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

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

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Abstract

Objectives:

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

Methods:.

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

Results:

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

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

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

Conclusion:

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

Introduction

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

Acquired resistance to macrolides/azalides may be caused by several different mechanisms of resistance.[13] They include (i) target site modification by methylases encoded by *erm* genes,[14,15] (ii) modifying enzymes such esterases encoded by *ere*A and B genes or phosphotransferases encoded by *mph*A,B and D genes,[16,17] (iii) efflux pumps, e.g. *mef*A and *msr*A found mainly in Gram positive bacteria, with *mef*A also identified in Gram negative strains,[15] (iv) Mutations in the *rrl* and *rpl* genes encoding ribosomal proteins L22,L4 and 23S rRNA also confer resistance in Gram positive bacteria.[18] Full cross resistance between erythromycin and azithromycin can be confered between these genes.[14]

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

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

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

including *Salmonella*, by either the Clinical and Laboratory Standards Institute (CLSI) or EUCAST leading to delays in early detection of azithromycin resistance. However the epidemiological cut-off (ECOFF) for azithromycin has been accepted as ≤16 mg/L for *Salmonella enterica*.[11,20]

The recent advancement in WGS technologies for routine microbiology is well documented.[21] Sequence data allows rapid identification of *Salmonella* serotypes by Multilocus Sequence Typing (MLST) as proposed by Achtman *et al* (2012).[22] In addition, availability of the whole genome sequences allows *in silico* prediction of antimicrobial resistance that should be validated by phenotypic antimicrobial testing prior to being applied.[23,24,25]

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

Methods

Bacterial isolates and phenotypic typing

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

DNA extraction for WGS

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

DNA from the treated cells were then extracted on the Qiasymphony SP platform (Qiagen) and eluted in 100 uL of water.

DNA concentration using the GloMax system (Promega) and quality (optimal OD260/230 = 1.8 -2.0) using the LabChip DX system (Perkin Elmer) were determined for the following sequencing steps.

DNA sequencing

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

MinION sequencing was also carried out to define the complete structure of the genomic drug island in the S. Blockley isolate H123780513. A library was prepared using Genomic DNA Sequencing Kit SQK–MAP006 according to the protocol from Oxford Nanopore Technologies (Version MN006_1115_revC_14Aug2015) and following the same principles as described in Ip *et al* 2015 [28] except the following: sheared DNA was repaired using FFP repair mix (New England Biolabs, Ipswich, Massachusetts) and then prepared using the NEBNext Ultra II End-Repair / dA-tailing Module (New England Biolabs). The final ligation of adapter and hairpin was performed using adapters and tethers from SQK–MAP006 sequencing kit (Oxford Nanopore Technologies, Oxford, UK) followed by purification of the adapted and tethered DNA using MyOne C1 beads (Life Technologies). Purified DNA was loaded for sequencing to the flow cell (R9 chemistry) by Oxford Nanopore Technologies (Oxford, UK).

Sequence assembly and detection of resistance genes

Genome assembly was carried out using Spades v.3.7.0(http://www.ncbi.nlm.nih.gov/pmc/articles/PMC3342519/) with the command line options '–k 21, 33, 55, 77' and '--careful'. Detection of resistance genes were as described by Doumith *et al* (2015).[29] Briefly reads were mapped to reference database of acquired genes including those conferring resistance to macrolides that were collated from the Comprehensive Antibiotic Resistance Database (http://arpcard.mcmaster.ca).

Spades v.3.7.0 hybrid assembly was used to combine the MinION reads with the Illumina reads. MinION reads were mapped back to the hybrid assembly and this mapping was used to confirm the contiguity of key parts of the hybrid assembly

Phylogenetic analysis

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

Location and characterization of drug resistance region

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

Nucleotide sequence accession number

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

In silico plasmid detection

PlasmidFinder (http://cge.cbs.dtu.dk/services/PlasmidFinder/) was used to detect known plasmid replicons types of plasmids in the isolates studied.[36]

Plasmid extraction

Plasmid DNA was isolated as previously described[37] in accordance to the methods of Kado and Liu (1981).[38]

Phenotypic and PCR susceptibility testing

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

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

Results

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

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 *mph*A 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 IS*6100* and IS*26* 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,[40,41]
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* $_{\text{CMY-2}}$ 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.

