White Paper

Detecting antimicrobial resistance in extensively drug-resistant *Salmonella enterica* serovar Typhi from Pakistan

Authors

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Introduction

Infections caused by drug-resistant bacteria are being reported at an increasing rate. Antibiotic resistance is, now, recognized as one of the major threats to global public health. Whole genome sequencing has become an important method for understanding antimicrobial resistance determinants and for surveillance of the emergence and spread of resistant bacteria, and the underlying genetic mechanisms of resistance.

In this white paper, we demonstrate how CLC Microbial Genomics Module 4.0 (or later) can be used to identify antimicrobial resistance markers from the genomes of pathogenic bacteria. In our study, we employed three different methodologies for characterizing antimicrobial resistance determinants:

- Identifying resistance genes
- Identifying antimicrobial resistance via protein markers
- Identifying point mutations determined as driving antimicrobial resistance

Additionally, we demonstrated how to distinguish between chromosomal and plasmid-encoded resistance determinants.

For demonstration, we chose the data reported by Klemm and colleagues [1] that describe the emergence of an extensively drug-resistant clone of *Salmonella enterica* serovar Typhi in Pakistan in 2016. The study characterizes 88 extensively drug-resistant and 12 multidrug-resistant isolates of S. Typhi by whole genome sequencing and standard antimicrobial susceptibility testing. All isolates were sequenced using Illumina® 250bp pair-end sequencing chemistry.

Methods

The bioinformatics analysis presented here was conducted using CLC Genomics Workbench along with CLC Microbial Genomics Module (QIAGEN Bioinformatics). Details on this platform are provided at the end of this white paper. Specific software tools will be referred to below in italics, e.g., '*De Novo Assemble Metagenome*' refers to the tool of the same name within the Microbial Genomics Module. The analysis pipeline we created for this study consists of two parts: first, separating the microbial genome into chromosome and plasmid content; and second, detecting the antimicrobial resistance markers present (Figure 1).

Determining which parts of a microbial genome is of chromosomal or plasmid origin can be achieved by first assembling reads into contigs using the tool '*De Novo Assemble Metagenome*'. As we need to recollect all reads at a later stage, it is important not to restrict the minimum contig length during assembly. Restricting the minimum contig length will filter out a subset of the reads that may be of interest. After assembly, the contigs can be separated into "bins" based on their taxonomic assignment using the tool *Bin Pangenomes by Taxonomy*. The tool requires two databases to find the binning of contigs upon – one database containing chromosome references and another database containing plasmid references. Both sets of references can be downloaded via the tool *Download Microbial Reference Database*. For optimal resolution, it is recommended to use a small set of closely related reference genomes. If it is not clear which reference is most suitable for use, the tool *Find Best Matches using K-mer Spectra* can be run to help identify reference genomes that would be good for use with the binning tool. *Bin Pangenomes by Taxonomy* will separate contigs into bins according to the assigned taxonomic labels during read mapping at the level specified by the user. Accordingly, the output contains both binned contigs and binned reads. Both the contig output and the read output can be used to identify antimicrobial resistance markers.

The CLC Microbial Genomics Module toolbox currently includes three different tools designed for detecting antimicrobial resistance markers in genomic data.

- *Find Resistance with PointFinder* detects point mutations known to mediate antimicrobial resistance using a read mapping approach. Our implementation and the associated database is based on the PointFinder tool by Zankari and colleagues (2017) [2]. The input for *Find Resistance with PointFinder* is NGS reads.
- *Find Resistance with ShortBRED* also take reads as input but predicts antimicrobial resistance based on peptide markers. The tool is based on the ShortBRED pipeline by Kaminski et al. [3] and runs DIAMOND [4] against a database of antimicrobial resistance associated peptide markers. The marker database is regularly updated by the QIAGEN CLC development team and is currently based on the most recent version of ARG-ANNOT [5]. In addition to detecting the presence of peptide markers, it also quantifies the abundance of each marker.

