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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*

Key words: whole genome sequencing, multilocus sequence typing, *Salmonella*, azithromycin resistance, MinION
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 mphA, mphB or mefB. These genes were either plasmid or chromosomally mediated.

Azithromycin resistant S. Blockley isolates harboured a macrolide inactivation gene cluster mphA- mrx-mpfr(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 mphA 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. Widespread resistance to amoxicillin, chloramphenicol, trimethoprim-sulfamethoxazole and fluoroquinolones 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. It is also used to treat infections with multidrug resistant non-typhoidal *Salmonella* (NTS) in vulnerable patients who have prolonged or invasive infections. 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. However, reports are emerging of azithromycin resistance in cases of enteric fever as well as invasive NTS infection.

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

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. 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°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°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 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). 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.SNPs were then called using GATK2 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) (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). 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.

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.

Plasmid extraction

Plasmid DNA was isolated as previously described in accordance to the methods of Kado and Liu (1981).
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. 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. 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). NTS isolates were classified as multidrug resistant if they were resistant to three or more antimicrobial agents. 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).

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 metB (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).
Strains were confirmed to be NTS by MLST (MLST database: http://mlst.warwick.ac.uk/mlst/) and classical serology of which three were S. Typhimurium (ST19 and ST34), three S. Blockley (ST52), and one each of S. Thompson (ST26), S. Ridge (novel ST not found in the MLST database) and S. Kentucky (ST198) (Table 1). Table 1 shows the epidemiological data and analysis of the mechanism of drug resistance, in particular to azithromycin resistance detected by phenotypic (MIC), genetic (WGS) and molecular (PCR) methods in the nine NTS isolates studied.

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

When the genomic context of the resistance genes was investigated, it was found that they were on contigs that showed homology to either chromosomes or plasmids. (Supp. Table 2). Bandage, Blast, Prokka and Artemis analysis of the nine S. Blockley isolates harbouring the mphA gene and the ten mphA negative S. Blockley isolates shows mphA being inserted downstream from a livF gene on a chromosomal contig (Fig. 2 and 3). The chromosomally mediated macrolide inactivation gene cluster mphA-mrx-mpfr(A) which is flanked by IS6100 and IS26 elements is part of a larger composite transposon inserted within the coding sequence of the ribokinase gene (rbsK) in all the nine S.
Blockley isolates (Fig.2 and 3). However, we were not able to resolve the full island structure using Illumina data, so long read technology was used for a representative isolate.

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

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

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

The age of the nine cases from whom the isolates were acquired ranged from 5 to 79 years and five were males (Table 1). The nine isolates were recovered from urine (n=1) and stool (n=8) and were multidrug resistant. One isolate was acquired from a case with history of recent travel to Egypt and was identified as a S. Kentucky. The isolate was confirmed to be an AmpC producer by both phenotypic (ceftoxamine MIC> 1 mg/L and cefoxitin MIC> 8mg/L) and molecular methods (PCR demonstrated bla_CMY-2 gene). Another multidrug resistant isolate identified as S Ridge, was acquired from a case with underlying immunosuppression (post bone marrow transplant) and had recent 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. 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. 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. 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. A Canadian study showed that strains of S flexneri isolated from MSM harbourled mphA gene and had azithromycin MIC > 64 mg/L. 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, 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). 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.
The single isolate of *S*. Kentucky which was a AmpC producer and carried the *bla* _cmy-2_ gene was associated with travel to Egypt, a finding consistent with previous studies. This isolate was typed as ST198 Kentucky and this serovar has been reported to be an ESBL producer. It is interesting to note that the 15 *Salmonella* isolates were multidrug resistant and the presence of plasmids and mobile genetic elements may have played a crucial role in acquisition of resistance to multiple agents. There were various mechanisms involved in high level azithromycin resistance in the different *Salmonella* serovars studied, conferred either on the chromosome or plasmid. In *S*. Blockley azithromycin resistance was not associated with the presence of a plasmid but rather a chromosomally mediated macrolide inactivation gene cluster *mphA-mrx-mphr(A)*. The macrolide inactivation gene cluster was part of a novel SARGI which was inserted in the same chromosomal _rbsK_ gene in all the azithromycin resistant *S*. Blockley isolates (Fig.3). This chromosomal *mphA-mrx-mphr(A)* gene cluster has not been described previously in Salmonellae but has been recently characterised in a genomic island in *Proteus mirabilis*. The complete structure of the azithromycin drug island in *S*. Blockley (Fig.3) was deduced by hybrid genome assembly of long MinION reads and short Illumina reads. Recently the *mphA* gene was shown to be present on a plasmid encoded drug island in *Salmonella* Corvallis. This plasmid drug island differed from the one described in the current study as it did not have the same macrolide inactivation cluster. Azithromycin resistance in *S*. Typhimurium, *S*. Thompson and *S*. Ridge was associated to the presence of the *mphA* gene located on a plasmid. The plasmids associated to the *mphA* gene in each of these serovars, *S*. Typhimurium (incQ1 or incHI2), *S*. Ridge (incA/C2), *S*. Thompson (incFIB(K)) differed from the one described by Villa et al, 2015 for *S*. Corvallis as well as the incFII plasmid associated with azithromycin resistance in *Shigella*, thus providing further evidence of multiple modes of transmission for azithromycin resistance.

