

1 **WGS for surveillance of antimicrobial resistance: A pilot study to detect the prevalence and**  
2 **mechanism of resistance to azithromycin in a UK population of Non-Typhoidal *Salmonella***

3 Satheesh Nair\*, Philip Ashton, Michel Doumith, Steve Connell, Anais Painset, Solomon Mwaigwisya<sup>1</sup>,  
4 Gemma Langridge<sup>1</sup>, Elizabeth de Pinna, Gauri Godbole, Martin Day

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6 Gastrointestinal Bacteria Reference Unit, Public Health England, London, NW9 5EQ

7 <sup>1</sup>Norwich Medical School, University of East Anglia, Norwich NR4 7UQ

8 \*Corresponding Author

9 Satheesh Nair

10 Gastrointestinal Bacteria Reference Unit, Public Health England, 61 Colindale Ave, London NW9 5EQ

11 E-mail : [satheesh.nair@phe.gov.uk](mailto:satheesh.nair@phe.gov.uk)

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14 Running title: WGS for the detection of azithromycin resistance in *Salmonella*

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17 resistance, MinION

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19

20 **Abstract**

21 Objectives:

22 Whole genome sequencing (WGS) and phenotypic methods were used to determine the prevalence  
23 of azithromycin resistance in *Salmonella enterica* isolates from the UK and to identify the underlying  
24 mechanisms of resistance.

25 Methods:

26 WGS by Illumina HiSeq was carried out on 683 isolates of *Salmonella* spp. .Detection of known  
27 acquired resistance genes associated with azithromycin resistance were determined from WGS using  
28 a mapping-based approach. Macrolide resistant determinants were identified and the genomic context  
29 of these elements assessed by various bioinformatics tools. Susceptibility testing was in accordance  
30 with the EUCAST methodology (MIC $\leq$ 16mg/L).

31 Results:

32 Fifteen isolates of non-typhoidal *Salmonella enterica* (NTS) belonging to serovars S.Blockley, S.  
33 Typhimurium, S. Thompson, S. Ridge and S. Kentucky showed resistance or decreased susceptibility  
34 to azithromycin (from 6 to >16mg/L) due to the presence of macrolide resistance genes *mphA*, *mphB*  
35 or *mefB*. These genes were either plasmid or chromosomally mediated.

36 Azithromycin resistant S. Blockley isolates harboured a macrolide inactivation gene cluster *mphA*-  
37 *mrx-mphr(A)* within a novel Salmonella Azithromycin Resistance Genomic Island (SARGI), the full  
38 structure determined by long read MinION sequencing .To our knowledge this is the first  
39 chromosomally mediated *mphA* gene cluster in Salmonellae. Based on phylogenetic analysis and  
40 epidemiological information, the *mphA* S.Blockley isolates were not derived from a single  
41 epidemiological related event.

42 The azithromycin MICs of the 15 *Salmonella* spp. isolates showed that the presence of the *mphA*  
43 gene was associated with MIC $\geq$ 16mg/L, while presence of *mefB* or *mphB* was not .

44 Conclusion:

45 Resistance to azithromycin, due to acquisition of known macrolide resistance genes was seen in four  
46 different *Salmonella* serovars and can be either plasmid or chromosomally encoded.

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## 50 Introduction

51 The increased resistance to a broad range of antibiotics in both *Salmonella* strains that cause enteric  
52 fever and non-typhoidal *Salmonella* (NTS) are an emerging threat.<sup>1,2,3,4,5,6,7,8</sup> Widespread of  
53 resistance to amoxicillin, chloramphenicol, trimethoprim-sulfamethoxazole and fluoroquinolones has led  
54 to azithromycin being used as the preferred antimicrobial agent to treat cases of uncomplicated  
55 enteric fever reporting travel to the Indian subcontinent and South East Asia.<sup>4</sup> It is also used to treat  
56 infections with multidrug resistant non-typhoidal *Salmonella* (NTS) in vulnerable patients who have  
57 prolonged or invasive infections.<sup>7</sup> Azithromycin is an azalide and has excellent tissue penetration,  
58 concentrates in the reticuloendothelial cells and has the advantage of oral administration and a long  
59 half-life. Clinical trials have shown it to be the equivalent or superior to chloramphenicol,  
60 fluoroquinolones, and third generation cephalosporins for the management of uncomplicated typhoid  
61 fever.<sup>9,10,11</sup> However, reports are emerging of azithromycin resistance in cases of enteric fever as well  
62 as invasive NTS infection.<sup>1,10,11,12</sup>

63 Acquired resistance to macrolides/azalides may be caused by several different mechanisms of  
64 resistance.<sup>13</sup> They include (i) target site modification by methylases encoded by *erm* genes,<sup>14,15</sup> (ii)  
65 modifying enzymes such as esterases encoded by *ereA* and *B* genes or phosphotransferases encoded  
66 by *mphA*, *B* and *D* genes,<sup>16,17</sup> (iii) efflux pumps, e.g. *mefA* and *msrA* found mainly in Gram positive  
67 bacteria, with *mefA* also identified in Gram negative strains,<sup>15</sup> (iv) Mutations in the *rrl* and *rpl* genes  
68 encoding ribosomal proteins L22, L4 and 23S rRNA also confer resistance in Gram positive bacteria.<sup>18</sup>  
69 Full cross resistance between erythromycin and azithromycin can be conferred between these  
70 genes.<sup>14</sup>

71 The Gastrointestinal Bacteria Reference Unit (GBRU), Public Health England (PHE) is the national  
72 reference laboratory for *Salmonella* in England and Wales. Each year, approximately 10,000 isolates  
73 are referred to the Salmonella Reference Service (SRS). WGS is currently used as the primary test for  
74 identification and typing of isolates received by SRS.

75 (<http://biorxiv.org/content/early/2015/11/29/033225.abstract>).<sup>19</sup> These isolates are also tested  
76 phenotypically for resistance to a wide range of antimicrobial agents

77 The European Committee on Antimicrobial Susceptibility Testing (EUCAST) is responsible for  
78 defining clinical breakpoints for new and existing drugs within the European Union and affiliated  
79 nations. Currently, no clinical breakpoints for azithromycin have been defined for *Enterobacteriaceae*,

80 including *Salmonella*, by either the Clinical and Laboratory Standards Institute (CLSI) or EUCAST  
81 leading to delays in early detection of azithromycin resistance. However the epidemiological cut-off  
82 (ECOFF) for azithromycin has been accepted as  $\leq 16$  mg/L for *Salmonella enterica*.<sup>11,20</sup>  
83 The recent advancement in WGS technologies for routine microbiology is well documented.<sup>21</sup>  
84 Sequence data allows rapid identification of *Salmonella* serotypes by Multilocus Sequence Typing  
85 (MLST) as proposed by Achtman *et al* (2012).<sup>22</sup> In addition, availability of the whole genome  
86 sequences allows *in silico* prediction of antimicrobial resistance that should be validated by  
87 phenotypic antimicrobial testing prior to being applied.<sup>23,24,25</sup>

88 Here, we used available WGS data to determine the prevalence and underlying mechanisms of  
89 resistance of azithromycin resistance among *Salmonella* in the UK.

