pneumoniae)

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

Clinical Urine 6 (E. coli)

MinION and Illumina both found $bla_{\text{CTX-M-15}}$ and $bla_{\text{OXA-1}}$, again congruent with an ESBL phenotype and amoxicillin-clavulanate resistance. Both also indicated $bla_{\text{TEM-1.}}$ MinION

232 flagged several acquired *ampC* genes whilst Illumina indicated *bla*$_{\text{CMY-113}}$ and *bla*$_{\text{MIR-14}}$, albeit
233 below the 90% threshold. It is likely that all these *ampC* calls really corresponded to *E. coli*
234 chromosomal *ampC,* as the cefoxitin MIC for the isolate was only 8 mg/L, whereas cefoxitin
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 *aadA*5, 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*$_{\text{CTX-M-15}}$, agreeing with an ESBL phenotype; *bla*$_{\text{OXA}}$ 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*$_{\text{TEM-1}}$. 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*$_{\text{ACT-24}}$)
248 was flagged by MinION, not Illumina. As with CU5, however, a low cefoxitin 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.

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

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

Clinical Urine 10 (K. pneumoniae)

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

Spiked urines

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

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

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

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

Discussion

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

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

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

Most sequence analysis was post-run, using BLAST search and CARD database. However the Metrichor WIMP and ARMA software, adopted late in these studies, allowed real-time analysis. With this approach, adding together times for analysis (1h), sequencing (1h), library preparation (3h), DNA extraction and sample transport (2.5h) suggests a total 7-8h turnaround, equating to one dosage interval for a 'typical' q8h antibiotic. Further acceleration is feasible using the 15-min library preparation kit (as with spiked urine Run 4) reducing turnaround to *c.* 4h (Fig. 3). This is similar to PCR methodology, and would inform much earlier de-escalation and refinement of therapy than now. During the WIMP/ARMA-based analysis *c.* 32 MB of 2-D sequencing data were generated, with almost 7x depth of coverage after 1h (57 MB of 2-D data with 11.37x depth were available after 2h). Based on Lander and Waterman's[36] equation, we calculate that 7x depth covers 99.905% of the *E. coli* genome (4.6 MB), leaving little risk of missing an acquired resistance gene. Moreover, MinION sequencing error rates are diminishing rapidly (>90% identity with recent R9 pore chemistry)[37].

Although the approach has great potential, challenges remain:

Firstly, we used heavily-infected urines (>10^7cfu/mL) to deliver the *c.*1 µg of DNA required for sequencing, whereas significant bacteriuria is defined as >10^5 cfu/mL. Low-input procedures have been described and should address this issue[38]; these reduce the DNA requirement for nanopore sequencing to 20 ng.

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

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

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

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

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

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

multiple species in metagenomic samples[41]. Multiple strains of the same species would be harder to distinguish, but all their resistance genes would be represented in the sequence, whereas conventional culture would be liable to randomly select and test one of the strains present.

