Rapid whole genome amplification and sequencing of low cell numbers in a bacteraemia model

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Words count: Abstract; 154, Text; 2530

Abstract

Whilst next generation sequencing is frequently used to whole genome sequence bacteria from cultures, it is rarely achieved direct from sample, and even more rarely performed in a clinically relevant time frame. To demonstrate the potential of direct from blood sequencing a bacteraemia model was developed, using defibrinated horse blood to model whole human blood infections. Sample processing included removal of erythrocytes and lysis of white blood cells, before rapid and accurate none targeted amplification. The rapid approach to allow direct from sample sequencing, allowed greater than 92% genome coverage of pathogens of interest whilst limiting the sequencing of host genome (less than 7% of all reads). Analysis of *de novo* assembled reads allowed accurate genotypic antibiotic resistance prediction. The sample processing would be easily applicable to multiple sequencing platforms. Overall this model provides evidence that it is currently possible to rapidly produce whole genome bacterial data from low cell number sterile site infections.

Introduction

Bacterial sequencing of clinical isolates has been used in many settings, including for virulence determinants[1–4], population structure[5 6] and outbreak investigations [7–10]. Almost exclusively current applications of sequencing rely either upon culture or targeted amplification imparting a diagnostic bias towards known pathogens and bacteria with known and relatively simple growth requirements. Bacterial whole genome sequencing is most often performed at reference or research centres to study population structures and evolution, with results rarely available in a clinically relevant time. One exception to this is the application of next generation sequencing to the diagnosis of tuberculosis[11,12], where WGS was shown, in some cases, to be quicker than current routine methods for predicting resistance; however this process still relies on culturing of the bacteria prior to sequencing. Direct from sample sequencing of pathogens using an untargeted (metagenomic) approach has the potential to lower turnaround times and lower diagnostic bias. Metagenomic sequencing techniques have

39 already been applied to ecological studies [13–16], gut microbiomes [17–19] and investigation and
40 identification of viruses [20–23].

41 In order to sequence a bacterial genome, at least 1 ng of DNA is needed, which is the equivalent
42 of over a 200000 copies of the *E. coli* K12 genome. To directly sequence pathogens present at low
43 levels (as few as 1 bacterial cell per ml blood) such as those found in sterile site infections an
44 unbiased and high fidelity amplification enzyme is needed. Multiple displacement amplification
45 (MDA) using ϕ 29 is an alternative method to PCR for the production of DNA in high enough
46 amounts for sequencing. ϕ 29 MDA has the potential to decrease our reliance on primers and increase
47 the lengths of DNA produced through amplification. This method has the advantage of being able to
48 produce large lengths of DNA with lower errors than conventional PCR. ϕ 29 MDA has been applied
49 to samples with very low starting DNA amounts from single cells (both prokaryotic and eukaryotic)
50 and provided DNA in levels high enough to perform sequencing [24]. The high fidelity and 3'–5'
51 proofreading activity reduces the amplification error rate to 1 in 10^6–10^7 bases compared to
52 conventional Taq polymerase with a reported error rate of 1 in 90,000 [25]. A single binding reaction
53 can incorporate over 70kb [26], meaning that this method is not limited by the length of the initial target
54 like conventional PCR. It has been demonstrated that this method is suitable for use with single
55 bacterial cells[24] and more recently malaria parasites directly from blood samples [27].Bacteraemia is a
56 major global cause of morbidity and mortality, with a large range of aetiologies and is a particular
57 problem in healthcare settings[28,29]. The current microbiological diagnostic process is culture based
58 often involving specialised equipment, with time to positivity varying due to aetiology and pathogen
59 load. Although recent advances in MALDI-TOF processing has allowed direct identification of
60 bacteria from positive blood cultures [30,31,32], antibiotic sensitivities take a further 18 hours. The direct
61 application of whole genome sequencing to blood samples would allow rapid pathogen diagnosis,
62 along with simultaneous pathogen typing and genotypic resistance prediction. Furthermore, by
63 applying unbiased pathogen detection method the diagnostic bias would be lowered. In order to allow
64 to allow direct from sample sequencing, a two stage host cells removal was performed firstly red
65 blood cells were removed using HetaSep®, which has previously been used to isolate nucleated cells

66 in the blood, particularly granulocytes but has not previously been applied to aid in pathogen
67 isolation. Secondly a selective white blood cell lysis was undertaken using saponin[33] in order to
68 release intracellular pathogens and aid in host nucleic acid removal.