## 90 **Methods**

### 91 ***Bacterial isolates and phenotypic typing***

92 Six hundred and sixty seven *Salmonella* isolates from 2012 that were part of a six month (April –  
93 September 2013) WGS validation project were selected for this retrospective study (nine isolates  
94 shown in Table 1). A further 16 S. Blockley isolates from 2012 -2015 were used as comparators for  
95 phylogenetic analysis (Supp. Table 1). Selected isolates were identified and confirmed by serotyping  
96 and/or phage typing.<sup>26,27</sup>

### 97 **DNA extraction for WGS**

98 DNA extraction of *Salmonella* isolates was carried out using a modified protocol of the Qiasymphony  
99 DSP DNA midi kit (Qiagen). In brief, 0.7 mL of overnight *Salmonella* broth culture was harvested.  
100 Bacterial cells were pre-lysed in 220  $\mu$ L of ATL buffer (Qiagen) and 20  $\mu$ L Proteinase K (Qiagen), and  
101 incubated shaking for 30 mins at 56°C. Four  $\mu$ L of RNase at 100 mg/mL (Qiagen) was added to the  
102 lysed cells and re-incubated for a further 15 mins at 37°C. This step increases the purity of the DNA  
103 for downstream sequencing.

104 DNA from the treated cells were then extracted on the Qiasymphony SP platform (Qiagen) and eluted  
105 in 100  $\mu$ L of water.

106 DNA concentration using the GloMax system (Promega) and quality (optimal OD<sub>260/230</sub> = 1.8 -2.0)  
107 using the LabChip DX system (Perkin Elmer) were determined for the following sequencing steps.

### 108 **DNA sequencing**

109 Extracted DNA was then prepared using the NexteraXT sample preparation method and sequenced  
110 with a standard 2x101 base protocol on a HiSeq 2500 Instrument (Illumina, San Diego).

111 MinION sequencing was also carried out to define the complete structure of the genomic drug island  
112 in the S. Blockley isolate H123780513. A library was prepared using Genomic DNA Sequencing Kit  
113 SQK-MAP006 according to the protocol from Oxford Nanopore Technologies (Version  
114 MN006\_1115\_revC\_14Aug2015) and following the same principles as described in Ip *et al* 2015<sup>28</sup>  
115 except the following: sheared DNA was repaired using FFP repair mix (New England Biolabs,  
116 Ipswich, Massachusetts) and then prepared using the NEBNext Ultra II End-Repair / dA-tailing  
117 Module (New England Biolabs). The final ligation of adapter and hairpin was performed using  
118 adapters and tethers from SQK-MAP006 sequencing kit (Oxford Nanopore Technologies, Oxford,  
119 UK) followed by purification of the adapted and tethered DNA using MyOne C1 beads (Life  
120 Technologies). Purified DNA was loaded for sequencing to the flow cell (R9 chemistry) by Oxford  
121 Nanopore Technologies (Oxford, UK).

### 122 **Sequence assembly and detection of resistance genes**

123 Genome assembly was carried out using Spades  
124 v.3.7.0(<http://www.ncbi.nlm.nih.gov/pmc/articles/PMC3342519/>) with the command line options '-k 21,  
125 33, 55, 77' and '--careful'. Detection of resistance genes were as described by Doumith *et al* (2015).<sup>29</sup>  
126 Briefly reads were mapped to reference database of acquired genes including those conferring  
127 resistance to macrolides that were collated from the Comprehensive Antibiotic Resistance Database  
128 (<http://arpcard.mcmaster.ca>).  
129 Spades v.3.7.0 hybrid assembly was used to combine the MinION reads with the Illumina reads.  
130 MinION reads were mapped back to the hybrid assembly and this mapping was used to confirm the  
131 contiguity of key parts of the hybrid assembly

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### 135 **Phylogenetic analysis**

136 Raw FASTQs were processed as previously described.<sup>30</sup> These processed reads were mapped to a  
137 *de novo* assembled *S. Blockley* strain (73626) (Fig.1) using BWA mem.<sup>31</sup> SNPs were then called  
138 using GATK2<sup>32</sup> in unified genotyper mode. Core genome positions that had a high quality SNP (>90%  
139 consensus, minimum depth 10x, GQ >= 30, MQ >=30) in at least one strain were extracted and  
140 RAxML v8.1.17 used to derive a maximum likelihood tree for the *S. Blockley* genomes.

141

### 142 **Location and characterization of drug resistance region**

143 *De novo* assembly graphs (in fastg format) produced by Spades v.3.7.0 of isolates were visualised  
144 using Bandage (<http://github.com/rrwick/Bandage>)<sup>33</sup> (Fig.2). Blast analysis  
145 ([blast.ncbi.nlm.nih.gov/Blast.cgi](http://blast.ncbi.nlm.nih.gov/Blast.cgi)) was conducted to detect the macrolide resistant genes and location  
146 in the assembled contigs. Prokka was used to annotate genome sequences  
147 (<http://www.ncbi.nlm.nih.gov/pubmed/24642063>).<sup>34</sup> Artemis  
148 ([www.sanger.ac.uk/resources/software/artemis](http://www.sanger.ac.uk/resources/software/artemis)) was used to visualise the resistant region and  
149 annotated contigs of the genomic resistant island was then drawn using EasyFig.<sup>35</sup>

150

### 151 **Nucleotide sequence accession number**

152 The nucleotide sequence of the Salmonella Azithromycin Resistance Genomic Island (SARGI) was  
153 assigned a GenBank accession number KX237654

154

155

### 156 ***In silico* plasmid detection**

157 PlasmidFinder (<http://cge.cbs.dtu.dk/services/PlasmidFinder/>) was used to detect known plasmid  
158 replicons types of plasmids in the isolates studied.<sup>36</sup>

159

### 160 **Plasmid extraction**

161 Plasmid DNA was isolated as previously described<sup>37</sup> in accordance to the methods of Kado and Liu  
162 (1981).<sup>38</sup>

163

## 164 Phenotypic and PCR susceptibility testing

165 Susceptibility testing for isolates harbouring azithromycin resistance determinants was performed  
166 using a well established breakpoint agar dilution method using Iso-sensitest agar or Muller Hinton  
167 agar to determine if the isolate is susceptible or resistant to a known concentration of the  
168 antimicrobial(1). The antimicrobial concentrations used for screening of resistance were: ampicillin  
169 8mg/L, chloramphenicol 8 and 16mg/L, colomycin 2mg/L, sulphonamide 256mg/L, gentamicin 2mg/L,  
170 tobramycin 8mg/L, amikacin 8mg/L, streptomycin 16mg/L, tetracycline 8mg/L , trimethoprim 2mg/l ,  
171 nalidixic acid 16mg/L, ciprofloxacin 0.064 and 0.5 mg/L, ceftazidime 1 and 2 mg/L, cefotaxime 0.5 and  
172 1 mg/L, cefoxitin 8 mg/L, ceftiofime 8mg/L , ertapenem 0.064 and 0.5 mg/L, and temocillin 128  
173 mg/L.<sup>1,20,39</sup> Azithromycin susceptibility testing was performed using E tests(ABiodisk/Biomeriux,  
174 France) and MIC  $\leq$  16mg/L according to the EUCAST guidelines were used for interpretation of  
175 resistance.<sup>20</sup> Antimicrobial susceptibility testing was subjected to internal quality assurance (QA) in  
176 accordance with the published methods and to external quality assurance in collaboration with  
177 laboratories within the European Union Reference Laboratory Antimicrobial Resistance (EURL-  
178 AMR).<sup>1,39</sup>

179 NTS isolates were classified as multidrug resistant if they were resistant to three or more antimicrobial  
180 agents.<sup>6</sup> Isolates which were resistant to cefotaxime 1mg/L were subjected to an in house PCR assay  
181 to detect mechanisms of  $\beta$ -lactam resistance (CTX-M extended spectrum  $\beta$ -lactamases, and genes  
182 encoding for Amp C, SHV, TEM, GES, VEB, PER  $\beta$ -lactamases<sup>3</sup>).