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

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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 *E. coli* from CU6**	**Clinical Urine 7**	**Clinical Urine 8**	**Clinical Urine 9**	**Clinical Urine 10**	**Urine spiked with *E. coli* H141480453 Run 1**	**Urine spiked with *E. coli* H141480453 Run 2**	**Urine spiked with *E. coli* 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	*K. pneumoniae* CG43	*E. coli* JJ1886	*E. coli* JJ1886	*E. coli* PMV-1	*E. coli* 536	*E. cloacae* NCTC 9394	*K. pneumoniae* CG43	*E. coli* APEC O78	*E. coli* APEC O78	*E. coli* APEC O78
Best species match to Illumina sequence data	*K. pneumoniae* MGH 78578	*E. coli* JJ1886	*E. coli* JJ1886	*E. coli* IHE3034	*E. coli* 536	*E. cloacae* NCTC 9394	*K. pneumoniae*	*E. coli* ST410	*E. coli* ST410	*E. coli* 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 *K. pneumoniae*	MICs	>64	16	8	128	64	≤0.06	16	0.25	8	≤0.06	4	16	2	2	16	32	R	R
	MinION	*bla*OXA-1			*bla*CTX-M-15, *bla*SHV-32									*qnrB, aac(6')-Ib-cr*	*aac(6')-Ib-cr, aacC2, strA*				*dfrA14*
	Illumina	*bla*TEM-1, *bla*OXA-1			*bla*CTX-M-15, *bla*SHV-27, *bla*LEN-12									*qnrB, aac(6')-Ib-cr*	*aac(6')-Ib-cr, aacC2, strA, strB*				*dfrA14*
CU6 *E. coli*	MICs	>64	16	4	128	32	≤0.06	16	0.25	8	≤0.06	8	32	>8	4	16	16	S	R
	MinION	*bla*TEM (mv*), *bla*OXA-1			*bla*CTX-M gp1 (15),*ampC* (*bla*CMY mv*, *bla*ACC-4, *bla*MIR-9, *bla*DHA-22)									*aac(6')-Ib-cr*	*aac(6')-Ib-cr, aacC2,aadA5*				*dfrA17*
	Illumina	*bla*TEM-1, *bla*OXA-1			*bla*CTX-M-15									*aac(6')-Ib-cr; gyrA* (83:SL;87:D-N); *parC* (80:S-I; 84:E-V)	*aac(6')-Ib-cr, aacC2,aadA5*				*dfrA17*
CU7 *E. coli*	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	*bla*TEM (mv*)			*bla*CTX-M gr1,ampC (*bla*ACT-24)										*aadA1, aadA3,strA, strB*				*dfrA1*
	Illumina	*bla*TEM-1			*bla*CTX-M-15									*gyrA* (83:S-L)	*aadA1, strA, strB*				*dfrA1*
CU8 *E. coli*	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	*ampC* (*bla*CMY mv*, *bla*ACC-4, *bla*MIR-4, *bla*DHA-6, *bla*FOX4)																	
	Illumina																		
CU9 *E. cloacae*	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	*ampC* (*bla*CMY mv*,*bla*ACT-18, 24)																	
	Illumina	*ampC* (*bla*ACT-24)																	
CU10 *K. pneumoniae*	MICs	>64	32	>64	>256	256	0.125	128	1	64	≤0.06	16	>64	>8	8	>32	>32	R	R
	MinION	*bla*TEM (mv*), *bla*OXA-1			*bla*CTX-M gr1, *bla*SHV(mv*),									*aac(6')-Ib-cr, qnrB*	*aac(6')-Ib-cr, aacA4, aacC2, aadA3, strA, strB*				*dfrA14*
	Illumina	*bla*TEM-1, *bla*OXA-1			*bla*CTX-M-15, *bla*SHV-28, *bla*LEN-12									*gyrA* (83:S-I), *parC* (80:S-I),*aac(6')-Ib-cr,qnrB*	*aac(6')-Ib-cr, aacC2, strA, strB*				*dfrA14*

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, cefoxitin; CIP, ciprofloxacin; AMK, amikacin, TOB- tobramycin; GEN, gentamicin; STREP, streptomycin; TRIM, trimethoprim. All β-lactamase inhibitors were used at 4 mg/L. White text **(R)**: resistant; Black text **(I):** intermediate**;** Black text **(S):** susceptible based on EUCAST criteria; Black text: acquired genes found only by Illumina; White text**:** 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		
bla$_{TEM}$	1[a]	1, mv*	1, mv*	1, mv*	1, mv*
bla$_{CTX-M}$	group-1 (15)	group-1 (1, 3, 15, 52, 114)	group-1 (15, mv*)	mv* not including *bla*$_{CTX-M-15}$	mv* not including *bla*$_{CTX-M-15}$
bla$_{OXA}$	1, 181	31 (=1,30), 181	2, 7, 30, 232 (=181)	1, 181, mv*	181, mv* not *bla*$_{OXA-1}$
bla$_{NDM}$	4	4, 6, 7	4, 5, 7, 12, 13	1	mv*
bla$_{CMY}$	2	34, 45, 111	mv* not including *bla*$_{CMY-2}$	mv* not including *bla*$_{CMY-2}$	mv* not including *bla*$_{CMY-2}$
others	-	-	-	-	*bla*$_{LAT-1}$
			Aminoglycoside resistance genes		
aacC	*aacC2*	*aacC2*	*aacC2*	*aacC2*	*aacC2, aacC8*
aadA2,aadA, aadA5	*aadA2, aadA3, aadA5*	*aadA2, aadA3*	*aadA5*	*aadA2, aadA3, aadA5,* mv*	mv* not including *aadA2,A3, A5*
rmtB	*rmtB*	*rmtB*	*rmtB*	*rmtB*	*rmtA*
aac6'-1b-cr	*aac6'-1b-cr*	*aac6'-1b-cr*	*aac6'-1b-cr*	*aac6'-1b-cr*	*aac6'-1b*
strA/B	*strA/B*	*strA/B*	*strA/B*	*strA/B*	*strA*
			Quinolone resistance genes		
qnr	*qnrS1*	*qnrS3*	*qnrS3, qnrS7*	*qnrS1*	*qnrS*
aac(6')-Ib-cr	*aac(6')-Ib-cr*	*aac(6')-Ib-cr*	*aac(6')-Ib-cr*	*aac(6')-Ib-cr*	*aac(6')-Ib*
			Trimethoprim resistance genes		
dfrA	*dfrA-12, dfrA-17*	not detected	*dfrA-12, dfrA-17*	*dfrA-12, dfrA-17*	*dfrA7 (A17) , A12, A21, A22*
			Others		
cat	not detected	*catB3*	*catB3*	*catB3*	*catB3/B6*
sul	*sul1*	*sul1, sul2*	*sul1, sul2*	*sul1, sul2*	*sul1, sul2*
tet	*tetA, tetR*	*tetA, tetB, tetC*	*tetE*	*tetA, tetR*	*tetA, tetR*