69 **Methods**

70 ***Model process***

71 Clinical isolates were collected from the Royal Free Hospital Hampstead, where they had been
72 stored at -80°C after isolation from septic patients. Phenotypic data was produced using the BD
73 phoenix and was retrieved from final hospital reports. Bacteraemia models were set up by adding an
74 estimated ten bacterial cells, (*S. aureus* or *E. coli*) calculated using serial dilutions to 1ml horse blood.
75 The workflow depicted in Figure 1was then applied, with samples being cultured at each stage to
76 assess bacterial survival, sample processing was repeated three times. 200 µl HetaSep® was added
77 and the sample vortexed and incubated at 37°C for 10 minutes. 550 µl supernatant was removed and
78 200 µl 5% saponin was added to a final 2% solution, and incubated at room temperature for 5
79 minutes. 700µl sterile water was added for a water shock and incubated at room temperature for 30
80 seconds before salt restoration with the addition of 21µl 5M NaCl. The sample was centrifuged at
81 4000xg for 5 minutes and the supernatant discarded before addition of 2 µl turbo DNase1 and 5 µl of
82 10x buffer (Ambion). The sample was vortexed and incubated at 37°C for 15 minutes EDTA was
83 added to a final concentration of 15nM. The sample was then centrifuged for 5 minutes at 4000xg and
84 the supernatant removed and discarded. The pellet was washed in decreasing volumes of PBS,
85 initially 200µl then 100µl followed by 20 µl with each stage being centrifuged at 6000xg for three
86 minutes.

87 Bacteria were suspended in a total of 4 µl sterile PBS. Extraction was performed using alkaline
88 method; briefly cell suspensions were added to 200 mM potassium hydroxide (Qiagen) and 50mM
89 dithiothreitol (Qiagen) and incubated at 65°C for 10 minutes. The reaction was then neutralised using
90 neutralisation buffer (Qiagen). The sample was then briefly vortexed and placed on ice.

91 Amplification was performed using ϕ29 MDA (Repli-g Single Cell Kit Qiagen). A master mix
92 was prepared on ice in a total volume of 40 µl, with 29 µl reaction buffer, containing endonuclease
93 resistant hexamer primers and 2 µl (40 U) of ϕ29polymerase (Qiagen, REPLI-g Single Cell Kit). The
94 extracted DNA was then added to the master mix and the sample was then incubated at 30^0C for 2
95 hours, and the reaction stopped by heating to 65^0C for 3 minutes.

96 ***Sequencing***

97 DNA was quantified using Qubit BR kit and 3 µg of ϕ29 MDA DNA was de-branched using S1
98 nuclease in a 90 µl reaction as follows, 3 µl 10x buffer, 3 µl 0.5M NaCl, 10 µl S1 nuclease (1U/ µl)
99 with water to make the volume to 90µl. The digestion reaction was left at room temperature for 30
100 minutes and the enzyme deactivated by incubating at 70^oC with 6 µl 0.5M EDTA. The DNA was then
101 fragmented by nebulisation at 30psi for 180 seconds. The DNA was sequenced using the 454 Junior
102 using the manufacturer's recommended methods. A negative library was produced by using sterile
103 PBS in the ϕ29 MDA, this was then sequenced using the same method.