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

Discussion

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

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

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

280 The single isolate of *S*. Kentucky which was a AmpC producer and carried the *bla* $_{cmy-2}$ gene was
281 associated with travel to Egypt, a finding consistent with previous studies.[3] This isolate was typed as
282 ST198 Kentucky and this serovar has been reported to be an ESBL producer.[47]

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*.[48] 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.[12]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[12] for *S*. Corvallis as well as the incFII plasmid associated with azithromycin resistance in
301 *Shigella*,[46] 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

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

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

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

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

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

Conclusion

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

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Transparency declaration

None to declare

Supplementary Data

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

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Figures

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

Figure 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 susceptible *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.

Figure 3 :
The chromosomal insertion site of the azithromycin resistance gene (*mphA*) and possible 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 . Postulated structure of drug island,

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.

Table

Table 1 :

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

Supplementary Tables and Figures

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.

Supp. Table 2 :

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

Supplementary Figure 1 4:
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

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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 typhiodal *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	*mphA*	*aac-lb-cr*;*aac-ly*;*aadA16*;*qnrB6*;*arr-3*;*sul3*;*dfrA27*;*sul1*	16	>0.064		SUL-TMP	
SAL2	67	M	Nil	Urine	Typhimurium	19	*mphB*	*aac-Iaa*;*aph-Id*;TEM-98,TEM-1;*sul3*;*sul2*;*tet(A)-1*;*dfrA1*	6	<0.064		AMP-SUL-STR-TET-TMP	
SAL3	58	F	Nil	Faeces	Typhimurium	34	*mphA*	*aac-IId*;*aac-Iaa*;*aadA17*;*aph-Id*;TEM-98,TEM-1;*qnrS1*;*lnu(F)*;*arr-2*;*sul2*;*tet(A)*;*dfrA5*;*dfrA14*;*dfrA25*	96	>0.064	>0.5	AMP-SUL-GEN-TOB-STR-TET-TMP-NAL-FOX	
SAL4	79	M	Nil	Faeces	Typhimurium	34	*mefB*	*aac-Iaa*;aadA2;*aph-Id*;TEM-98,TEM-1;*sul2*;*tet(A)*;*dfrA12*	8	<0.064		AMP-SUL-STR-TET-TMP	

