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Comparative genome analysis and global phylogeny of the toxin variant *Clostridium difficile* PCR Ribotype 017 reveals the evolution of two independent sub-lineages.

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Abstract

The diarrhoeal pathogen *Clostridium difficile* consists of at least six distinct evolutionary lineages. The RT017 lineage is anomalous as strains only express toxin B, compared to strains from other lineages that produce toxins A and B and occasionally binary toxin. Historically, RT017 were initially reported in Asia but have now been reported worldwide. We used whole genome sequencing and phylogenetic analysis to investigate the patterns of global spread and population structure of 277 RT017 isolates from animal and human origins from six continents, isolated between 1990 and 2013. We reveal two distinct evenly split sub-lineages (SL1 and SL2) of *C. difficile* RT017 that contain multiple independent clonal expansions. All 24 animal isolates were contained within SL1 along with human isolates suggesting potential transmission between animals and humans. Genetic analyses revealed an over representation of antibiotic resistance genes. Phylogeographic analyses show a North American origin for RT017 as has been found for the recently emerged epidemic RT027 lineage. Despite only having one toxin, RT017 strains have evolved in parallel from at least two independent sources and can readily transmit between continents.

Introduction

Clostridium difficile is a spore-forming obligate anaerobe that continues to be the leading cause of healthcare-associated infections in the developed world (1, 2). There are six main lineages that broadly split into PCR ribotypes (RTs) associated with RT027, RT023, RT017, RT078, a grouping of diverse RTs and the recently identified novel lineage containing RT131 (3). The global emergence of the RT027 strain was responsible for multiple outbreaks and increased disease severity in Canada and the United States in 2001 (4). This strain has since spread to South America (5-7), China (8), Japan (9), Hong Kong (10), Korea (11, 12), Taiwan (13), Singapore (14), Australia (15, 16), Saudi Arabia (17), Israel (18), New Zealand (19) and throughout Europe (5, 20-28). Although RT027 remains the dominant clone in the United States, Europe has seen a decline in RT027 with a simultaneous increase in other virulent RTs such as RT017 and RT078 (29).

Using whole genome sequencing (WGS) and phylogenetic analysis, He *et al*., (4) identified the presence of two genetically distinct sub-lineages of RT027 through single nucleotide polymorphism (SNP) analysis; both had emerged in North America within a relatively short period after acquiring the same fluoroquinolone resistance conferring mutation encoding an alteration in *gyrA* and a highly related conjugative transposon (4). The two epidemic sub-lineages showed distinct patterns of global spread, with one lineage spreading more widely and causing healthcare-associated outbreaks globally (4).

Traditionally, virulent *C. difficile* strains are characterised and identified in diagnostic laboratories by the presence of two potent toxins TcdA and TcdB (30). These genes

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are located on a 19.6 kb pathogenicity locus (PaLoc). There is genetic variation in this region which can be exploited and which has revealed 30 different toxinotypes including six A-B+ toxinotypes. The most common and clinically relevant is toxinotype VIII and these isolates belong to RT017 (31). It is well known that the *tcdA* gene of this type contains a 1.8 kb deletion at the 3' end and a nonsense mutation at *tcdA* amino acid 47 that introduces a stop codon leading to a truncated *tcdA* gene (31). RT017 strains also lack the binary toxin (CDT) found in for example pathogenic RT027 strains that produce all three toxins. Despite lacking two toxins, clinically significant *C. difficile* infection (CDI) has been reported worldwide for the RT017 lineage (32-41).

Historically, these strains were initially identified in CDI outbreaks in Asia and are thought to have spread to Europe and other continents. RT017 strains have been reported in: Canada (35, 42) China (34, 43), Korea (33, 44, 45), Argentina (46), Australia (47, 48), Israel (49), Japan (50) South Africa (51) and throughout Europe (36, 39, 41, 52, 53). These strains have also been isolated from non-human sources including equine, bovines (54) and rabbits (55). We recently performed WGS on 35 human and two hospital environmental isolates of RT017 circulating in London, United Kingdom and identified three SNP variants (39). One variant was found to be clonal and had persisted in a London hospital ward for at least five years (39).