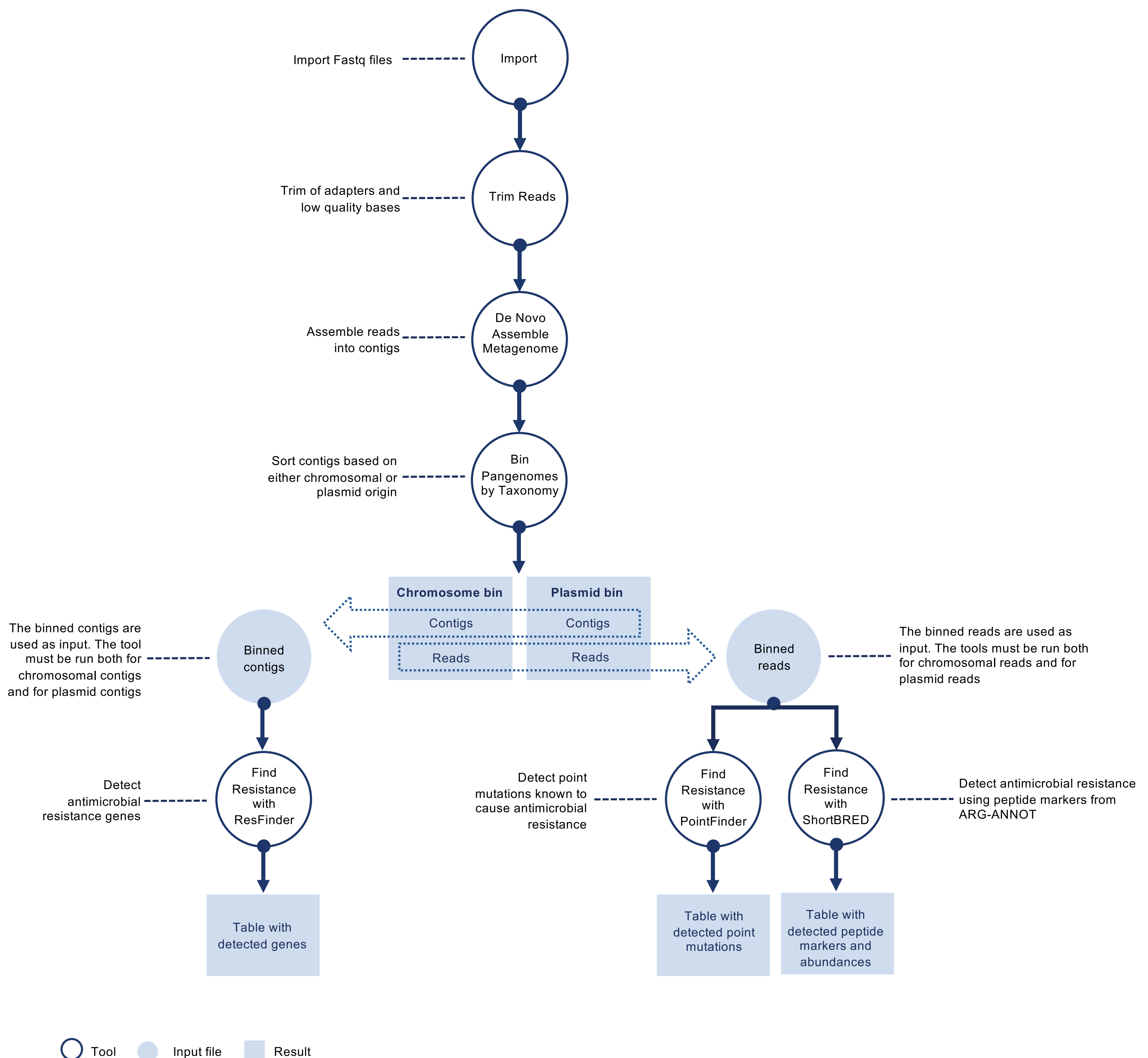

Figure 1. The demonstrated workflow used to detect antimicrobial resistance in isolates of *S.* Typhi distinguishing between chromosomal and plasmid regions.