104 ***Data analysis***

105 Data analysis was performed in three stages, firstly host and contaminating reads were removed
106 before abundance trimming, secondly reads were classified using lowest common ancestor (LCA)
107 analysis and finally classified reads were assembled and analysed. Initially reads were mapped using
108 Newbler standard parameters against host and a local contamination library consisting of sequenced
109 negative controls and reads identified as contamination in previous runs. Unmapped reads were
110 written into a new fastq file using a custom python script (supplementary material) and taken forward
111 for further analysis. The remaining reads were abundance trimmed using Khmer[34] using two passes
112 to a maximum depth of 50, to remove over represented reads produce by the ϕ29 MDA method.
113 Following this the reads were error trimmed using Prinseq[35], with ends being trimmed to a Q20 cut
114 off. Blastn was then used to assign the reads against the nr database. Once completed lowest common
115 ancestor (LCA) analysis was performed using MEGAN[36], and reads associated with the bacterial
116 species of interest were extracted to a new fastq file. These reads were then *de novo* assembled using
117 standard parameters in SPAdes[37] assembly outputs were assessed using QUAST (Quality Assessment

118 Tool for Genome Assemblies)[35]. Reference assemblies were performed using the closest reference
119 sequence identified using the LCA analysis. Antibiotic prediction was performed using Mykrobe[11]
120 for *S. aureus* and ResFinder[38] for *E. Coli* using the *de novo* assembled reads.

121 **Results**

122 ***Sample processing***

123 When the full blood processing method was applied to *E. coli* and *S. aureus*, good survival rates
124 was found for both bacteria through-out all stages with the final survival rate being 100%. Details of
125 survivial at each stage of processing can be found in Table 1

126 ***Sequencing results S. aureus***

127 After processing and sequencing the horse blood spiked with *S. aureus* the number of reads
128 passing basic filter was 128500. Once known contaminants were removed and error and abundacnce
129 trimming complete 124,145 reads remained. Using Blastn and MEGAN, 62.1% reads were identified
130 as *S. aureus*. 6.76% of the reads were identified as the genus Equus, and 1.72% were identified as
131 *Parascaris equorum*. When examining the *S. aureus* reads closer 4254 reads were identified to the
132 subspecies level, (*Staphylococcus aureus subsp. aureus* HO 5096 0412), this subspecies has a
133 complete genome available (GenBank GCA_000284535.1) and was used as a reference for reference
134 mapping. Reference mapping produced 451 contigs and covered 92% of the reference genome. When
135 the reads identified as *Staphylococcus* using the LCA analysis were extracted and the reads *de novo*
136 assembled 1212 contigs were produced with an N50 of 3882. When this was compared to the
137 reference sequence 83% of the genome was covered with 10 misassemblies. Mykrobe analysis using
138 the *de novo* assembly gave genotypic result for 12 antibiotics. When comparing the genotypic and
139 phenotypic (BD Phoenix™) results matched in 11 of the 12 antibiotics. The results for ciprofloxacin
140 were inconclusive in genotypic tests, but resistant by phenotypic methods.

141 ***Sequencing results E. coli***

142 Post sequencing 173597 reads passed the initial filter, once contaminants were removed and the
143 reads error and abundance trimmed 170243 reads remained. Overall 73% of all reads remaining after

144 the analysis pipeline were identified as *E. coli.* 31959 (18%) reads had no identity. Unlike *S. aureus* it
145 was not possible to type the *E. coli*, as 150 reads were assigned to O7:K1 and 211 reads were assigned
146 to JJ1886. Reads which were identified as *Enterobacteriaceae, Escherichia* and *E. coli* by BLAST
147 and LCA analysis were extracted from the fastq produced after pipeline completion. The genome of
148 JJ1886 was available from the Integrated Microbial Genomes database (ID 2558309052), and the
149 chromosomal sequence was used as the reference against which the extracted reads were assembled.
150 After reference mapping assembly 93.5% of the genome was covered in 548 contigs. When the reads
151 were *de novo* assembled, 89% of the same reference was covered in 1334 contigs. The *de novo*
152 assembly was used to identify several resistance markers using ResFinder. The resistance markers
153 included dfrA, conferring trimethoprim resistance, gyrA conferring resistance to fluoroquinolones.
154 mdtK an efflux pump conferring resistance to norfloxacin and blaCMY-2 which is an AmpC,
155 conferring resistance to beta-lactams including cephalosporins. Additionally, eight drug efflux
156 systems were identified. These results were concordant to the phenotypic data.

157 **Discussion**

158 Direct diagnostics using WGS from clinical samples would, in many ways, provide the ideal
159 diagnostic method. By providing all the information from whole genome data with the speed of direct
160 sample testing. However, there are various obstacles, including low pathogen numbers, high host
161 background and difficulty in interpreting genotypic data. Here, a model was prepared to demonstrate
162 the potential for direct from sample sequencing from whole blood.

