Single gene targeted nanopore sequencing enables simultaneous identification and antimicrobial resistance detection of sexually transmitted infections

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Abstract

Objectives

To develop a simple DNA sequencing test for simultaneous identification and antimicrobial resistance (AMR) detection of multiple sexually transmitted infections (STIs).

Methods

A total of 200 vulvo-vaginal swab samples from 200 female sex workers in Ecuador were initially tested by real-time PCR for *Neisseria gonorrhoeae* (NG), *Chlamydia trachomatis* (CT), *Mycoplasma genitalium* (MG) and *Trichomonas vaginalis* (TV). Samples positive for these STIs and controls were subjected to single gene targeted PCR MinION-nanopore sequencing by using the smartphone operated MinIT.

Results

Among 200 vulvo-vaginal swab samples 43 were positive for at least one of the STIs by real-time PCR. Single gene targeted nanopore sequencing generally yielded higher pathogen specific reads in positive samples than negative controls. Of the 26 CT, NG or MG infections identified by real-time PCR, 25 were clearly distinguishable from the negative controls using sequence read count. Discrimination of TV positives from controls was poorer as many had low pathogen loads (cycle threshold > 35) with associated low sequence read counts. AMR analysis workflow indicated that 11% of the classified reads aligned with antibiotic resistance genes, all of which identified fluoroquinolone resistance in NG.

Conclusions

Single gene targeted nanopore sequencing for diagnosing and simultaneously identifying key antimicrobial resistance markers for four common STIs, associated with genital tract discharge in women shows promise. Further work to optimise accuracy, reduce costs and improve speed may allow sustainable approaches for managing STIs and emerging AMR in resource poor and laboratory limited settings.

Introduction

Sexually transmitted infections (STIs) remain a major public health problem worldwide, with an estimated 357 million new cases of *Chlamydia trachomatis* (CT), *Neisseria gonorrhoeae* (NG), *Treponema pallidum* (TP) and *Trichomonas vaginalis* (TV) per year.[1] High rates of the STI, *Mycoplasma genitalium* (MG), have also been reported worldwide.[2] CT, NG, MG and TV commonly cause a genital discharge syndrome and are generally curable with existing, effective single-dose antibiotic regimens but, if left undiagnosed and/or untreated, can result in serious long-term reproductive health sequelae, particularly for women. Antimicrobial resistance (AMR) among some STIs to multiple classes of antibiotics has spread rapidly in recent years. For NG, loss of extended spectrum cephalosporins as first line empirical treatment is a major concern[3] as circulating multi- and extensive-drug resistance clones have been detected internationally.[4] For MG, macrolide resistance is now widely but not universally reported with increasing rates of resistance to fluoroquinolones also detected.[5] These developments have made treatment, and particularly empirical treatment challenging.

New World Health Organization (WHO) guidelines reinforce the need to treat these STIs with the right antibiotic, at the right dose, and the right time to reduce spread and improve sexual and reproductive health. For both NG and MG, accurate and rapid diagnostics which also predict antibiotic susceptibility are likely to be needed to achieve this. Laboratory based nucleic-acid amplification tests (NAAT) for detection of NG, CT, TV and MG are the current gold standard for detection, providing high sensitivity and specificity. NAAT are widely used in high-income countries but are often unavailable in resource-poor settings. Where testing is available, it is still frequently expensive and geographically inaccessible.[6] Culture-based antimicrobial susceptibility testing (AST) for NG remains gold standard for phenotypically predicting AMR and takes usually two to five days to obtain a result. This in practical terms may be too late to initiate targeted antibiotic therapy, particularly for hard to reach vulnerable populations. MG is difficult to cultivate and requires cell culture, not usually feasible in most clinical settings.