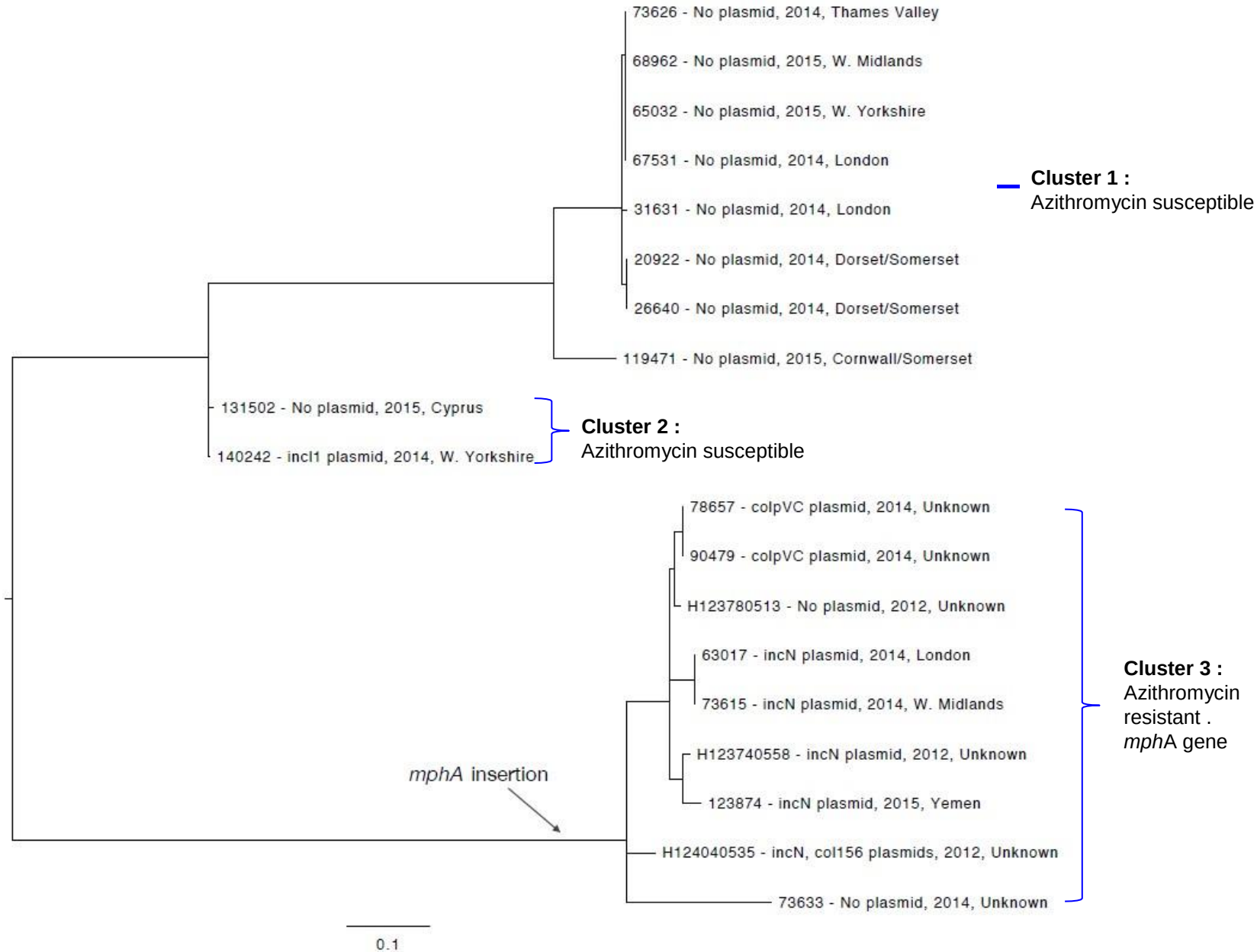
SAL5	58	M	Nil	Faeces	Ridge	Novel ST	*mphA*	*aac-IIa*;*aac-Iy*;*aph-Id*;TEM-98,TEM-1;*sul2*;*dfrA14*;*tet(A)-1*	16	<0.064		AMP-SUL-GEN-TOB-STR-TET-TMP	
H1237 40558	5	M	Nil	Faeces	Blockley	52	*mphA*	*aac-Iy*;*aph-Id*;*tet(A)-1*	24	>0.064	>0.5	STR-TET-NAL	
H1237 80513	78	F	Not known	Faeces	Blockley	52	*mphA*	*aac-Iy*;*tet(A)-1*	24	>0.064	>0.5	TET-NAL	
H1240 40535	59	F	Nil	Faeces	Blockley	52	*mphA*	*aac-Iy*;*aph-Id*;*tet(A)-1*	48	>0.064		STR-TET-NAL	
SAL6	38	F	Egypt	Faeces	Kentucky	198	*mphB*	*aac-Ie*;*aac-Ib*;*aac(6')-Iaa*;*aadA7;aph-Id;*CMY-2;OXA-10;TEM-98,TEM-1;*cmlA1*;*sul3*;*tet(A)-1*;*aadA1*;*floR*	8	>0.064	>0.5	AMP-SUL-GEN-TOB-STR-TET-NAL-CAZ2-CTX1-FOX	Amp C *bla* $_{\text{CMY-2}}$