Here, WGS and phylogenetic analysis was used to define the population structure of a collection of 277 RT017 isolates from six continents of human and non-human origins with isolation dates between 1990 and 2013. Analyses reveal that RT017 strains have evolved in parallel from at least two independent sources and can readily transmit

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149 between continents. Genotypic and phenotypic antimicrobial susceptibilities were also
150 compared.
151
152 **METHODS**
153 The 277 isolates described in this study are shown in table 1 and included 37 isolates
154 from a previous study (ENI study accession number ERP009770) (39) and the
155 remaining new to this study (ENA study accession number PRJEB11868). These were
156 of human (*n* = 251), environmental/hospital ward (*n* = 2), equine (*n* = 4), canine (*n* =
157 11) and bovine (*n* = 9) origin with isolation dates between 1990 and 2013. These
158 isolates were subjected to genomic DNA extraction as previously described by Stabler
159 *et al.,* (56). WGS data for the isolates was obtained using either the HiSeq 2000
160 Sequencing System or the MiSeq Sequencing System (Illumina, California, USA) and
161 libraries were created as previously described (57) or using Nextera XT kit (Illumina,
162 California, USA) respectively. The sequence data was processed and quality
163 controlled according to a standard pipeline as previously described (58). Briefly,
164 FASTQ formatted sequencing reads were quality controlled with a minimum quality
165 phred-score of 30 (as a rolling average over 4 bases) using trimmomatic (59). The
166 resulting reads were mapped using the BWA-MEM (60) software against the M68 *C.*
167 *difficile* reference strain and the majority of post-trimmed reads (>92% for all samples
168 passing quality control) were mapped to the reference. SNPs were called using
169 Samtools/VCFtools (61).
170
171 Velvet (62) and Velvet Optimiser (63) were used to *de novo* assembly the trimmed
172 reads into contigs producing 277 assemblies. Optimal k-mers fell between 53 bp and
173 97 bp and the mean n50 was over 928,000 bp. The mean longest contig was 1,067,000

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bp, with 71 samples producing contigs that covered over half of the genome (greater than ~2.15 Mbp) and 16 samples assembled to contigs greater than 4 Mbp (equivalent greater than 90% of the genome). Pipeline, post-analyses, genetic, phylogenetic, phylogeographic and cluster analysis were carried out using Perl, R, abacas, prokka, RaXML, Bayesian Evolutionary Analysis Sampling Trees (BEAST) and mclust software (64-68). A minor allele frequency (MAF) of less than 1% was used and to remove any SNPs that may be associated with recombination and which would mask the true phylogeny, SNPs within 1 bp distance of an insertion or deletion site were excluded from further analysis. We used BEAST (67) to produce a SNP phylogeny from the SNPs as well as geographical and temporal data combined in phylogeographic analysis and mclust software for maximum likelihood cluster analysis.

To determine the minimum inhibitory concentrations (MICs) of 7/277 isolates, dilutions for the antibiotics; chloramphenicol, rifampicin, tetracycline, erythromycin, naladixic acid, gentamicin, teicoplanin and ampicillin were made as previously described (69). Briefly, 10 ml pre-equilibrated Brain Heart Infusion broth, supplemented with yeast (Oxoid), L-Cysteine (Sigma) and *C. difficile* supplement (Oxoid) (BHIS) were inoculated with three colonies of 48 h culture on BHIS agar plates. Once the OD reached 0.3 nm, 24-well plates containing the antibiotic dilutions were inoculated with 1/100 of the BHIS broths and incubated. The ODs were measured 24 h post inoculation and MIC data were categorised as susceptible, intermediate and resistant following the Clinical and Laboratory Standards Institute (CLSI) and the European Committee on Antimicrobial Susceptibility Testing

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(EUCAST) guidelines. The reference strain M68 was used as a control as were appropriate negative controls.

RESULTS

WGS was performed on a global collection of 277 *C. difficile* RT017 isolates. Collectively, these were isolated from human (n=251), bovine (n=9), canine (n=11), equine (n=4) and hospital ward environments (n=2) between 1990 and 2013 (Supplementary Information 1). All isolates belonged to multilocus sequence type 37. After sequence quality control and mapping to the M68 RT017 reference genome (GenBank accession number FN668375), we identified 1288 high quality bi-allelic SNPs with 311 present in greater than 1% of samples and greater than 1 bp from an insertion or deletion. Of these non-rare SNPs, 65.6% (n=204) were non-synonymous, 17.7% (n=55) synonymous and 16.7% (n=52) were present in non-coding regions of the genome (non-synonymous SNPs are shown in Supplementary Information 2). Twelve SNPs affected stop-codons; eleven non-synonymous and one synonymous (Table 1).

SNP data revealed 109 haplotypes containing between 0 and 52 SNPs (with respect to the M68 reference) with 76.5% (212/277) of isolates having between 10 and 35 SNPs (Table 2).