Advances in understanding AMR in NG, MG and TV have allowed for development of NAAT-based AMR detection. For example, absence of *gyrA* mutations in NG accurately predicts fluoroquinolone susceptibility,[7,8] presence of *23S rRNA* mutations in MG is associated with failure of treatment with azithromycin,[9-11] and *ntr6* mutations in TV may have diagnostic value for metronidazole resistance.[12] Use of NAAT-based AMR tests, however, may have limitations due to continually changing mutations and novel mechanisms of resistance evolving under ongoing treatment selection pressures. Whole genome sequencing (WGS) using high

throughput sequencing platforms may address this challenge to some degree and give added value in identifying phylogenetic relationships in identified infections.[13,14] However, for diagnostic purposes WGS itself can be constrained by need to first culture from clinical samples or to use nucleic acid capture techniques, increasing turnaround time and cost, and making them additionally unsuitable for near patient applications.

Oxford Nanopore Technologies' (ONT) portable MinION DNA sequencer, together with its recent MinIT hand-held processor, may offer advantages as an accurate diagnostic in resource-limited settings. Herein we report early phase evaluation among female sex workers (FSWs) in Ecuador where the STIs are prevalent,[15] of the MinION, controlled by a smartphone-operated MinIT to detect the four common STIs and profile AMR to ciprofloxacin in NG, azithromycin in MG and metronidazole in TV utilising a single gene targeted PCR approach, followed by long read sequencing in clinical samples.

Materials and Methods

Clinical sample collection and DNA preparation

A cross-sectional study of STIs was conducted among FSWs in a primary health centre in Quito, Ecuador, during the last quarter of 2017. Written and verbal informed consent was obtained from all participants all of whom were 18 years or older and none of whom received any compensation for their participation. Participants were interviewed by a local study investigator using a questionnaire designed for the study. The study was conducted according to the Declaration of Helsinki and approved by the Ethical Committee of Universidad Internacional del Ecuador (02-02-17).

Vulvo-vaginal swab samples were collected by clinicians using Xpert® CT/NG Patient-Collected Vaginal Swab Specimen Collection Kit (Cepheid). DNA from swab samples was prepared using PureLink Genomic DNA Mini Kit (Thermo Fisher Scientific) according to the manufacturer's instructions and quantified using a NanoDrop Spectrometer. All nanopore sequencing and experiments were done in Ecuador.

STI identification of swab samples by real-time PCR

DNA prepared from the swab samples was initially tested by real-time PCR for NG, TV, MG and CT infections, with gene targets and primers for real-time PCR detection shown in Table 1a. Real-time PCR was performed using Applied Biosystems 7500 Fast Real-Time PCR System in a volume of 10 µl containing 5 µl of TaqMan™ Fast Universal PCR Master Mix, 1 µl of 10x Exogenous Internal Positive Control (IPC) Mix, 0.2 µl of 50x IPC DNA, 250 nM

each of the primers, 100 nM each of the probes and 50 ng of template DNA. Cycling parameters were 95˚C for 10 min, followed by 40 cycles of 95°C for 15 sec, 60°C for 1 min.

