Nanopore + Ion Torrent sequencing, assembly, and annotation of culture-free streambed plasmids reveals hitchhiking genes for resistance to multiple human clinical antibiotics

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1. BACKGROUND

- Transmissible plasmids affect environmental ecosystems via exchange and recombination of antibiotic resistance genes.
- Exchange occurs between native bacterial populations and introduced fecal pathogens selected for resistance in farm animals.
- **Public Health Relevance:** Native bacteria in aquatic and soil habitats may act as incubators and sites for recombination of genes that are subsequently transferred to human pathogens.

2. PROCEDURE

PLASMID CAPTURE AND SEQUENCING

- Sediment collected from streams flowing through heavily-utilized cattle pastures and near high density poultry farms and a poultry processing plant in central Virginia.
- Plasmids captured from stream sediment samples as shown in Fig. 1.
- Two plasmids (1-1 and 1-20) sequenced using the Oxford Nanopore MinION and Ion PGM DNA sequencers.

SEQUENCING DATA PROCESSING & ANALYSIS

- Nanopore MinION data: extracted passing 2D reads using poretools.
- Ion PGM data: filtered using ea-utils removing reads with ≥4 Ns, avg quality <25, read lengths 100-400 bp.
- Nanopore-only assembly with PBcR (Celera assembler).
- Aligned reads back to assembly with bwa-mem.
- Polished assembly with pilon.
- Annotate with prokka.
- Search for antibiotic resistance using abricate (assembled contigs) and SRST2 (unassembled reads).

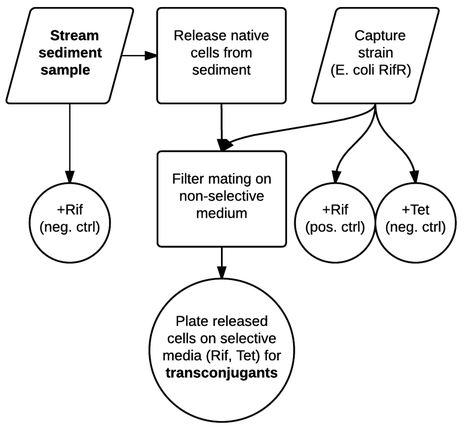

Figure 1. Method for capturing tetracycline resistance plasmids from sediment. Plasmids captured from stream sediment samples by releasing cells from sediment and conjugating with a rifampicin-resistant strain of E. coli. Transconjugants selected on tetracycline-and rifampicin-amended medium. Plasmids purified and electroporated into an electrocompetent E. coli strain and tested for decreased antibiotic susceptibility to 12 antibiotics relative to un-electroporated strain.

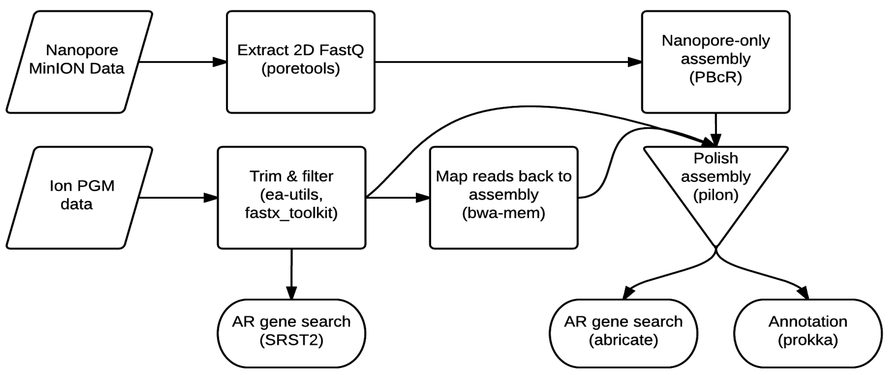

Figure 2. Sequencing and data analysis pipeline. Plasmids were sequenced using both Oxford Nanopore MinION and Ion Torrent PGM sequencers. An assembly derived from only nanopore data was polished with Ion PGM reads. The assembly was annotated with genes, and the assembly and unassembled reads were queried for antibiotic resistance genes.

	Version	URL
metrichor	1.12	https://metrichor.com/
poretools	0.5.1	https://github.com/arq5x/poretools
PBcR	8.3rc2	http://wgs-assembler.sourceforge.net/wiki/index.php/PBcR#Assembling_a_MinION_dataset
bwa-mem	0.7.12	https://github.com/lh3/bwa
pilon	1.13	https://github.com/broadinstitute/pilon
prokka	1.11	https://github.com/tseemann/prokka
abricate	0.2	https://github.com/tseemann/abricate
SRST2	0.1.5	https://github.com/katholt/srst2

Table 1: Software stack used in the data analysis pipeline.

3. RESULTS

	Plasmid 1-1	Plasmid 1-20
MinION 2D # reads	1684	2103
MinION 2D base pairs	2.6 Mbp	8.5 Mbp
MinION 2D mean length	1550 bp	4019 bp
Ion PGM # reads	2.0M	2.1M
Ion PGM base pairs	388 Mbp	455 Mbp
Ion PGM mean length	192 bp	215 bp

Table 2: Sequencing run statistics.