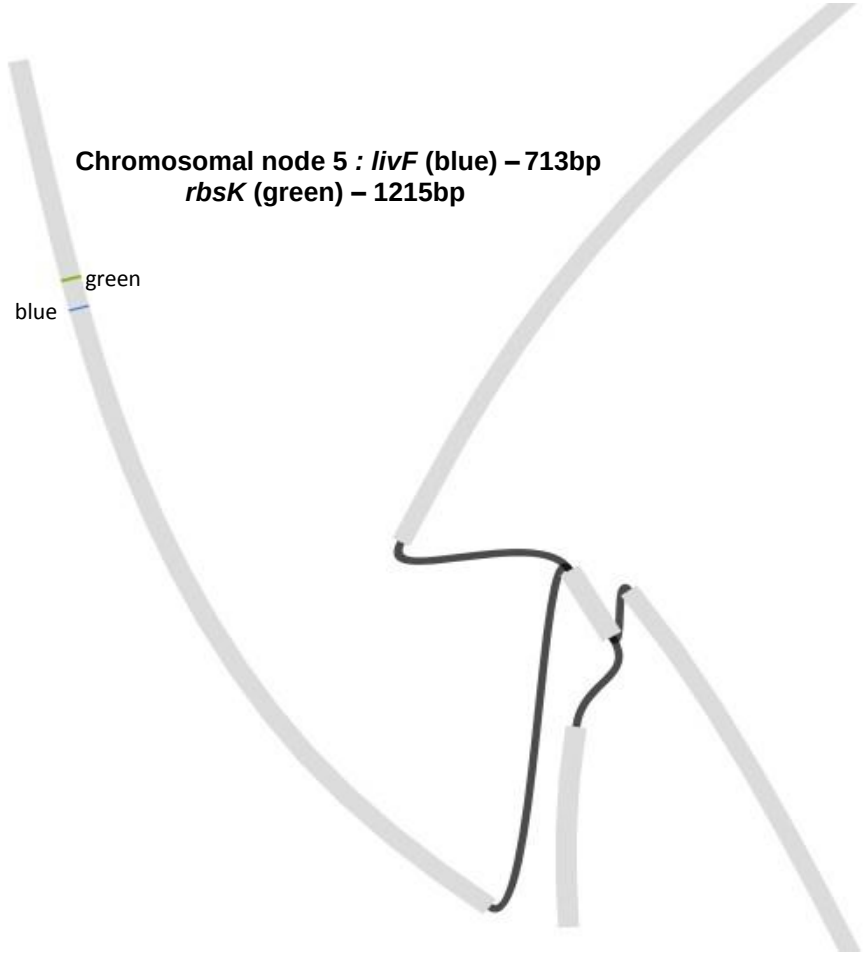
Breakpoints for antibiotics: AMP- Ampicillin 8mg/L, CHL chloramphenicol 8 and 16mg/L, SUL sulphonamide 256mg/L, GEN gentamicin 2mg/L, TOB tobramycin 8mg/L, STR streptomycin 16mg/L, TET tetracycline 8mg/L , TMP trimethoprim 2mg/l , NAL nalidixic acid 16mg/L, CIP ciprofloxacin 0.064 and 0.5 mg/L, CAZ ceftazidime 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* (aminoglycosides), *qnrB6/S1* (quinolone), *arr* (rifampin), *sul* (sulfonamide*), dfr* (trimethoprim), TEM-1 (Beta-lactam, ampillicin), TEM-98(Beta-lactam, ampillicin), *tet* (tetracycline), *lnu* (oxazolidinone), CMY-2 (Amp C Beta lactam), OXA-10 (Beta-lactam), *cmlA1*(Chloramphenicol*), floR* (Chloramphenicol), *mph,mef*(macrolide)