Table 1 Gene targets, primers and probes used in this study

a) Initial real-time PCR for STI testing on samples
Trichomonas vaginalis: *T. vaginalis* specific repeat DNA fragment[16] TV_F: AAAGATGGGTGTTTTAAGCTAGATAAGG TV_R: TCTGTGCCGTCTTCAAGTATGC TV_P: [6FAM]AGTTCATGTCCTCTCCAAGCGTAAGT[BHQ1]
Mycoplasma genitalium: MgPa adhesin[17] MG_F: GAGAAATACCTTGATGGTCAGCAA MG_R: GTTAATATCATATAAAGCTCTACCGTTGTTATC MG_P: [6FAM]ACTTTGCAATCAGAAGGT[MGBNFQ]
Chlamydia trachomatis: The cryptic plasmid[18] CT_F: CATGAAAACTCGTTCCGAAATAGAA CT_R: TCAGAGCTTTACCTAACAACGCATA CT_P: [6FAM]TCGCATGCAAGATATCGA[MGBNFQ]
Neisseria gonorrhoeae: opa[19] NG_F: TTGAAACACCGCCCGGAA NG_R: TTTCGGCTCCTTATTCGGTTTGA NG_P: [6FAM]CCGATATAATCCGCCCTTCAACATCAG[BHQ1]
b) Targeted PCR for nanopore sequencing*
Trichomonas vaginalis: Nitroreductase family protein (ntr6) TV_NTR6_BC_F: 5’TTTCTGTTGGTGCTGATATTGCCTTCATTGAATTTATTCGTTCAAAATT TV_NTR6_BC_R: 5’ACTTGCCTGTCGCTCTATCTTCTTATTCAATGTATGTAACCTTTCTAA
Mycoplasma genitalium: 23S ribosomal RNA Mg 23S_BC_1992F: 5’ TTTCTGTTGGTGCTGATATTGCCCATCTCTTGACTGTCTCGG Mg 23S_BC_2679R: 5’ ACTTGCCTGTCGCTCTATCTTCTCCTCTCGTACTAGAAGCAAAG
Chlamydia trachomatis: Major outer membrane protein 1 (omp1) CT_OMP1_BC_F: 5’ TTTCTGTTGGTGCTGATATTGCTTTGCCGCTTTGAGTTCTGCT CT_OMP1_BC_R: 5’ ACTTGCCTGTCGCTCTATCTTCCAATACCGCAAGATTTTCTAGATTTC
Neisseria gonorrhoeae: gyrA NG_gyrA_BC_F: 5’ TTTCTGTTGGTGCTGATATTGCATCCGCCACGACCACAAATT

NG_gyrA_BC_R: 5' ACTTGCCTGTCGCTCTATCTTCATATTGGACAGTGCGACGGC
*developed in this study

Targeted PCR amplification and barcoding

DNA of NG, MG, TV and CT positive samples was amplified by PCR, targeting genes *gyrA* (NG), *23S rRNA* (MG), *ntr6* (TV) and *omp1* (CT) with the primers listed in Table 1b. PCR reactions for each target were performed in a 50 µl volume consisting of 25 µl LongAmp® Taq 2X Master Mix (NEB), 200 nM each of PCR primers and 100 ng DNA template as follows: 95 °C for 3 min followed by 35 cycles of 95 °C for 30 sec, 58 °C for 30 sec, 65 °C for 1 min 30 sec, and one cycle of 65 °C for 5 min. PCR amplicons were purified and subjected to a second PCR for barcoding using PCR Barcoding Expansion 1-96 - EXP-PBC096 (Oxford Nanopore Technologies) according to the manufacturer's instructions.

Nanopore sequencing and data analysis

A DNA sequencing library was constructed from a pool of barcoded PCR amplicons of different clinical samples using Ligation Sequencing Kit - SQK-LSK108 and sequenced using FLO-MIN106 R9 Version Flow Cell MK I Spot-ON on portable DNA sequencer MinION MK I which was controlled by a smartphone operated MinIT (Oxford Nanopore Technologies) according to the manufacturer's instructions. Sequencing data were uploaded onto Metrichor Epi2ME (Oxford Nanopore Technologies) and analysed using the workflow Fastq Antimicrobial Resistance r3.3.2 which included three components: QC and Barcoding [rev. 3.10.4], WIMP [rev. 3.4.0] and ARMA CARD [rev. 1.1.6]. QC and Barcoding component contains quality score (qscore) and barcode filter, cutting off reads with a qscore below the threshold (min qscore: 7) which will not be uploaded for analysis, and demultiplexing barcodes if the sequenced samples are barcoded. A qscore is calculated as Phred scores, ie -10log(Pe), where Pe is the probability of a particular base call. The mean qscore for a read directly corresponds to the alignment accuracy. WIMP (what's In My Pot) allows for species identification by classifying read sequences against the standard Centrifuge database (including RefSeq complete genomes for bacteria). ARMA (antimicrobial resistance mapping application) performs antimicrobial resistance identification by aligning input reads with minmap2 against all reference sequences available in the CARD database (CARD version 1.1.3). TV, an anaerobic, flagellated protozoan parasite, was not included in RefSeq for bacteria and therefore a TV G3 nitroreductase family protein (TVAG_354010, ntr6) reference was uploaded using

Fasta Reference Upload r3.2.2 and TV identification was performed using Fastq Custom Alignment r3.2.2 against the *ntr6* reference sequence. Read counts were log-transformed and compared between positive and negative samples by t-test.