We generated a maximum-likelihood phylogenetic tree based on the 1288 SNPs, which demonstrates the presence of two genetically diverse sub-lineages; SL1 and SL2 (Figures 1 and 2). Of the 1288 SNPs, 76% (977/1288) had a minor allele frequency (MAF) of ≤1% and/or were within 1 bp of an insertion or deletion. To

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control for false positive identification of SNPs (these SNPs may mask the true phylogeny of RT017) phylogenetic trees with and without these SNPs were generated. The inclusion of 977 SNPs only had a minor effect on the overall phylogenetic tree. Four SNPs were found to differentiate the two sub-lineages; one present in a non-coding region and three non-synonymous SNPs (Table 3). SL2 is the most distantly related to the reference M68 strain of the two sub-lineages and both sub-lineages are geographically and temporally widespread. All isolates from the previously reported study on London isolates fell into SL2 (39).

The RT017 strains are documented to have a higher level of antibiotic resistance compared to other *C. difficile* RTs (37, 70). Fluoroquinolone resistance in *C. difficile* has been associated with mutations in codon 82 of the *gyrA* gene and codon 426 of the *gyrB* gene. The common SNP found in the *gyrA* gene is T82I and the *gyrB* gene are A426V and A426A (71). Remarkably, we found 64.6% (179/277) to have the amino acid substitution found in the *gyrA* gene (T82I). A substitution in the *gyrB* gene (V426N) was present in 4.7% of strains (13/277) and an additional 10.1% (28/277) including M68 harboured a valine at position 426 of the predicted *gyrB* product (Table 2 and Supplementary Information 1). The T82I substitution was globally distributed in both sub-lineages. Additionally, substitutions in the 81-bp rifampicin resistance determining region of the *rpoB* gene; R505K, H502N and S485F were found in 32.5% (90/277), 33.2% (92/277) and 1.1% (3/277) respectively (Table 2 and Supplementary Information 1).

To investigate horizontal gene transfer, a key mechanism driving *C. difficile* evolution, we performed programmatic and visual inspection of the comparisons

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248 which revealed 56 regions of DNA between ~4 and ~61.5 kb that were absent in the
249 M68 strain but present in other strains. These had 34 different insertion sites (Table 2,
250 Figure 3 and Supplementary Information 1 and 4). Additionally, we found regions of
251 DNA of between ~8 and ~29 kb present in the M68 strain at six sites but absent from
252 multiple samples (Table 2 and Supplementary Information 1 and 3). These insertions
253 and deletions were associated with erythromycin, teicoplanin, tetracycline,
254 chloramphenicol and beta-lactam resistance genes and their products potentially
255 associated with virulence such as a two-component response regulator, a SAM
256 protein, an AntA/AntB antirepressor, a cell surface protein and a sporulation-specific
257 glycosylase (Supplementary Information 3 and 4). The deletions and insertions were
258 well distributed geographically and temporally and a 49 kb insertion found only in a
259 clonal cluster of 23/37 London isolates in our previous study (39) was also found to
260 insert at a different site in single isolates from Canada, USA and the UK with
261 isolation dates of 2006, 2006 and 2011 respectively (Figure 3). Only one SNP was
262 found in the toxin pathogenicity locus region, which was synonymous and present in
263 the non-functioning *tcdA* gene fragment from five Korean isolates in SL2 isolated
264 between 2004 and 2008. Visual inspection of the comparisons revealed both *tcdA* and
265 *tcdB* genes to be highly conserved; no sequence variations were found.
266
267 MICs were determined for eight *C. difficile* isolates (including M68 as a control)
268 against the antibiotics; chloramphenicol, rifampicin, tetracycline, erythromycin,
269 naladixic acid, gentamicin, teicoplanin and ampicillin. Their MIC values are shown in
270 table 4. All isolates were resistant to naladixic acid, gentamicin and ampicillin, either
271 resistant or intermediate resistance to tetracycline and all were sensitive to

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teicoplanin. Two (2/8) isolates were resistant to chloramphenicol, four (4/8) were resistant to rifampicin and 7/8 were resistant to erythromycin.

DISCUSSION

The RT017 lineage, with its unique toxin profile and unusual global prevalence, has been overshadowed by the global outbreak of the RT027 lineage. Reminiscent of the RT027 lineage, two distinct sub-lineages of *C. difficile* RT017 that contain multiple independent clonal expansions were revealed in this study. This division demonstrates that toxin variant strains emerged on at least one occasion, suggesting that a full toxin repertoire is not essential for efficient human-to-human transmission.

Based on our *gyrA* and *gyrB* SNP data, we would predict up to 76.2% (211/277) of isolates to be resistant to the fluoroquinolone class of antibiotics. Interestingly, the T82I SNP found in *gyrA* is the same mutation reported in the global outbreak of RT027 (4). Based on our MIC data, all eight isolates were resistant to naladixic acid indicating resistance to the fluoroquinolone class of antimicrobials.