- *Find Resistance with ResFinder* identifies antimicrobial resistance genes from assembled genomes and contigs. The tool is inspired by ResFinder by Zankari et al. 2012 [6] and uses BLAST to identify antimicrobial resistance genes based on the associated database.

To distinguish antimicrobial resistance markers located chromosomally and on plasmids, the resulting files (contigs and/or reads) from the contig binning step must be used for further analysis. The binned contigs can be used to search for resistance genes with Resfinder, and the binned reads can be used to identify resistance markers with ShortBRED and PointFinder (Figure 1).

Results

Using *Find Resistance with ResFinder*, we identified nine resistance genes in the S. Typhi genomes:

- *dfrA7, sul1* and *sul2* – conferring resistance towards Sulfonamides (trimethoprim-sulfamethoxazole)
- *strA* and *strB* conferring resistance towards Aminoglycosides (streptomycin)
- beta-lactamases *bla*TEM-1 and *bla*CTX-M-15 conferring resistance towards Beta-lactams (ampicillin, ceftriaxone)
- *catA* conferring resistance to Chloramphenicol
- *qnrS* conferring resistance towards Fluoroquinolones (ciprofloxacin)

The set of genes detected by ResFinder is in complete agreement with resistance genes detected in the original study of Klemm and colleagues [1] (Table 1).

Using ShortBRED to identify antimicrobial resistance markers 12 genes were detected in the *S.* Typhi genomes - the nine antimicrobial resistance genes detected in the study by Klemm et al. ***plus an additional three***. The newly identified antimicrobial resistance genes, *ampH*, PBP2 and *aac6-laa*, are located on the chromosome of the *S.* Typhi genomes and mediate beta-lactam and aminoglycoside resistance, respectively.

Using *Find Resistance with PointFinder* we identified *gyrA* mutations in all analyzed isolates (n=100). A single mutation in *gyrA* is predictive of intermediate susceptibility of S. Typhi towards ciprofloxacin. The *gyrA* substitution S83F was detected in all 100 isolates. In addition to *gyrA* mutations, the *qnrS* gene is also responsible for increased resistance towards ciprofloxacin in S. Typhi, and resistance produced by *qnrS* and *gyrA* are additive [7]. In 87 of 89 ciprofloxacin-resistant isolates, we detected *qnrS* in combination with a mutation in *gyrA*. In one of the remaining two resistant isolates, we detected in addition to the *gyrA* mutation, a second *gyrA* substitution, D87N, plus a mutation in parC (S80I). Concurrent mutation of *gyrA* and/or *parC* is known to increase the MIC for ciprofloxacin [8].

Table 1. Comparison of genes detected using different tools

Resistance gene	Antibiotic class	Detection method		Gene detected in original study
		ResFinder	ShortBRED	
catA1	Chloramphenicols	x	x	x
blaTEM-1	Beta-lactam	x	x	x
dfrA7	Sulfonamides	x	x*	x
sul1	Sulfonamides	x	x	x
sul2	Sulfonamides	x	x	x
strA (aph(3)-lb)	Aminoglycosides	x	x	x
strB (aph(6)-lb)	Aminoglycosides	x	x	x
blaCTX-M-15	Beta-lactam	x	x*	x
qnrS	Fluoroquinolones	x	x	x
ampH	Beta-lactam		x	
Penicillin Binding Protein 2	Beta-lactam		x	
aac6-laa	Aminoglycoside		x	

*Subgroup not identified

In Table 2, we show the comparison of the presence of genetic determinants of antimicrobial resistance with the measured phenotypic resistance as reported in the original publication. Generally, the positive predictive value (PPV) was high for the analysed genetic loci highlighting that genomic prediction of antimicrobial resistance can be of great importance. For only one isolate, the phenotype could not be explained by the detected ciprofloxacin mediating genes and mutations. In this isolate, a single *gyrA* mutation was detected, but not the *qnrS* gene or a second point mutation in either *gyrA* or *parC*. This means that genotypically the isolate should have intermediate susceptibility towards ciprofloxacin and not be resistant. In a few cases, we did not detect resistance genes to account for the phenotypic resistance reported or we detected the presence of a resistance gene in a susceptible isolate. *bla*CTX-M-15 was not detected in one ceftriaxone-resistant isolate, *bla*TEM-1 was detected in one isolate susceptible towards ampicillin, *catA1* was not detected in three chloramphenicol-resistant isolates but was, however, detected in three susceptible isolates.*dfrA7*, *sul1* and *sul2* were all not detected in three trimethoprim-sulfamethoxazole resistant isolates but were then detected in a single susceptible isolate.