BLAST search

NG, MG and TV read DNA sequences were extracted from read FASTQ files and BLAST-aligned against the reference sequences: *Neisseria gonorrhoeae* FA 1090 *gyrA* reference (NC_002946.2:c621189-618439), *Mycoplasma genitalium* strain G-37 *23S rRNA* gene (NR_077054.1) and *Trichomonas vaginalis* G3 nitroreductase family protein reference (TVAG_354010) respectively. Part of the aligned read sequences flanking AMR gene mutations were aligned to construct a consensus sequence using MultAlin.[20] AMR gene mutations were confirmed by manually comparing the consensus sequence against the reference.

Results

STI identification of swab samples by real-time PCR

A total of 200 vulvo-vaginal swab samples from 200 FSWs were initially tested by real-time PCR, and 43 were found positive, including 37 single infections (11 MG, 19 TV and 7 CT) and six co-infections (two CT/TV and one each of CT/NG, NG/TV, CT/NG/TV and CT/TV/MG). These positive samples together with their Ct values are shown in Table S1 (Supplementary data).

STI identification and AMR detection by single gene targeted PCR nanopore sequencing

All 43 PCR positives plus 12 negatives (3 for each pathogen) and an NG positive control were amplified by PCR. The amplicons from each sample were barcoded and combined in one sequencing library. A sequencing run of 10 hours produced 1,499,872 reads of which 1,127,703 (76%) reads passed QC (min qscore: 7), with total yield of 1.2 gigabases, average qscore 8.34 (Figure S1, Supplementary data) and average sequence length 816 bases. To identify MG, NG and CT, the 1,127,703 reads were analyzed by WIMP, resulting in 810,683 (72%) classified reads of which 43,491 (5.4%) were non-barcoded. For TV identification, Fastq Custom Alignment workflow was used, producing 148,458/1,127,703 (13%) read alignments with TV *ntr6* sequence of which 66,500 (5.9%) were non-barcoded.

Targeted amplicon sequencing from clinical samples, as expected, still non-specifically produced some sequence reads from other microbial and/or host DNA in the sample. However,

for all target pathogens, PCR positive samples generally yielded higher pathogen specific read counts than PCR negative controls, all of the latter of which had fewer than 15 reads (Figure 1). Mean $\log_{10}$ read counts were higher in positive NG, MG and CT samples compared to negative by t-test (Table S2, Supplementary data) but not significantly for TV as 14/24 TV read counts in TV positive samples were very low (<20 reads). For NG, MG and CT read counts clearly separated PCR positive and negative samples except for one MG positive sample that had an absolute read count of 1 (see Figure 1). The samples having <20 reads generally had a low load of pathogen, as evidenced by their Ct values being >35 (Table S1, Supplementary data).

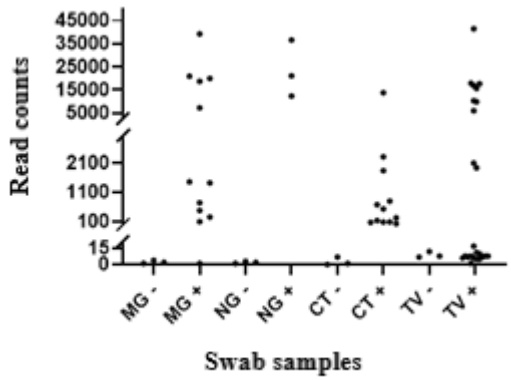

Figure 1. Pathogen read counts in the clinical swab samples analyzed by single gene targeted PCR nanopore sequencing

All the classified reads were analyzed by the ARMA workflow and 86,430/810,683 (11%) classified reads aligned with antibiotic resistance genes in the CARD database, > 99.99% of which were identified to be fluoroquinolone resistant *gyrA* gene in NG, using protein variant model. This analysis indicated that 3/3 NG clinical strains identified were resistant to fluoroquinolones, while none of the MG strains were resistant to macrolide antibiotics. Surprisingly, the NG positive control, which was phenotypically susceptible to fluoroquinolones, and genotypically wild-type by BLAST (see below) was also identified to be fluoroquinolone resistant by ARMA. TV antimicrobial resistance was not profiled due to the absence of TV *ntr6* entries in the databases.