Based on our rifampicin SNP data, we would predict 34.7% (96/277) of isolates in this study to be resistant to the rifampicin class of antibiotics. Interestingly, 82% (152/185) of these substitutions were found in SL1. R505K, H502N have previously been associated with rifampicin resistance in *C. difficile* (72), however, based on our MIC data, only two (2/8) isolates were sensitive to rifampicin with one of the isolates containing the R505K and H502N SNP indicating that these alone do not always lead to phenotypic resistance. Interestingly, S485F was found in three historical isolates from Wrexham, UK. This resistance conferring SNP has not previously been reported

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297 in *C. difficile*, only in *Mycobacterium tuberculosis* (73). All three isolates were
298 phenotypically resistant to rifampicin, however, all three isolates also contained the
299 R505K SNP and so confirming this SNP's contribution to resistance was not possible.
300 The multiple haplotypes revealed is similar with that found for the RT027 global
301 study where >100 distinct genotypes were found in 151 isolates. Despite SNPs and
302 insertion and deletions, there was no variation on susceptibility to ampicillin,
303 teicoplanin, gentamicin, or naladixic acid. However, there was some variation with
304 chloramphenicol, rifampicin, tetracycline and erythromycin. Whether the insertions
305 carrying chloramphenicol o-acetyltransferase, TetR-family transcriptional regulator or
306 the *ermB* gene played a role in this variation is unknown.
307
308 Figure 4 depicts the phylogeny of the isolates by source. Interestingly, the 24 animal
309 strains, which were all isolated from a similar location (Ontario, Canada) over a
310 relatively short time period (2002 and 2005), are distributed amongst human isolates
311 in SL1 only. This suggests there is possible transmission between humans and
312 animals.
313
314 The ready global distribution of RT017 suggests determinants independent of toxin B
315 are important in transmission. This could be related to the ready acquisition of
316 antibiotic resistance determinants, efficient germination and/or spore formation. This
317 study provides the basis to further investigate factors important for the epidemic
318 spread of *C. difficile.*
319
320 The deletions and insertions were well distributed geographically and temporally
321 suggesting either the rapid dissemination of strains or the multiple independent

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322 acquisition and loss of DNA regions (Figure 2 and Supplementary Information 1).
323 The insertion of different clusters of genes at the same site suggests 'hot-spot' regions
324 for the uptake of DNA (Supplementary Information 4) and a 49 kb insertion found
325 only in a clonal cluster of 23/37 London isolates in our previous study (39) was also
326 found to insert at a different site in single isolates from Canada, USA and the UK with
327 isolation dates of 2006, 2006 and 2011 respectively (Figure 3). This suggests these
328 isolates have independently acquired this insertion.
329
330 Similar to RT027, our analyses support a North American origin for RT017 with
331 multiple, global transmission events with its earliest movement into Europe in 1986
332 (Figures 4 and 5). The North American health system and practices appears to
333 facilitate the ready evolution and epidemic spread of *C. difficile* for RT027 (4) and
334 now in this study with RT017. Our data shows that it was Europe that introduced
335 RT017 to Asia and Australia, with subsequent spread from Asia to the Middle East,
336 South America and South Africa. The analysis indicates over 40 movements back and
337 forth over the span of 30 years, consistent with population movements of a globalised
338 society. Traditionally, it has been considered that RT017 strains emerged from Asia
339 due to the reported high incidence of this RT, that could not relate to nor depend on
340 toxin A-based assays for diagnosis (40). However, our analysis does not support an
341 "out of Asia" hypothesis and supports a North American origin (Figures 4 and 5).
342
343 This study investigated the genetic diversity of 277 *C. difficile* RT017 isolates with
344 temporal, geographical and source variation. Phylogeographic analysis of the SNPs
345 identified through WGS of the isolates suggests that there are two main sub-lineages
346 of RT017 that share a common ancestry and are globally disseminated. Both sub-

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lineages contain isolates from diverse geographical locations and isolation dates, with animal isolates spread amongst human isolates in SL1. Together with the haplotype diversity and geographically and temporally diverse presence of the transposable elements, these data suggest widespread transcontinental spread and recombination with independent acquisition and loss within different clusters.