183

## 184 Results

185 Genomes of 667 *Salmonella* isolates were screened for known acquired resistance genes including  
186 those previously associated with resistance to azithromycin in *Enterobacteriaceae*. The presence of  
187 azithromycin resistance determinants *mphA* (n=6), *mphB* (n=2) and *mefB* (n=1), amongst other  
188 resistance determinants conferring resistance to  $\beta$ -lactams, aminoglycosides, quinolones,  
189 tetracycline, and sulphonamides were identified in only nine genomes as detailed in Table 1.  
190 Phenotypic susceptibility testing confirmed the multidrug-resistance phenotypes of the corresponding  
191 nine isolates and had MICs for azithromycin ranging from  $6\geq$  or  $\geq 16$  mg/L (Table 1).

192

193 Strains were confirmed to be NTS by MLST (MLST database : <http://mlst.warwick.ac.uk/mlst/>) and  
194 classical serology of which three were *S. Typhimurium* (ST19 and ST34), three *S. Blockley* (ST52),  
195 and one each of *S. Thompson* (ST26), *S. Ridge* (novel ST not found in the MLST database) and *S.*  
196 *Kentucky* (ST198) (Table 1).

197 Table 1 shows the epidemiological data and analysis of the mechanism of drug resistance, in  
198 particular to azithromycin resistance detected by phenotypic (MIC), genetic (WGS) and  
199 molecular(PCR) methods in the nine NTS isolates studied.

200

201 In addition to the sequence type, we also investigated the whole genome similarity between the three  
202 *S. Typhimurium* and three *S. Blockley* isolates. The phylogenetic analysis and metadata of the *S.*  
203 *Typhimurium* isolates indicated that they were not closely related (data not shown) and different  
204 genes (*mphA*, *mphB* and *mefB*) associated with azithromycin resistance/partial resistance were  
205 involved suggesting it had been acquired following separate events . In addition to the three  
206 *S. Blockley* isolates harbouring *mphA*, a further 16 *S. Blockley* isolates not included in the initial  
207 screening process were used as background isolates in the *S. Blockley* phylogenetic analysis (Supp.  
208 Table 1 and Fig.1). Six of the additional 16 *S. Blockley* isolates harboured the *mphA* gene. The  
209 resultant phylogeny separated the azithromycin resistant *S. Blockley* isolates harbouring *mphA*  
210 (cluster 3) from the azithromycin sensitive isolates (cluster 1 and 2) (Fig. 1). The diversity between  
211 the azithromycin resistant isolates was not consistent with them being derived from a single  
212 epidemiologically related event with SNP differences ranging from 0 – 50 (Fig. 1). The inferred point  
213 of insertion of the *mphA* gene in the azithromycin resistant *S. Blockley* population is indicated in  
214 Fig.1.

215

216 When the genomic context of the resistance genes was investigated, it was found that they were on  
217 contigs that showed homology to either chromosomes or plasmids. (Supp. Table 2 ). Bandage , Blast,  
218 Prokka and Artemis analysis of the nine *S. Blockley* isolates harbouring the *mphA* gene and the ten  
219 *mphA* negative *S. Blockley* isolates shows *mphA* being inserted downstream from a *livF* gene on a  
220 chromosomal contig (Fig. 2 and 3). The chromosomally mediated macrolide inactivation gene cluster  
221 *mphA-mrx-mphr(A)* which is flanked by IS6100 and IS26 elements is part of a larger composite  
222 transposon inserted within the coding sequence of the ribokinase gene(*rbsK*) in all the nine *S.*



223 Blockley isolates (Fig.2 and 3). However, we were not able to resolve the full island structure using  
224 Illumina data, so long read technology was used for a representative isolate.

225 We generated a total of 10913 2D MinION reads (both pass and fail were used) with a mean length of  
226 3133 bp. When mapped using bwa mem, 9076 reads (83%) mapped back to the Illumina only  
227 assembly of H123780513 giving an average depth of 5.8x. This depth of coverage is not sufficient for  
228 de novo assembly, so a hybrid assembly approach was used. The hybrid Illumina-MinION assembly  
229 resolved the complete structure of the c.17kb Salmonella Azithromycin Resistance Genomic island  
230 (SARGI)(Fig.3).The island harboured tetracycline and aminoglycoside resistance genes as well as  
231 phage and plasmid remnants.

232 There are various ways of detecting plasmids from WGS sequence data using bioinformatic tools,<sup>40,41</sup>  
233 but in this study we used classical plasmid extraction analysis to show that all the azithromycin  
234 sensitive isolates (except for 140242 which is a MDR isolate) were plasmid free (Fig.1 and sup Fig.1)  
235 . PlasmidFinder confirmed the absence of known replicon sequence types in the respective genomes.

236 Two of the *mphA* positive S. Blockley isolates (H123780513 and 73633) did not harbour any plasmids  
237 while the other isolates had an *incN*,*colpVC* or *col156* plasmid which did not seem to be associated  
238 with the azithromycin resistance (Fig.1). Preliminary Bandage, Blast, Prokka , Artemis and  
239 PlasmidFinder analysis also suggests that the *mphA* gene is present on a *incFIB(K)* plasmid in S.  
240 Thompson, *incA/C2* plasmid in S. Ridge and on either an *incQ1* or *incH12* plasmid in S. Typhimurium  
241 (data not shown). Characterisation of the complete resistance regions in each of the plasmids  
242 belonging to the different serovars were not carried out as it was beyond the remit of this study.

243

244 The age of the nine cases from whom the isolates were acquired ranged from 5 to 79 years and five  
245 were males (Table 1). The nine isolates were recovered from urine (n=1) and stool (n=8) and were  
246 multidrug resistant. One isolate was acquired from a case with history of recent travel to Egypt and  
247 was identified as a S. Kentucky. The isolate was confirmed to be an AmpC producer by both  
248 phenotypic (ceftoxamine MIC> 1 mg/L and cefoxitin MIC> 8mg/L) and molecular methods (PCR  
249 demonstrated *bla*<sub>CMY-2</sub> gene). Another multidrug resistant isolate identified as S Ridge, was acquired  
250 from a case with underlying immunosuppression (post bone marrow transplant) and had recent  
251 exposure to antibiotics.

252 The azithromycin MICs of the six non *S. Blockley* and nine *S. Blockley* isolates showed that the  
253 presence of the *mphA* gene was associated with MIC $\geq$ 16mg/L, while presence of *mefB* or *mphB* was  
254 not associated with MIC $\geq$ 16 mg/L (Table 1 and supp. Table 1).