Confirmation of antimicrobial resistance by manual BLAST search

A manual BLAST search was performed to ascertain the accuracy of the pathogen AMR identified through the sequence analysis of the ARMA workflow. The manual BLAST search confirmed, as shown in Table 2, that all (3/3) of NG had fluoroquinolone-resistant mutations, 2/10 (20%) of TV had mutations related to metronidazole resistance, and none of MG had macrolide resistance-associated mutations. However, the manual BLAST search confirmed that the NG positive control was incorrectly identified to be fluoroquinolone resistant by ARMA workflow.

Table 2. BLAST confirmation of antimicrobial resistance associated mutations identified by single gene targeted PCR nanopore sequencing*

Gene	Sample	Sequence of antimicrobial resistance region
NG *gyrA*	Wild type	-T**C**CGCAGTTTACG**A**C-
	All NG samples	-T**T**CGCAGTTTACG**C**C-
	Positive control	-T**C**CGCAGTTTACG**A**C-
MG *23S rRNA*	Wild type	-CGGGACGG**AA**AGACC-
	All MG samples	-CGGGACGG**AA**AGACC-
TV *ntr6*	Wild type	-AATGCAA**A**AGCAGAC-
	2 TV samples	-AATGCAA**T**AGCAGAC-
	8 TV samples	-AATGCAA**A**AGCAGAC-
*One or more changes of the bold bases in the wild type cause antimicrobial resistance of strains		

Discussion

We demonstrated the potential to test four common STIs responsible for genital discharge syndrome and simultaneously detect AMR in three of them among vulnerable FSWs in Quito, Ecuador using the portable MinION DNA sequencer, controlled by smartphone, through a single gene targeted sequencing approach.

Single gene approaches for detection of infection, such as 16S rRNA sequencing have been increasingly described,[21,22] particularly with the advent of long read sequencing such as PacBio and ONT[23], which has greater likelihood of taxonomically identifying organisms at species level. We intentionally evaluated the accuracy of sequencing of a single AMR gene, as opposed to a two-stage gene approach,[24] to both diagnose infection and predict AMR simultaneously in order to evaluate potential for use in resource poor field settings where simplicity and cost become increasingly important factors to consider.

Our approach appeared to have some advantages over NAAT. We demonstrated initially that genes responsible for AMR have potential to be as useful as 16S rRNA genes for diagnosis and thus may serve as a ‘two-in-one’ target. The approach is most advantageous for antimicrobials

for which the number of essential AMR genes are limited. We chose for both pathogen and AMR detection the NG *gyrA*, MG *23S rRNA* and TV *ntr6*, all gene mutations in which have been shown to be predictive of AMR,[7-12,25,26] and CT *omp-1* for CT detection.[27,28] Although for *gyrA* in NG and to some extent *23S rRNA* in MG, mutations that confer resistance to ciprofloxacin and azithromycin respectively appear to be constrained to limited possibilities, for other genes not assessed here, such as *penA* in which changes may give rise to penicillin and extended spectrum cephalosporin resistance, there appears to be continuous evolution under selection pressure.[29] As sequencing allows for identification of evolving mutations in the same genes without changing the test, our approach will therefore have value as it is adapted to other gene targets, although for antibiotics where AMR involves multiple mechanisms, multiple genes would need to be sequenced alongside vigilance over any changing nature of phenotypic to genotypic association. Although previously the possibility of identifying NG and AMR markers on enriched NG positive urine samples has been demonstrated,[24] we did not perform WGS directly from clinical samples because of the costly and complex process of enrichment, host DNA depletion[30] and the need for sufficient pathogen load. Approaches that directly sequence from clinical samples involving capture probe technology, immunomagnetic separation and optimal extraction may in future allow for sequencing without amplification routinely.[31-33]