Resistance analysis of contigs sorted into bins according to either their chromosomal or plasmid origin showed us that the genes *bla*CTX-M-15, *bla*TEM-1, *qrnS*, *strA*, *strB*, and *sul2* were all located on a plasmid. Genes *catA1, dfrA7, sul1, sul2, strA, strB, bla*TEM-1, *ampH*, PBP2, and *aac6*-Iaa were found located on the chromosome. Several genes were shared between the plasmid and the chromosome, including *bla*TEM-1, *strA, strB*, and *sul2*. By visualizing the genetic content of the chromosome, we can see that *sul2, strA, strB, bla*TEM-1, *sul1*, *dfrA7* and *catA1* form an antimicrobial resistance cassette that is harbored on a transposon. The transposon is integrated into the *yidA* site (Figure 2).

Note: Open reading frames can be predicted from contigs or genomes with the tool *Find Prokaryotic Genes* and annotated using one of three gene annotation tools (*Annotate CDS with Best BLAST Hit, Annotate CDS with Best DIAMOND Hit*, and *Annotate CDS with Pfam domains*).

Table 2. Congruence of detected resistance genes and point mutations, and phenotypic test results

Resistance mechanism	No isolates carrying gene	No phenotypically resistant isolates	PPV [%]
Ceftriaxone			
blaCTX-M-15	87	88	100
Ampicillin			
blaTEM-1	92	91	98.91
Chloramphenicol			
catA1	92	92	96.74
Trimethoprim-Sulfamethoxazole			
dfrA7	92	94	98.91
sul1	92	94	98.91
sul2	92	94	98.91
Ciprofloxacin			
qnrS + gyrA mutation	83	73	87.95
gyrA and parC mutations	1	1	100
gyrA mutation*	12	11	91.67
Streptomycin			
strA (aph(3)-Ib)	92	ND	ND
strB (aph(6)-Ib)	92	ND	ND

* A single gyrA mutation is predictive of intermediate susceptibility towards Ciprofloxacin

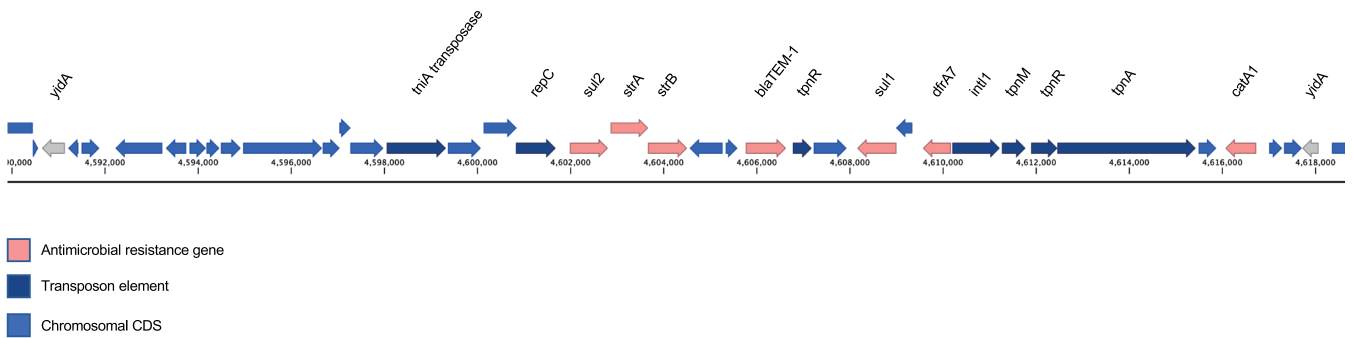

Figure 2. The genetic region of *S.* Typhi with an antimicrobial resistance cassette harbored on a chromosomally integrated transposon.