The presence of *S*. Blockley isolates with chromosomally mediated high levels of azithromycin resistance in the UK population is a cause of concern. This stable chromosomal resistance may lead to the dissemination of resistant clones that can cause outbreaks. Phylogenetic analysis of the nine azithromycin resistant and ten background susceptible *S*. Blockley isolates studied indicated that the majority of the resistant isolates are not clonally related and the probable point of insertion of the *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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Funding declaration

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Transparency declaration
None to declare

Supplementary Data

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

References


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**Figures**

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529 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 *S. Blockley* population indicated. Presence (inc group)/absence of plasmid, year of isolation and location shown beside isolate number.

536 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.

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.

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

<table>
<thead>
<tr>
<th>N</th>
<th>Age</th>
<th>Sex</th>
<th>Travel</th>
<th>Origin of isolate</th>
<th>Serotype</th>
<th>MLST</th>
<th>Azithromycin Resistance gene (WGS)</th>
<th>Other resistance genes (WGS)</th>
<th>Phenotypic resistance profile</th>
<th>β-lactamase (PCR)</th>
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<td>SAL1</td>
<td>28</td>
<td>M</td>
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<td>Thompson</td>
<td>26</td>
<td>mphA</td>
<td>aac-Ib-cr; aac-Ia; aadA16; qnrB6; arr-3; sul3; dfrA27; sul1</td>
<td>AZT16 CIP&lt;0.064 CIP &gt; 0.5</td>
<td>Other antibiotics</td>
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<td>mphB</td>
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<td>Typhimurium</td>
<td>34</td>
<td>mphA</td>
<td>aac-lld; aac-laa; aadA17; aph-ld; TEM-98, TEM-1; qnrS1; lnu(F); arr-2; sul2; tet(A); dfrA2; dfrA5; dfrA14; dfrA2; dfrA5</td>
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<td>mefB</td>
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<td>Faeces</td>
<td>Ridge</td>
<td><strong>Novel ST</strong></td>
<td><strong>mphA</strong></td>
<td><strong>aac-llb; aac-ld; aph-lid:TEM-98; TEM-1; sul2; dfrA14; tet (A)-1</strong></td>
<td><strong>16</strong></td>
<td><strong>&lt;0.064</strong></td>
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<td>&gt;0.064</td>
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<td><strong>8</strong></td>
<td><strong>&gt;0.064</strong></td>
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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 0.5mg/L, CAZ ceftazidime 1 and 2mg/L, CTX cefotaxime 0.5 and 1mg/L, FOX cefoxitin 8mg/L, AZT Azithromycin 16mg/L

aac, aph, aad (aminoglycosides), qnrB6/S1 (quinolone), arr (rifampin), sul (sulfonamide), dfr (trimethoprim), TEM-1 (Beta-lactam, ampicillin), TEM-98 (Beta-lactam, ampicillin), 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 *mphA* into the *S. Blockley* population indicated. Presence (inc group)/absence of plasmid, year of isolation and location shown beside isolate number.

Cluster 1: Azithromycin susceptible
- 73626 - No plasmid, 2014, Thames Valley
- 69962 - No plasmid, 2015, W. Midlands
- 65032 - No plasmid, 2015, W. Yorkshire
- 67531 - No plasmid, 2014, London
- 31631 - No plasmid, 2014, London
- 20922 - No plasmid, 2014, Dorset/Somerset
- 26640 - No plasmid, 2014, Dorset/Somerset
- 119471 - No plasmid, 2015, Cornwall/Somerset

Cluster 2: Azithromycin susceptible
- 131502 - No plasmid, 2015, Cyprus
- 140242 - incl plasmid, 2014, W. Yorkshire

Cluster 3: Azithromycin resistant. *mphA* gene
- 73657 - ccolVC plasmid, 2014, Unknown
- 90479 - ccolVC plasmid, 2014, Unknown
- H123780513 - No plasmid, 2012, Unknown
- 73615 - incN plasmid, 2014, W. Midlands
- H123740558 - incN plasmid, 2012, Unknown
- 123874 - incN plasmid, 2015, Yemen
- H124040535 - incN, col156 plasmids, 2012, Unknown
- 73633 - No plasmid, 2014, Unknown
Chromosomal node 5: 
- livF (blue) – 713bp
- rbsK (green) – 1215bp

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

Node 23: red
Node 27: IS26
Node 13: rbsK (red) – 953bp

(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.

(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, 20) are 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 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.


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.

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<th>Origin</th>
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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')-ly (probably kanamycin not tested in the lab).

parC(57:T-S) – single point mutation does not confer resistance to nalidixic acid or fluoroquinolone.
**Supplementary Table 2**: Contigs harbouring genes associated to azithromycin (AZT) resistance and its association to either chromosomal or plasmid regions in *Salmonella* isolates

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<th>Isolate</th>
<th>Serotype</th>
<th>AZT resistant gene</th>
<th>Contig</th>
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**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.