255

## 256 Discussion

257 Multidrug resistance in typhoidal and non-typhoidal *Salmonella* is an emerging threat to public  
258 health.<sup>3,4,5,6,8</sup> Azithromycin is being used as the preferred antimicrobial agent to treat cases of  
259 uncomplicated enteric fever from Asia and multidrug drug resistant NTS in the immunosuppressed or  
260 with invasive infections. However there are emerging reports of azithromycin resistance in cases of  
261 enteric fever as well as invasive NTS infection.<sup>9,10,12,42,43</sup>

262 The incidence of azithromycin resistance is increasing in *E coli*, *Klebsiella* and *Shigella*. Azithromycin  
263 resistant *Shigella* spp isolated from men who have sex with men (MSM) who had previous multiple  
264 exposures to azithromycin have been reported.<sup>44</sup> Decreased susceptibility to azithromycin (DSA) is  
265 defined as a strain of *Shigella* with azithromycin MIC $>$ 16mg/L; such strains often harbour genes *ermB*  
266 and *mphA* which are plasmid encoded and are associated with clinical failure.<sup>44,45,46</sup> A Canadian study  
267 showed that strains of *S flexneri* isolated from MSM harboured *mphA* gene and had azithromycin  
268 MIC  $>$  64 mg/L.<sup>45</sup>

269 This study identified the presence of known azithromycin resistance determinants in 15 *Salmonella*  
270 isolates. Twelve out of the 15 isolates encoded the *mphA* gene and these isolates had azithromycin  
271 MIC between 16 mg/L to 96 mg/L, none of these isolates carried *ermB* (Table 1). Two isolates  
272 encoded only *mphB* while one isolate encoded only *mefB*; these three isolates all had azithromycin  
273 MICs less than 16 mg/L. These results indicate that carriage of only *mphB* or *mefB* may not lead to  
274 azithromycin resistance in *Salmonella*, as described previously in *S. flexneri*,<sup>44</sup> and that the presence  
275 of other genes, such as the *erm* cluster/genes or chromosomal mutations in the *rrl* ribosomal genes,  
276 may be required for a synergistic effect to produce higher resistance to azithromycin (or azalide  
277 group).<sup>14</sup> However larger studies with a more diverse set of *Salmonellae*, and more in depth functional  
278 characterisations, are needed to understand the resistance mechanisms associated with these  
279 genes.

280 The single isolate of *S. Kentucky* which was a AmpC producer and carried the *bla*<sub>cmv-2</sub> gene was  
281 associated with travel to Egypt, a finding consistent with previous studies.<sup>3</sup> This isolate was typed as  
282 ST198 Kentucky and this serovar has been reported to be an ESBL producer.<sup>47</sup>

283 It is interesting to note that the 15 *Salmonella* isolates were multidrug resistant and the presence of  
284 plasmids and mobile genetic elements may have played a crucial role in acquisition of resistance to  
285 multiple agents. There were various mechanisms involved in high level azithromycin resistance in the  
286 different *Salmonella* serovars studied, conferred either on the chromosome or plasmid. In *S. Blockley*  
287 azithromycin resistance was not associated with the presence of a plasmid but rather a  
288 chromosomally mediated macrolide inactivation gene cluster *mphA-mrx-mphr(A)*. The macrolide  
289 inactivation gene cluster was part of a novel SARGI which was inserted in the same chromosomal  
290 *rbsK* gene in all the azithromycin resistant *S. Blockley* isolates (Fig.3). This chromosomal *mphA-mrx-*  
291 *mphr(A)* gene cluster has not been described previously in *Salmonellae* but has been recently  
292 characterised in a genomic island in *Proteus mirabilis*.<sup>48</sup> The complete structure of the azithromycin  
293 drug island in *S. Blockley* (Fig.3) was deduced by hybrid genome assembly of long MinION reads and  
294 short Illumina reads. Recently the *mphA* gene was shown to be present on a plasmid encoded drug  
295 island in *Salmonella* Corvallis.<sup>12</sup> This plasmid drug island differed from the one described in the current  
296 study as it did not have the same macrolide inactivation cluster. Azithromycin resistance in  
297 *S. Typhimurium*, *S. Thompson* and *S. Ridge* was associated to the presence of the *mphA* gene located  
298 on a plasmid. The plasmids associated to the *mphA* gene in each of these serovars, *S. Typhimurium*  
299 (*incQ1* or *incHI2*), *S. Ridge* (*incA/C2*), *S. Thompson* (*incFIB(K)*) differed from the one described by Villa  
300 et al, 2015<sup>12</sup> for *S. Corvallis* as well as the *incFII* plasmid associated with azithromycin resistance in  
301 *Shigella*,<sup>46</sup> thus providing further evidence of multiple modes of transmission for azithromycin  
302 resistance.

303 The presence of *S. Blockley* isolates with chromosomally mediated high levels of azithromycin  
304 resistance in the UK population is a cause of concern. This stable chromosomal resistance may lead  
305 to the dissemination of resistant clones that can cause outbreaks. Phylogenetic analysis of the nine  
306 azithromycin resistant and ten background susceptible *S. Blockley* isolates studied indicated that the  
307 majority of the resistant isolates are not clonally related and the probable point of insertion of the  
308 *mphA* gene in the population is indicated on the phylogenetic tree (Fig.1) . Clonal relatedness were

309 observed for two resistant isolates (78657 and 90479) which were isolated from the same patient a  
310 few months apart (probable treatment failure), as well as 63017 and 73615 isolated from two separate  
311 cases in London and West Midland (possible undetected outbreak clone) (Supp. Table 1 and Fig.1).  
312 Screening for azithromycin resistance is not conducted routinely but should be encouraged as in the  
313 past 3 years there has been 16 -26 S. Blockley isolates submitted to GBRU each year with most  
314 being domestically acquired (PHE data).

315 There is also a cause of concern as plasmid mediated resistance to azithromycin is arising in multiple  
316 *Salmonella* serovars in the UK that may lead to easier and widespread onwards transmission of  
317 resistance. This rise of both chromosomally and plasmid mediated azithromycin resistance may be  
318 due to the increase of azithromycin usage and increase of azithromycin resistance in other  
319 Enterobacteriaceae populations.<sup>7,14,49</sup>

320

321 This study demonstrated the utility of WGS data as a rapid screening tool allowing many hundreds of  
322 isolates to be investigated for antimicrobial resistance determinants not routinely assayed using  
323 phenotypic tests. The availability of WGS data as well as phenotypic and epidemiological  
324 investigations allows emerging threats, such as azithromycin resistance in Enterobacteriaceae, to be  
325 monitored in a cost effective and timely manner. High throughput screening for surveillance is not  
326 only beneficial for public health purposes as it allows to detect the presence of azithromycin  
327 resistance in the population but enhanced surveillance of patients can be carried out to understand  
328 onwards transmission. Such data in turn can be used to inform clinicians to administer appropriate  
329 treatment. At present there is no clinical breakpoints for azithromycin that have been defined for  
330 Enterobacteriaceae by EUCAST or CLSI. However, further work on strains of NTS and those causing  
331 enteric fever needs to be undertaken to establish if 16 mg/L is the clinically relevant clinical MIC for  
332 azithromycin in *Salmonella* spp. This ECOFF established from wild type strains seems very high  
333 compared to clinical MICs for other Gram positive and Gram negative bacteria, however azithromycin  
334 has a very high tissue: serum concentration ratio.<sup>50</sup>

335

336 Using WGS for detection of antibiotic resistance also lends itself to data sharing, enabling  
337 international collaboration in the monitoring of this global threat. As part of this approach, continued  
338 phenotypic characterisation of antimicrobial resistance for a subset of isolates is vital to ensure that  
339 novel resistance mechanisms are discovered.