Many clinical STI samples in women contain a wide range of pathogen loads being drawn frequently from largely asymptomatic patients as in the case of the FSW cohort in this study. We found that except for TV, diagnosis accuracy by the single gene targeted nanopore sequencing appeared to be high and hardly limited by pathogen loads. Compared to the real-time PCR assay, the single gene approach correctly identified STI infections in samples with cycle thresholds (Ct) <35, that is with higher pathogen loads. In the group with lower pathogen loads (CT>35), PCR amplification did not enhance the absolute read counts of pathogen targets, possibly due to low PCR efficiency caused by the presence of a large quantity of background DNA and non-optimal PCR conditions. Further optimization of PCR conditions will be required to maximize the diagnostic sensitivity for this group of samples.

Regarding the Fastq Antimicrobial Resistance workflow used in this study, the component WIMP correctly identified each pathogen of interest, but we found the component ARMA CARD appeared to mis-profile the AMR of the NG control strain. This might be because the ARMA CARD used individual reads for matching AMR associated mutations, instead of a consensus sequence, supported by the result obtained with our manual BLAST search using

the consensus sequence. Any further development of ARMA could help improve accuracy of AMR prediction.

Our study although small suggested fluoroquinolone resistance in circulating NG strains may be high in Ecuador, consistent with neighboring Peru, where 82.3% of NG strains were fluoroquinolone resistant.[34] However, we did not find macrolide resistance associated mutations in *23S rRNA* gene of MG strains, even though macrolide-resistance of MG was prevalent in the U.S.[35] and common worldwide.[36] Interestingly, we also previously described lack of macrolide resistant MG in the Solomon islands[37] despite a high prevalence of infection in the islands. Among the TV strains detected, 2/10 had the *A238T* SNP which results in a premature stop codon in the *ntr6* gene that is associated with metronidazole resistance.[12] The presence of this mutation has potential to be useful in indicating where metronidazole may fail, but other mutations in a number of other genes are also likely to be important in confidently identifying susceptibility.[38] Adding other gene targets might therefore be useful to guide clinicians as to the reasons for treatment failure.

This study had some limitations. Firstly, there was a small sample size, impacting particularly on measures of specificity. Although future development is currently using larger sample sizes, species-specific sequencing gives inherent high specificity compared to real-time PCR. Secondly no further confirmatory sequencing or NAAT were performed to confirm diagnoses or mutations present, largely due to the local regulations which prevented transporting material out of Ecuador. Thus, this study should not be regarded as a formal diagnostic evaluation. Future work is required on larger sample sets with composite reference standards. Optimisation of amplification and target multiplexing will also be important.

Nanopore sequencing with MinION requires substantially lower infrastructure and startup cost compared to other high throughput sequencing platforms. Although arguably consumables are not optimally priced for resource poor settings, the promise of combining automated library preparations and use of disposable flow-cells has potential to use nanopore sequencing for field diagnostics. We recently demonstrated that even in developed settings implementation of rapid ciprofloxacin NAAT resistance tests for NG still requires net investment.[39] Small-scale local automated approaches have further potential to increase turn-around times to within time frames that may suite point-of care applications. More recently ONT has developed a tablet version, MinION™ Mk1C which enables full sequencing and analysis to be performed in the lab and field without need for internet connection. This development could reduce cost and improve speed further to use nanopore sequencing as a diagnostic tool.

In conclusion, this study demonstrated that single gene targeted nanopore sequencing for diagnosing and simultaneously identifying key antimicrobial resistance markers for four common STIs, associated with genital tract discharge in women shows promise. Further work to optimise accuracy, reduce costs and improve speed may allow sustainable approaches for managing STIs and emerging AMR in resource poor and laboratory limited settings.

Contributions

STS and LZ conceived the study. NRS, PC and STS conceived the wider study for FSWs and developed protocols for recruitment of patients with LML and ALR. ALR, LZ and LML performed PCR. LZ performed nanopore sequencing. LZ and STS performed analysis. LZ and STS wrote the paper with contributions from all authors.

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Transparency declarations

LZ and STS are inventors on patents in the field of STI diagnostics.

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