163 Fresh whole horse blood was used to model bacteraemia, as it was readily available. The process
164 was developed to remove RBCs early in the process, as they represent the largest proportion of the
165 cellular makeup of blood (up to 96%[39]), debulking the sample, and preventing release of oxidative
166 agents. Selective lysis on WBCs allowed the release of any intracellular pathogens and exposed host
167 nucleic acid to nucleases. *S. aureus* and *E. coli* were chosen due to different cell wall types and cell
168 morphology; both demonstrated good survival during the developed sample processing.

169 The majority of sequencing reads from spiked horse blood were associated with the spiked
170 bacteria (62.1% S. *aureus* and 73% *E. coli*), considering the horse genome is over 500 times larger
171 than the *E. coli* genome this shows that the vast majority of the host material was removed. Overall
172 there was good concordance of phenotypic and genotypic results showing the potential for rapid
173 genotypic prediction of antibiotic resistance from 10 bacterial cells in 1 ml of host blood. The
174 inconclusive ciprofloxacin results demonstrate the need for improved understanding of the
175 mechanisms of resistance. Ciprofloxacin resistance is harder to predict as it is chromosomal mutation
176 rather than gene acquisition. Three mechanisms of fluoroquinolone resistance have been proposed in
177 *S. aureus,* Topoisomerase IV gene mutations, DNA gyrase gene mutations and an active efflux pump
178 (NorA)[40]. The complexity of predicting ciprofloxacin resistance suggests that the database may be
179 lacking in its ability to predict ciprofloxacin resistance, and so this is the most likely cause of the
180 inconclusive result for ciprofloxacin resistance. Additionally the creators of Mykrobe (Bradley et al[11])
181 found a false negativity rate of 4.6% for ciprofloxacin resistance.

182 In addition to identifying the isolates resistance to beta-lactams the database was able to identify
183 the blaZ gene and MecA gene. Genotypic testing will never entirely replace phenotypic susceptibility
184 testing, due to its inability to identify novel resistance determinants and the comprehensive nature of
185 phenotypic testing. However, in this scenario, of invasive sepsis, the gain in speed provided by not
186 having to culture the organism to determine susceptibility could be life-saving.

187 Multiple antibiotic resistance factors were identified in the *E. coli*, which gave good concordance
188 with the phenotypic data. Using the genotypic data, it was possible to rapidly identify the beta-
189 lactamase present as BlaCMY-2. The rapid identification of the specific resistance genes in bacteria
190 could help identify outbreaks by providing more information than a simple antibiogram. Additionally,
191 it could help monitor novel resistance genes, or genes that are increasing in incidence. Large amounts
192 of horizontal genome transfer amongst Gram negative bacteria has the potential to cause outbreaks of
193 resistance bacteria through genes or plasmids[41], which would be more complex to track. Rapid
194 identification of genes causing the resistance in isolates could help inform epidemiological and
195 outbreak studies which could involve several species of bacteria. The output for the resistance

196 prediction was complex to interpret with several genes identified which didn't always have specific
197 drug resistance associated. This highlights a down side of generic databases, as they are often time-
198 consuming to interpret. A study of NGS sequencing data from bacteraemia isolates of *E. coli* have
199 shown resistance prediction specificity of 97%[2], if this was coupled with direct from sample
200 sequencing genotypic prediction could inform treatment more rapidly than phenotypic test.

201 Limitations of this model include differences in horse and human blood, and difference in sample
202 between spiked bacteria and bacteria causing a true infection. Additionally, factors in the blood such
203 as immune and inflammatory reactions could alter the efficiency of the preparation method. However,
204 this model shows proof of principal that direct unbiased sequencing directly from blood is possible
205 and could be used to diagnose and inform treatment of bacterial bloodstream infections.