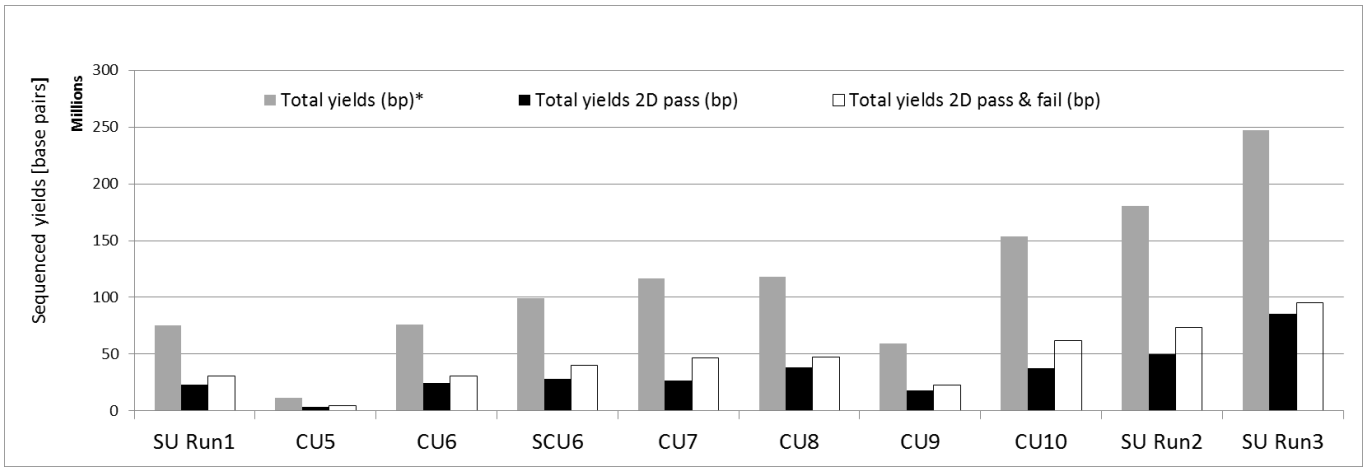
[a]β-Lactamase gene variant detected e.g. here '1' means *bla*$_{TEM-1}$

*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.

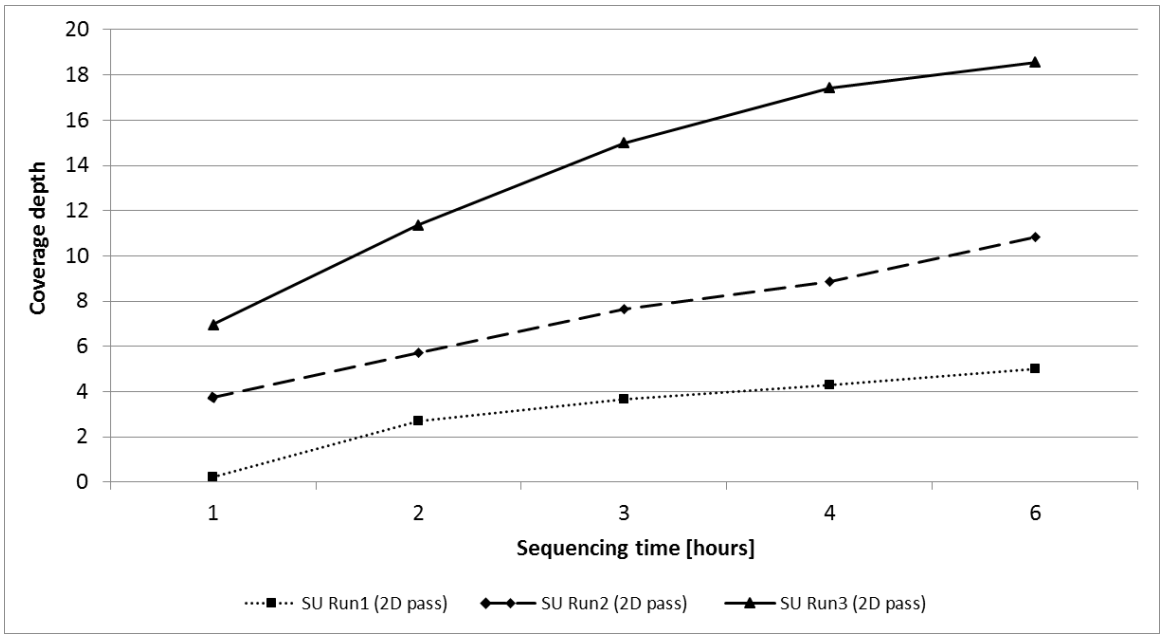
Fig. 1. Improvement of MinION sequencing performance and yields over 6h of sequencing run time



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