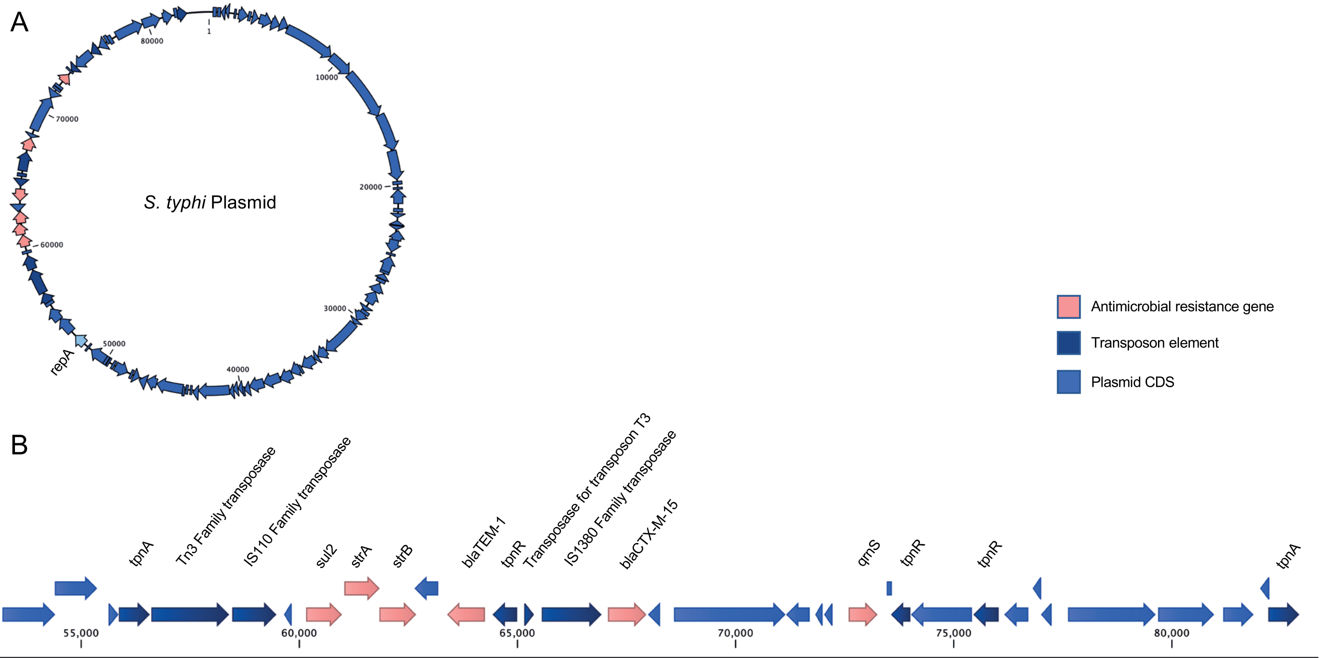

Figure 3. S. Typhi plasmid. A Circular view of the plasmid showing antimicrobial resistance genes. The *repA* gene is used for plasmid classification. **B** Genetic region of the plasmid containing antimicrobial resistance genes.

The genes *sul2, strA, strB, and bla*TEM-1 were located in a transposon on the plasmid along with genes *qnrS* and *bla*CTX-M-15 (Figure 3). By BLAST search of the *repA* gene sequence (Figure 3A) the plasmid was shown to be of type *IncY*.

Conclusions

In this white paper, we demonstrate how mediators of antimicrobial resistance can be determined from the genomes of pathogen isolates and their allocation on either the chromosome or plasmid resolved using tools of CLC Microbial Genomics Module.

The flexibility of our toolset allows the user to customize analysis with three different methodologies for detecting genetic determinants of antimicrobial resistance with direct access to databases of resistance markers. Furthermore, if desirable, the user can import or set up their specialized resistance marker database.

References

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