Gene	Resistance	Cov'g	Depth
AadA_AGly	aminoglycosides	100	5294
AadA9_AGly	aminoglycosides	100	4756
Aph3"Ia_AGly	aminoglycosides	100	4575
StrB_AGly	aminoglycosides	88	4330
AmpC1_Ecoli_Bla	beta-lactamases	100	21
AmpC2_Ecoli_Bla	beta-lactamases	100	26
AMPH_Ecoli_Bla	beta-lactamases	100	32
CARB_Bla	beta-lactamases	100	3785
Penicillin Binding Protein Ecoli Bla	beta-lactamases	100	66
FloR_Phe	fluoroquinolones	94	3512
SulI_Sul	sulfonamides	100	6820
TetC_Tet	tetracyclines	100	4177
TetG_Tet	tetracyclines	100	4546
TetRG_Tet	tetracyclines	96	4928

Table 3: AR genes found in unassembled PGM reads.

RESULTS HIGHLIGHTS:

- Good coverage of both plasmids from both MinION and PGM sequencing data.
- P1-1 assembly: 30kb (two ~15kb contigs)
- P1-20 assembly: **single ~90kb contig.**
- Polishing w/ PGM data corrected ~2000 sites (mostly SNPs/small indels).
- P1-20 assembly circular with unresolved complex repeats at ends.
- AR gene search on both unassembled reads and assembled contigs generally agree on AR genes co-located on same molecule.

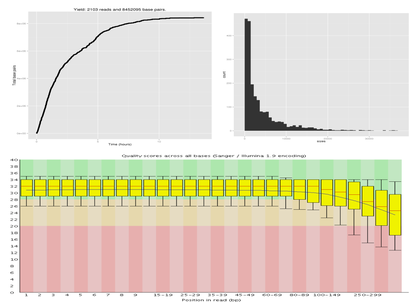

Figure 3: NGS read stats (top: nanopore throughput & length; bottom: Ion PGM quality distribution.

GENE	Resistance
aadA2	Aminoglycoside
aadA9	Aminoglycoside
aph(3')-Ic	Aminoglycoside
strB	Aminoglycoside
blaCARB-2	Beta-lactam
floR	Phenicol
sul1	Sulphonamide
tet(C)	Tetracycline
tet(G)	Tetracycline

Table 4: AR genes covered at >90% identity and coverage found in both assembled plasmids.

	Plasmid	
Drug susceptibility	**1-1**	**1-20**
Ticarcillin (tic)	***R***	***R***
Piperacillin (pip)	***R***	***R***
Tetracycline (tet)	***R***	***R***
Kanamycin (kan)	***R***	***R***
Tobramycin (tob)	***R***	s
Piperacillin / tazobactam (tzp)	***R***	s
Cefepime (fep)	***R***	s
Ciprofloxacin (cip)	s	***R***
Gentamicin (gent)	s	s
Sulfamethoxaxole / trimethoprim (sxt)	s	s
Imipenem (ipm)	s	s
Aztreonam (azt)	s	s

Table 5: Lab-confirmed AR profiles.

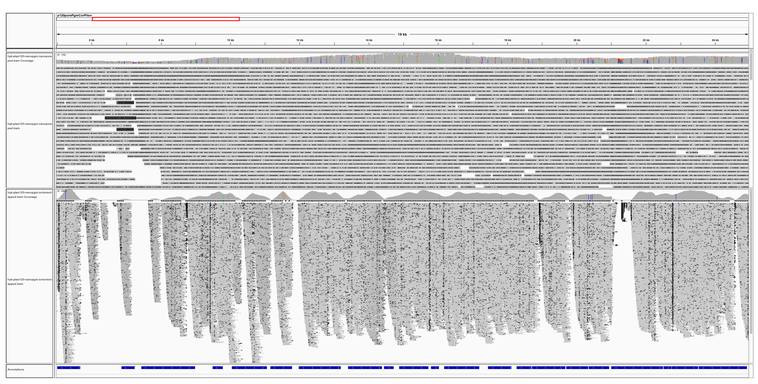

Figure 4: Reads mapped to assembly (top: MinION; bottom: PGM).

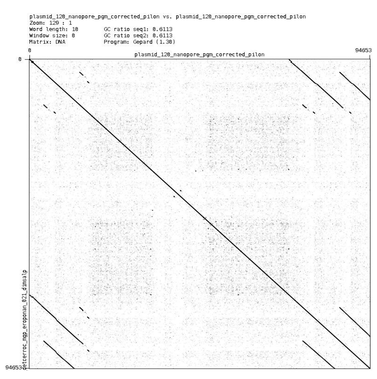

Figure 5: Assembly dot-plot.

4. CONCLUSIONS

- We confirmed presence of genes encoding resistance to multiple human clinical antibiotics travelling on tetracycline-resistant transmissible plasmids.
- There may be a significant reservoir of AR genes in streams capable of transmission to pathogenic *Enterobacteriaceae.*
- These resistance profiles can be detected and characterized cheaply and efficiently using portable DNA sequencing.

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