340

341 One of the limitations of this study is the absence of a complete clinical history of each case. This  
342 prevents us correlating our work with clinical outcome. Moreover most of the isolates were from stool  
343 specimens and the cases may not have received antimicrobials if they had self limiting infections.

#### **344 Conclusion**

345 Azithromycin resistance is probably under-reported in the UK and globally as front line laboratories do  
346 not test for azithromycin resistance in NTS due to the cost. This study has shown that WGS is an  
347 effective method for screening large numbers of isolates for known resistance determinants . Further  
348 clinical studies are needed to establish the role of various resistance genes in determination of clinical  
349 MIC in conjunction with WGS. Even though the numbers of azithromycin resistance in *Salmonella*  
350 spp. from the UK remained low (15/683 isolates studied), the detection of azithromycin resistance in  
351 multiple serovars of *Salmonella* is a matter of concern and regular monitoring and surveillance  
352 should be a priority .

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#### **361 Transparency declaration**

362 None to declare

### 363 Supplementary Data

364 Supp. Table 1 and 2. Supp. Fig.1

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## Figures

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529 Figure 1:

530 Phylogenetic relationship between azithromycin resistant and susceptible *S. Blockley* isolates  
531 circulating in the UK between 2012 - 2015. Phylogenetic tree generated by SNP analysis. 73626 was  
532 the reference strain used for de novo assembly for SNP detection. Insertion point of *mphA* into *S.*  
533 *Blockley* population indicated. Presence (inc group)/absence of plasmid, year of isolation and location  
534 shown beside isolate number.

535

536 Figure 2 :

537 Bandage assembly of nodes(contigs) from susceptible and resistant *S. Blockley* isolates. Bandage  
538 allows visualisation of how contigs (in gray) are possibly connected (in black) to each other. The  
539 genes of interest (in this case *livF*, *rhsK* and *mphA*) are then blasted against all the assembled contigs

540 and its location determined. Regions around the genes of interest can then be determined using  
541 Artemis.

542 (a) *livF* and *rbsK* located on a chromosomal node 5 in a azithromycin susceptible *S. Blockley*  
543 isolate. *mphA* not present.

544 (b) *mphA* gene (azithromycin resistance) and the other regions associated to resistance (node  
545 27, 23,20) is inserted in between *rbsK* in a azithromycin resistant *S. Blockley* isolate.

546 The figure appears in colour in the online version of JAC and in black and white in the print version of  
547 JAC.

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550 Figure 3 :

551 The chromosomal insertion site of the azithromycin resistance gene (*mphA*) and possible structure of  
552 the Salmonella Azithromycin Resistance Genomic Island (SARGI) .

553 Chromosomal nodes are based on bandage assembly (Fig. 2). Insertion site of  
554 drug island in *rbsK* depicted by blue lines . Postulated structure of drug island,

555

556 *neo* – aminoglycoside, *tet*-tetracycline,

557 *mphA*- macrolide (azithromycin), *mrx* – major facilitator protein, *mphr(A)* – macrolide repressor A,

558 *tnp* - transposase

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## 566 Table

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568 Table 1 :

569 Epidemiological features and analysis of azithromycin resistance mechanisms detected by  
570 phenotypic (MIC) , genotypic (WGS) and molecular(PCR) methods in nine Non typhoidal *Salmonella*  
571 *enterica* isolates

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## 574 Supplementary Tables and Figures

575

576 Supplementary Table 1 :

577 Epidemiological features of selected *S. Blockley* isolates between 2012 -2015 used for azithromycin  
578 resistance mechanisms detection and phylogenetic analysis. Isolates H123740558, H123780513 and  
579 H124040535 were used in the initial WGS screening process and the other isolates were used as  
580 background strains for the phylogenetic analysis.

581 Supp. Table 2 :

582 Contigs harbouring genes associated to azithromycin (AZT) resistance and its association to either  
583 chromosomal or plasmid regions in *Salmonella* isolates

584

585 Supplementary Figure 1 4:

586 Plasmid gel to show the absence of plasmids in azithromycin *mphA* resistance *S. Blockley*.

587 Lane 1 : *E. coli* marker, lane 2 : H123780513, a *mphA* positive plasmid free isolate,

588 lane 3 : H145040693, a *mphA* negative plasmid free isolate

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631 Table 1 : Epidemiological features and analysis of azithromycin resistance mechanisms detected by phenotypic (MIC) , genotypic (WGS) and  
 632 molecular(PCR) methods in nine Non typhoidal *Salmonella enterica* isolates

N	Age	Sex	Travel	Origin of isolate	Serotype	MLST	Azithromycin Resistance gene (WGS)	Other resistance genes (WGS)	Phenotypic resistance profile				β lactamase ( PCR)
									AZT 16	CIP<0.064	CIP > 0.5	Other antibiotics	
SAL1	28	M	Nil	Faeces	Thompson	26	<i>mphA</i>	<i>aac-lb-cr;aac-ly;aadA16;qnrB6;arr-3;sul3;dfrA27;sul1</i>	16	>0.064		SUL-TMP	
SAL2	67	M	Nil	Urine	Typhimurium	19	<i>mphB</i>	<i>aac-laa;aph-lt;TEM-98,TEM-1;sul3;sul2;tet(A)-1;dfrA1</i>	6	<0.064		AMP-SUL-STR-TET-TMP	
SAL3	58	F	Nil	Faeces	Typhimurium	34	<i>mphA</i>	<i>aac-llt;aac-laa;aadA17;aph-lt;TEM-98,TEM-1;qnrS1;lnu(F);arr-2;sul2;tet(A);dfrA5;dfrA14;dfrA25</i>	96	>0.064	>0.5	AMP-SUL-GEN-TOB-STR-TET-TMP-NAL-FOX	
SAL4	79	M	Nil	Faeces	Typhimurium	34	<i>mefB</i>	<i>aac-laa;aadA2;aph-lt;TEM-98,TEM-1;sul2;tet(A);dfrA12</i>	8	<0.064		AMP-SUL-STR-TET-TMP	

SAL5	58	M	Nil	Faeces	Ridge	Novel ST	<i>mphA</i>	<i>aac-IIa;aac-ly;aph-IId;TEM-98,TEM-1;sul2;dfra14;tet(A)-1</i>	16	<0.064		AMP-SUL-GEN-TOB-STR-TET-TMP	
H1237 40558	5	M	Nil	Faeces	Blockley	52	<i>mphA</i>	<i>aac-ly;aph-IId;tet(A)-1</i>	24	>0.064	>0.5	STR-TET-NAL	
H1237 80513	78	F	Not known	Faeces	Blockley	52	<i>mphA</i>	<i>aac-ly;tet(A)-1</i>	24	>0.064	>0.5	TET-NAL	
H1240 40535	59	F	Nil	Faeces	Blockley	52	<i>mphA</i>	<i>aac-ly;aph-IId;tet(A)-1</i>	48	>0.064		STR-TET-NAL	
SAL6	38	F	Egypt	Faeces	Kentucky	198	<i>mphB</i>	<i>aac-Ie;aac-Ib;aac(6')-Iaa;aadA7;aph-IId;CMY-2;OXA-10;TEM-98,TEM-1;cmlA1;sul3;tet(A)-1;aadA1;floR</i>	8	>0.064	>0.5	AMP-SUL-GEN-TOB-STR-TET-NAL-CAZ2-CTX1-FOX	Amp C <i>bla</i> <sub>CMY-2</sub>