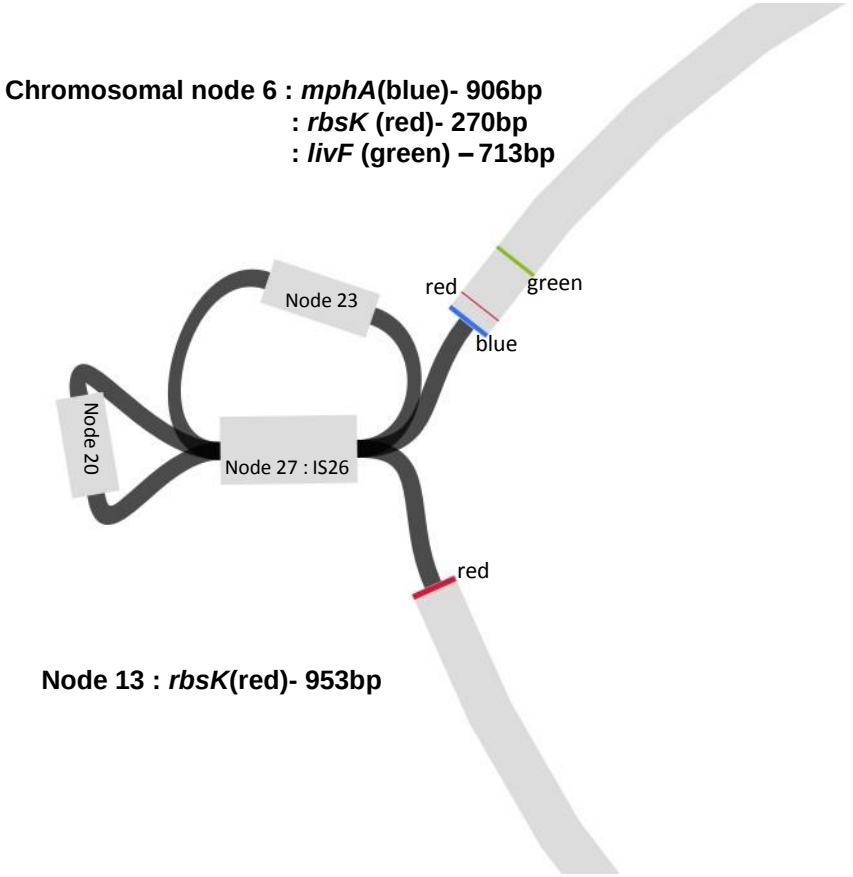
WGS – whole genome sequencing

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 *mph*A 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



(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..