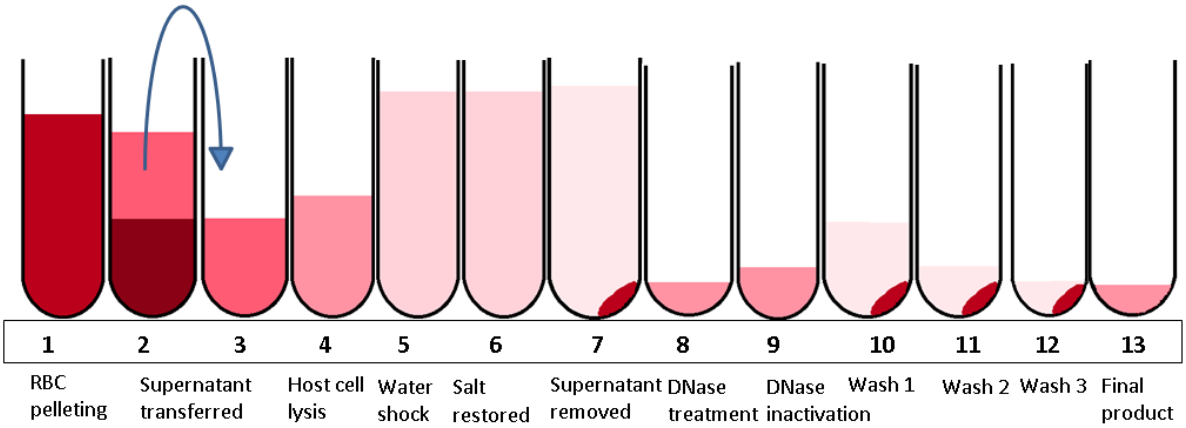
206 The method presented here is easily adapted to allow application to other sequencing platforms.
207 Overall the method presented allows sufficient DNA for whole genome sequencing of pathogens in
208 blood to be produced within a single day.

209

210

211

212 ***Tables and Figures***



213

214 **Figure 1** Illustration of the process for bacterial isolation from whole blood using HetaSep and

215 selective lysis with Saponin. Numbers refer to sampling points where bacterial recovery was

216 investigated.

217

Stage		*S. aureus*	*E. coli*
1	Spiked cells	10	11
2	RBC separation-bottom	0	0
3	RBC separation-top	10	9
4	Addition of Saponin	12	9
5	Water shock	12	12
6	Salt restoration	10	10
7	Cell pelleting-top Cell pelleting-bottom	0 9	0 9
8	DNAse treatment	11	12
9	EDTA and PBS	10	12
10	Wash 1-top Wash 1-bottom	0 9	0 11
11	Wash 2-top Wash 2–bottom	0 10	0 10
12	Wash 3-top Wash 3–bottom	0 8	0 9
13	Final pellet	11	9

218 **Table 1** the average number of CFU isolated at each processing stage for bacterial isolation from

219 whole blood using HetaSep and selective lysis with Saponin after workflow improvements. Numbers

220 refer to sampling points where bacterial recovery was investigated as illustrated in **Figure 1**

221

222

	Phenotypic-(MIC)	Genotypic	Comment 223
	E. coli		
Ampicillin	R (>8)	R	blaCMY-2 224
Ceftriaxone	R (>4)	R	blaCMY-2
Ciprofloxacin	R (>1)	R	gyrA 225
Colistin	S (<=0.5)	S	226
Gentamicin	S (<=0.25)	S	
Levofloxacin	R (>2)	R	gyrA 227
Meropenem	S (<=0.25)	S	
Nalidixic acid	(>16)	R	ermB
Nitrofurantoin	S (<=16)	S	
Trimethoprim	R (>4)	R	dfrA
	S. aureus		
Ciprofloxacin	R (>2)	Inconclusive	
Clindamycin	S (<=0.25)	S	
Erythromycin	S (<=0.25)	S	
Fusidic acid	S (<=1)	S	
Gentamicin	S (<1)	S	
Methicillin	R (oxacillin 2)	R	MecA
Mupirocin	S (<=1)	S	
Penicillin G	R (> 0.25)	R	blaZ
Rifampicin	S (<=0.25)	S	
Tetracycline	S (<=0.5)	S	
Trimethoprim	S (<=1)	S	
Vancomycin	S (1)	S	

Table 2 phenotypic and genotypic result for antibiograms of *E. coli* and *S. aureus*

228

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