633

634 Breakpoints for antibiotics: AMP- Ampicillin 8mg/L, CHL chloramphenicol 8 and 16mg/L, SUL sulphonamide 256mg/L, GEN gentamicin 2mg/L, TOB tobramycin 8mg/L, STR  
634 streptomycin 16mg/L, TET tetracycline 8mg/L, TMP trimethoprim 2mg/l, NAL nalidixic acid 16mg/L, CIP ciprofloxacin 0.064 and 0.5 mg/L, CAZ ceftazidime 1 and 2 mg/L, CTX  
635 cefotaxime 0.5 and 1 mg/L, FOX cefoxitin 8 mg/L, AZT Azithromycin 16mg/L

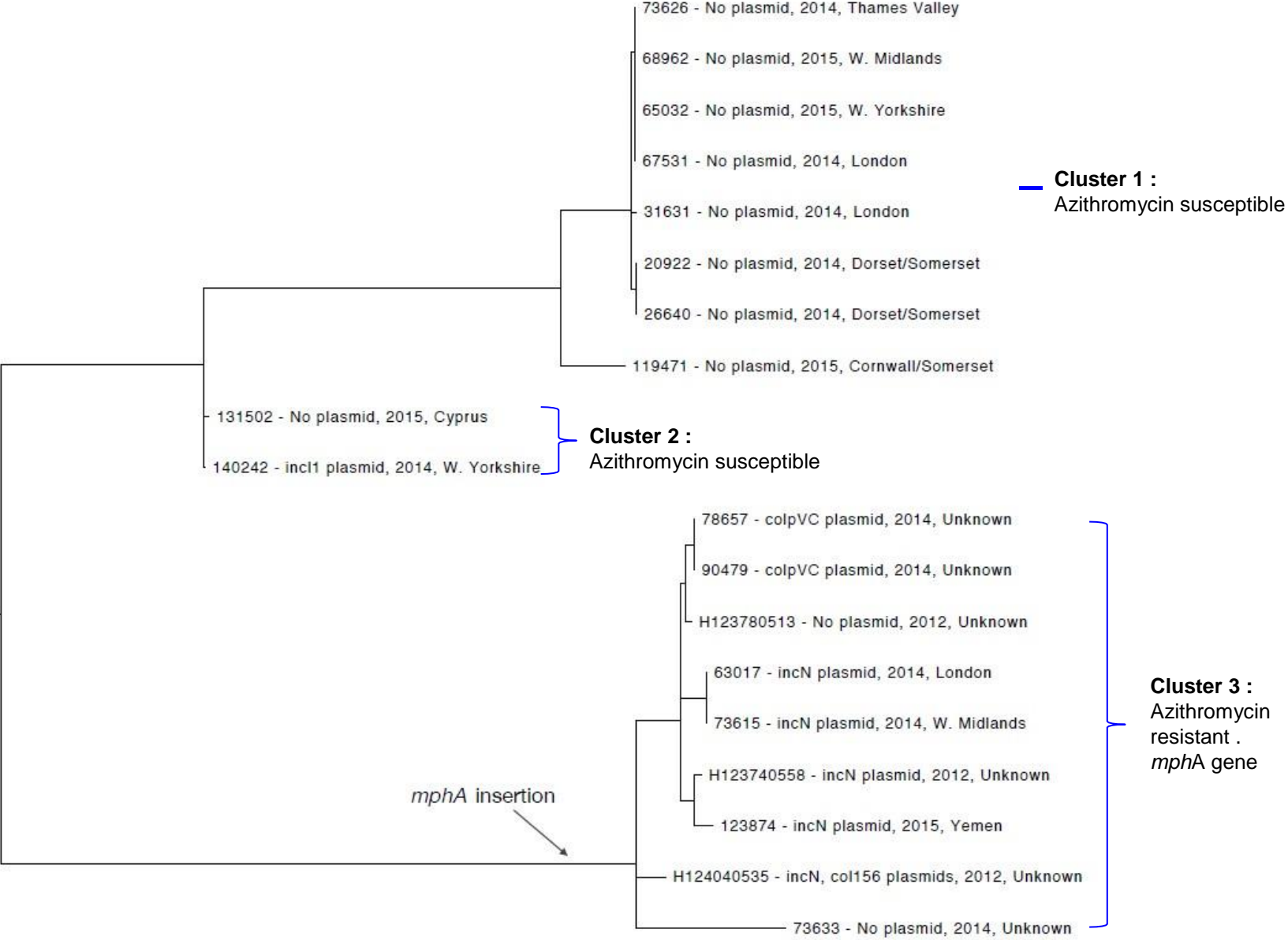
636 *aac*, *aph*, *aad* (aminoglycosides), *qnrB6/S1* (quinolone), *arr* (rifampin), *sul* (sulfonamide), *dfra* (trimethoprim), TEM-1 (Beta-lactam, ampicillin), TEM-98(Beta-lactam,  
637 ampicillin), *tet* (tetracycline), *Inu* (oxazolidinone), CMY-2 (Amp C Beta lactam), OXA-10 (Beta-lactam), *cmlA1*(Chloramphenicol), *floR* (Chloramphenicol),  
638 *mph*, *mef*(macrolide)