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

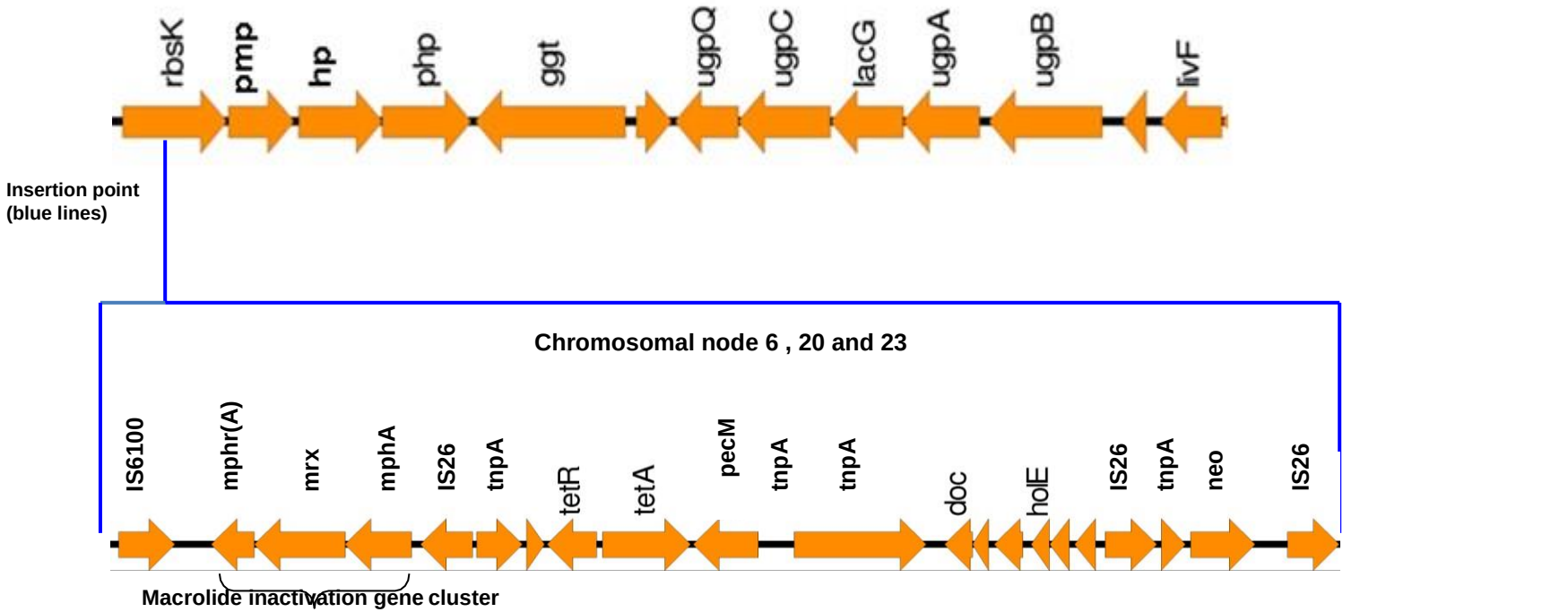

Figure 3 : The chromosomal insertion site of azithromycin resistance gene (*mphA*) and structure of the Salmonella Azithromycin Resistance Genomic Island (SARGI)

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

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73633	71	M		Sussex/Surrey	10/12/14	Faeces	Blockley	52	*mphA*	*aph(6')-Id;aac(6')-Iy;strB;strA;tet(A)-1*; *gyrA*(83:S-F); *parC*(57:T-S)	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	*aac(6')-Iy*; *parC*(57:T-S)	1	Sensitive
73626	76	M		Thames Valley	10/12/14	Faeces	Blockley	52	NP	*aac(6')-Iy*; *parC*(57:T-S)	1	Sensitive
68962	65	M		West Midlands	18/11/14	Faeces	Blockley	52	NP	*aac(6')-Iy*; *parC*(57:T-S)	2	Sensitive
31631	53	F		London	15/7/14	Faeces	Blockley	52	NP	*aac(6')-Iy*; *parC*(57:T-S)	2	Sensitive
26640	22	M		Dorset/Somerset	8/7/14	Faeces	Blockley	52	NP	*aac(6')-Iy*; *parC*(57:T-S)	2	Sensitive
20922	48	M		Dorset/Somerset	11/6/14	Faeces	Blockley	52	NP	*aac(6')-Iy*; *parC*(57:T-S)	4	Sensitive
119471	66	F		Devon,Cornwall, Somerset	2/6/15	Faeces	Blockley	52	NP	*aac(6')-Iy*; *parC*(57:T-S)	1	Sensitive
131502	25	M	Cyprus	West Midlands	21/7/15	Faeces	Blockley	52	NP	*aph(6')-Id;aac(6')-Iy;strB*	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), *dfr* (trimethoprim), *tet* (tetracycline), *lnu* (oxazolidinone), *par*,*gyr* (fluoroquinolone and nalidixic acid), mphA(macrolide)

WGS – whole genome sequencing

NP – Not present ; ND – Not determined

aac(6')-Iy (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	*mef*	*mefB*:active:NODE_37_length_50898_cov_51.726315:50956:347_1576	Plasmid + chromosome
H120620408	Thompson	*mph*	*mphA*:active:NODE_60_length_5225_cov_35.930717:5289:4251_5156	Plasmid
H121580347	Typhimurium	*mph*	*mphB*:active:NODE_7_length_96182_cov_30.584007:96256:79218_80126	Plasmid
H122040374	Typhimurium	*mph*	*mphA*:active:NODE_4_length_3611_cov_53.037663:3673:130_1035	Plasmid + chromosome
H122760596	Ridge	*mph*	*mphA*:active:NODE_31_length_6613_cov_24.291395:6673:129_1034	Plasmid
H123740558	Blockley	*mph*	*mphA*:active:NODE_84_length_114820_cov_25.853493:114894:113847_114752	Chromosome
H123780513	Blockley	*mph*	*mphA*:active:NODE_24_length_301287_cov_25.917475:301345:128_1033	Chromosome
H124040535	Blockley	*mph*	*mphA*:active:NODE_77_length_28024_cov_28.921745:28074:119_1024	Chromosome
H124580300	Kentucky	*mph*	*mphB*: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.