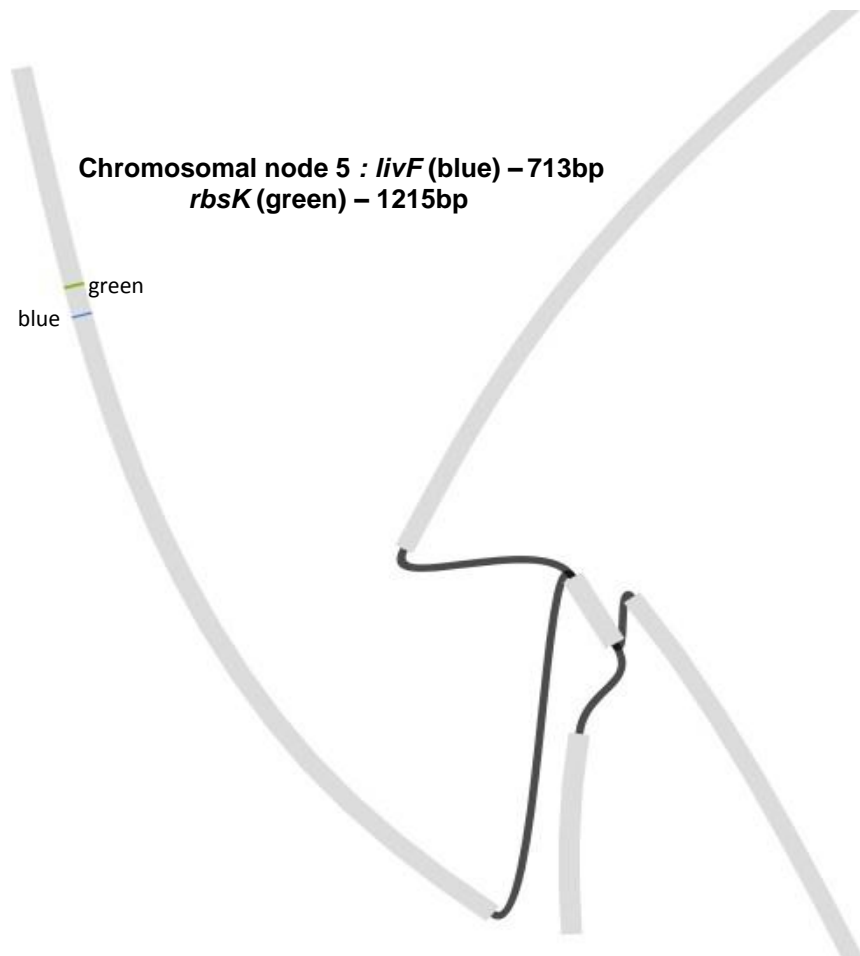
639 WGS – whole genome sequencing

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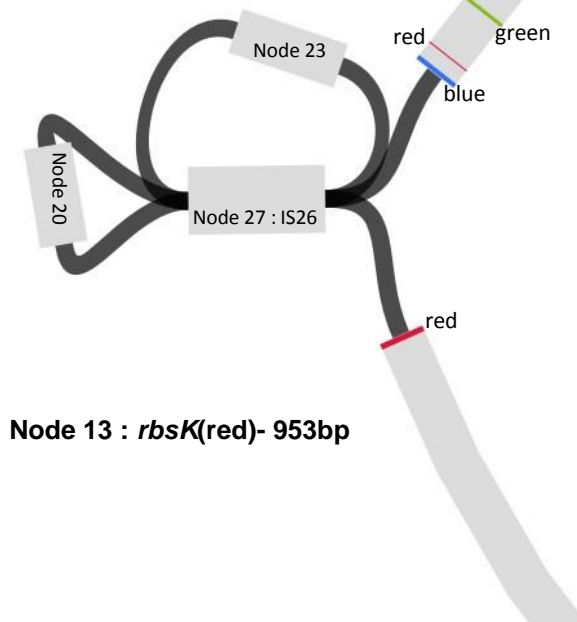
**Figure 1 :** Phylogenetic relationship between azithromycin resistant and susceptible *S. Blockley* isolates circulating in the UK between 2012 -2015. Phylogenetic tree generated by SNP analysis. 73626 was the reference strain used for de novo assembly for SNP detection. Insertion point of *mphA* into the *S. Blockley* population indicated. Presence (inc group)/absence of plasmid, year of isolation and location shown beside isolate number.





**(a) H144600627 AZT sensitive *S. Blockley***

**Chromosomal node 6 : *mphA*(blue)- 906bp  
: *rbsK* (red)- 270bp  
: *livF* (green) – 713bp**



**(b) H123780513 AZT resistant *S. Blockley***

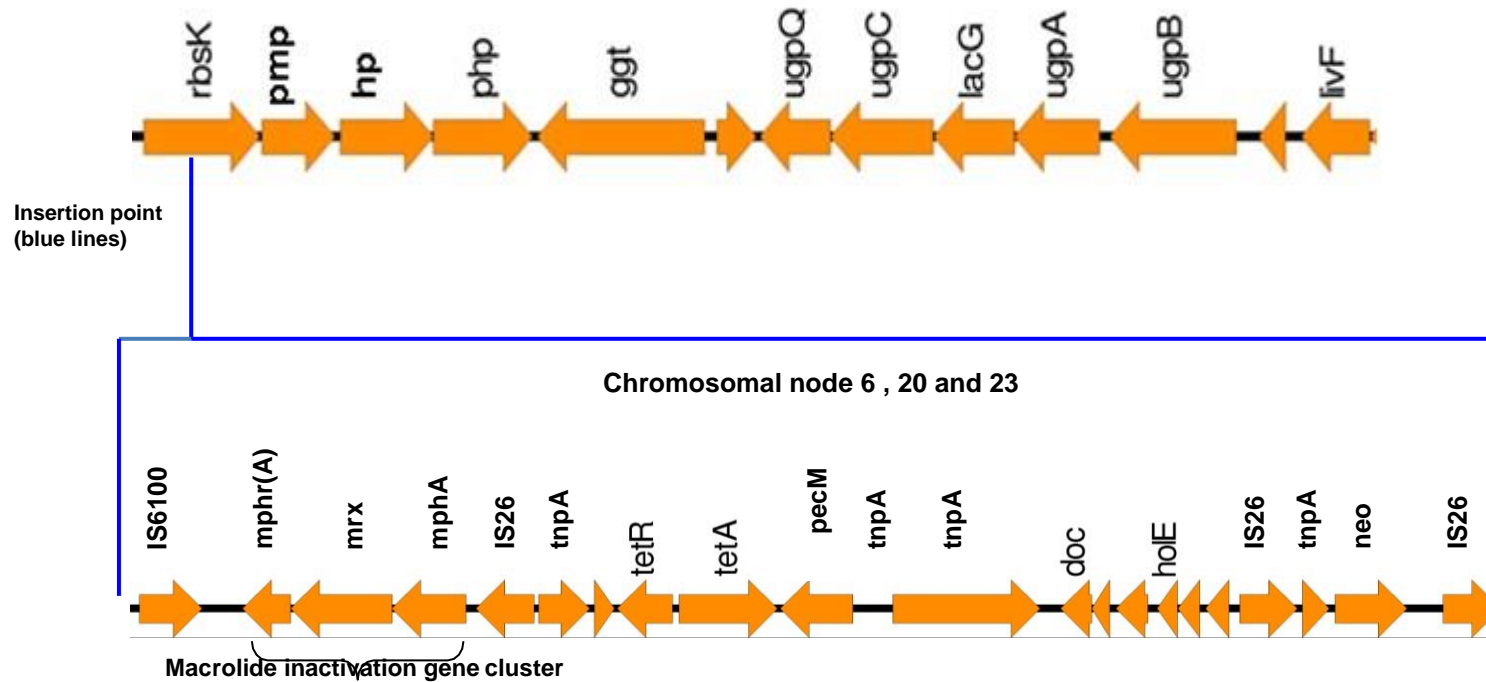
**Fig 2 :** Bandage assembly of nodes(contigs) from susceptible and resistant *S. Blockley* isolates.

Bandage allows visualisation of how contigs (in gray) are possibly connected (in black) to each other. The genes of interest (in this case *livF*, *rbsK* and *mphA*) are then blasted against all the assembled contigs and its location determined. Regions around the genes of interest can then be determined using Artemis..

(a) *livF* and *rbsK* located on a chromosomal node 5 in a azithromycin sensitive *S. Blockley* isolate. *mphA* not present.

(b) *mphA* gene (azithromycin resistance) and the other regions associated to resistance (node 27, 23, 20) is inserted in between *rbsK* in a azithromycin resistant *S. Blockley* isolate

The figure appears in colour in the online version of JAC and in black and white in the print version of JAC.



(b) H123780513 (AZT resistant S. Blockley)

**Figure 3 :** The chromosomal insertion site of azithromycin resistance gene (*mphA*) and structure of the Salmonella Azithromycin Resistance Genomic Island (SARGI)

Chromosomal nodes are based on bandage assembly (Fig. 2). Insertion site of drug island in *rbsK* depicted by blue lines .

*neo* – aminoglycoside, *tet*- tetracycline,  
*mphA*- macrolide (azithromycin), *mrx* – major facilitator protein, *mphr(A)* – macrolide repressor A, *tnp* – transposase  
 The figure appears in colour in the online version of JAC and in black and white in the print version of JAC.



**Supplementary Table 1** : Epidemiological features of selected *S. Blockley* isolates between 2012 -2015 used for azithromycin resistance mechanisms detection and phylogenetic analysis. Isolates H123740558, H123780513 and H124040535 were used in the initial WGS screening process and the other isolates were used as background strains for the phylogenetic analysis.

N	Age	Sex	Travel	Location	Year	Origin	Serotype	ML ST	AZT resistance gene (WGS)	Other resistance gene (WGS)	Phenotypic resistance	
											AZT	Other antibiotics
H123740558	5	M	Nil		12/9/12	Faeces	Blockley	52	<i>mphA</i>	<i>aac(6')-ly;aph(6')-ld;tet(A)-1</i>	24	STR-TET-NAL
H123780513	78	F	Not known		14/9/12	Faeces	Blockley	52	<i>mphA</i>	<i>aac(6')-ly;tet(A)-1</i>	24	TET-NAL
H124040535	59	F	Nil		3/10/12	Faeces	Blockley	52	<i>mphA</i>	<i>aac(6')-ly;aph(6')-ld;tet(A)-1</i>	48	STR-TET-NAL
78657	57	M			23/12/14		Blockley	52	<i>mphA</i>	<i>aac(6')-ly;tet(A)-1;gyrA(83:S-F,87:D-G);parC(57:T-S,80:S-R)</i>	64	TET-NAL
90479	57	M			17/2/15		Blockley	52	<i>mphA</i>	<i>aac(6')-ly;tet(A)-1;gyrA(83:S-F,87:D-G);parC(57:T-S,80:S-R)</i>	48	TET-NAL
63017	85	F		London	29/10/14	Faeces	Blockley	52	<i>mphA</i>	<i>aph(6')-ld;aac(6')-ly;strB;strA;tet(A)-1</i>	32	STR-TET-NAL
73615	56	F		West Midlands	9/12/14	Faeces	Blockley	52	<i>mphA</i>	<i>aph(6')-ld;aac(6')-ly;strB;strA;tet(A)-1</i>	32	STR-TET-NAL
123874	6	M	Yemen	London	17/6/15	Faeces	Blockley	52	<i>mphA</i>	<i>aph(6')-ld;aac(6')-ly;strB;strA;tet(A)</i>	48	ND

										-1		
73633	71	M		Sussex/Surrey	10/12/14	Faeces	Blockley	52	<i>mphA</i>	<i>aph(6')-IId;aac(6')-ly;strB;strA;tet(A)-1;gyrA(83:S-F);parC(57:T-S)</i>	32	STR-TET-NAL
65032	62	F		Sussex/Surrey	5/11/14	Faeces	Blockley	52	NP	ND	0.5	Sensitive
67531	65	M		Wales	10/11/14	Faeces	Blockley	52	NP	<i>aac(6')-ly;parC(57:T-S)</i>	1	Sensitive
73626	76	M		Thames Valley	10/12/14	Faeces	Blockley	52	NP	<i>aac(6')-ly;parC(57:T-S)</i>	1	Sensitive
68962	65	M		West Midlands	18/11/14	Faeces	Blockley	52	NP	<i>aac(6')-ly;parC(57:T-S)</i>	2	Sensitive
31631	53	F		London	15/7/14	Faeces	Blockley	52	NP	<i>aac(6')-ly;parC(57:T-S)</i>	2	Sensitive
26640	22	M		Dorset/Somerset	8/7/14	Faeces	Blockley	52	NP	<i>aac(6')-ly;parC(57:T-S)</i>	2	Sensitive
20922	48	M		Dorset/Somerset	11/6/14	Faeces	Blockley	52	NP	<i>aac(6')-ly;parC(57:T-S)</i>	4	Sensitive
119471	66	F		Devon,Cornwall, Somerset	2/6/15	Faeces	Blockley	52	NP	<i>aac(6')-ly;parC(57:T-S)</i>	1	Sensitive
131502	25	M	Cyprus	West Midlands	21/7/15	Faeces	Blockley	52	NP	<i>aph(6')-IId;aac(6')-ly;strB</i>	1	ND
140242	56	M		West Yorkshire	28/7/15	Faeces	Blockley	52	NP	ND	1	ND

Breakpoints for antibiotics: AMP- Ampicillin 8mg/L, CHL chloramphenicol 8 and 16mg/L, SUL sulphonamide 256mg/L, GEN gentamicin 2mg/L, TOB tobramycin 8mg/L, STR streptomycin 16mg/L, TET tetracycline 8mg/L, TMP trimethoprim 2mg/l, NAL nalidixic acid 16mg/L, CAZ ceftazidime 1 and 2 mg/L, CTX cefotaxime 0.5 and 1 mg/L, FOX cefoxitin 8 mg/L, AZT Azithromycin 16mg/L

*aac*, *aph*, *aad*, *str* (aminoglycosides), *sul* (sulfonamide), *dfp* (trimethoprim), *tet* (tetracycline), *lhu* (oxazolidinone), *par*, *gyr* (fluoroquinolone and nalidixic acid), *mphA*(macrolide)

WGS – whole genome sequencing

NP – Not present ; ND – Not determined

*aac(6')-ly* (probably kanamycin not tested in the lab).

*parC(57:T-S)* – single point mutation does not confer resistance to nalidixic acid or fluroquinolone.

**Supplementary Table 2** : Contigs harbouring genes associated to azithromycin (AZT) resistance and its association to either chromosomal or plasmid regions in *Salmonella* isolates

Isolate	Serotype	AZT resistant gene	Contig	Blast results
H122160478	Typhimurium	<i>mef</i>	<i>mefB</i> :active:NODE_37_length_50898_cov_51.726315:50956:347_1576	Plasmid + chromosome
H120620408	Thompson	<i>mph</i>	<i>mphA</i> :active:NODE_60_length_5225_cov_35.930717:5289:4251_5156	Plasmid
H121580347	Typhimurium	<i>mph</i>	<i>mphB</i> :active:NODE_7_length_96182_cov_30.584007:96256:79218_80126	Plasmid
H122040374	Typhimurium	<i>mph</i>	<i>mphA</i> :active:NODE_4_length_3611_cov_53.037663:3673:130_1035	Plasmid + chromosome
H122760596	Ridge	<i>mph</i>	<i>mphA</i> :active:NODE_31_length_6613_cov_24.291395:6673:129_1034	Plasmid
H123740558	Blockley	<i>mph</i>	<i>mphA</i> :active:NODE_84_length_114820_cov_25.853493:114894:113847_114752	Chromosome
H123780513	Blockley	<i>mph</i>	<i>mphA</i> :active:NODE_24_length_301287_cov_25.917475:301345:128_1033	Chromosome
H124040535	Blockley	<i>mph</i>	<i>mphA</i> :active:NODE_77_length_28024_cov_28.921745:28074:119_1024	Chromosome
H124580300	Kentucky	<i>mph</i>	<i>mphB</i> :active:NODE_48_length_71964_cov_24.346270:72018:7474_8382	Plasmid

**Supp. Fig. 1 :** Plasmid gel to show the absence of plasmids in azithromycin *mphA* resistance *S. Blockley*.  
Lane 1 : *E. coli* marker, lane 2 : H123780513, a *mphA* positive plasmid free isolate,  
lane 3 : H145040693, a *mphA* negative plasmid free isolate.