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686 **AUTHOR CONTRIBUTIONS**

687 M.D.C., R.A.S. and B.W.W. planned the experiments. M.D.C., performed
688 experiments and *de novo* analysis and M.D.C., R.A.S. and M.D.P. performed
689 bioinformatics analyses. C.L.H. performed MIC experiments. M.D.C., D.N.G.,
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691 provided strains and M.D.C. drafted the manuscript with contributions from R.A.S.,
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693

694 **COMPETING FINANCIAL INTERESTS STATEMENT**

695 No conflicting interests.

696

FIGURE LEGENDS

Figure 1: Maximum-likelihood Phylogenetic Analysis of 277 global RT017 isolates based on core-genome SNPs against the M68 reference. We used non-rare (>1% MAF) SNP's that were not in close proximity to insertions or deletions to determine the phylogenic tree. The SL1 and SL2 sub-lineages were differentiated by four SNP's (see table 3) with the reference strain M68 falling into SL2. The coloured nodes indicate the geographical source of isolates

Figure 2: Maximum-likelihood Phylogenetic Analysis of 277 global RT017 isolates based on core-genome SNPs against the M68 reference. The phylogeny is separated into individual panels corresponding to each continent. Data from five out of 7 continental designations (Africa, Europe, Asia, Oceania and North America) include SL1 and SL2 isolates indicating that both sub-lineages are global in nature.

Figure 3: Bayesian evolutionary analysis of 277 global RT017 isolates based on core-genome SNPs against the M68 reference. Using a geo-temporal model we can orient the evolution of the RT017s though time. The analysis indicates a split from SL1 (lower samples) into SL2 (upper samples) c1990, with the M68 reference in SL2. The introduction of resistance associated SNPs (such as in *rpoC*) fall within closely related groups in the phylogeny. The continents are coloured as in figures 1 and 2. The heat map depicts the sub-lineage, presence/absence of insertions and antimicrobial resistance associated SNPs in relation to the isolates and continent.

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Figure 4: Maximum-likelihood Phylogenetic Analysis of the global RT017 isolates based on core-genome SNPs against the M68 reference depicting the 24 animal isolates by coloured nodes. Note the three equine isolates are positioned (and masked) by the bovine and canine cluster on the left. The two bovine isolates on the right of the tree have SNP distance of 17 from the bovine, canine, and equine cluster. All animal isolates are from Ontario, Canada and isolated between 2002 and 2005.

Figure 5: Global transmission events inferred from Bayesian evolutionary analysis of RT017. From the geo-temporal analyses we can infer the first movements into each continent, with the date and originating continent. The analysis indicates a North American origin with an expansion into Europe in the mid-1980s, followed by a move into Asia and on to Africa and South America through the 1990s and early 2000s. RT017 was not identified in Oceania (Australia) until the late 2000s, via a jump from Europe.

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Table 1: Stop-codon associated SNPs.

Position in the M68 genome	M68 Reference Codon	Alternative Codon	Non-Synonymous / Synonymous / Non-Coding	Gene	Predicted Function and/or Potential Impact	No. of isolates with SNP
132573	TGG	TGA	NS	M68_00168	Amino acid aminotransferase	16
557896	TTC*	TAA*	NS	*feoB3*	Ferrous iron transport protein B	3
1204039	GGA	TGA	NS	M68_01144	Hydrolase	36
1359584	GGA	TGA	NS	M68_01270	Extracellular solute-binding protein	3
1907433	TAA	GAA	NS	*msrAB*	Peptide methionine sulfoxide reductase	256
1916756	AAT*	GAT*	S	M68_01782	Unknown	3
3304067	TCA*	GCA*	NS	*Sigma-54*	Controls expression of nitrogen related genes	29
3399853	TTG*	TAA*	NS	M68_03193	Ca2+/Na+ antiporter	13
3402470	CAA	TAA	NS	*plfB*	Formate acetyltransferase	3
3704987	CCA*	TGA*	NS	*sleB*	Spore-cortex-lytic protein	8
3784055	TTC*	TAA*	NS	M68_03513	Penicillin-binding protein	3
4157880	TTG*	TAA*	NS	M68_03851	PTS system, IIc component	6

* = encoded on reverse strand

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Table 2: Summary details of 277 *C. difficile* study isolates and their genotypic characteristics

Sub-lineage	Total No. of Isolates	Country of Origin	Isolation Dates	No. of Haplotypes	No. of SNPs	No. of isolates with a insertion	No. of isolates with a deletion	Rifampicin resistance			Fluoroquinolone resistance			Resistance inferred
								34,687	34,697	34,747	112,752	113,641	113,642	Position
								rpoB	*rpoB*	*rpoB*	*gyrA*	*gyrB*	*gyrB*	Gene
								R505K	H502N	S485F	T82I	V426D	V426I	*Amino acid change
1	163	Argentina, Australia, Bulgaria, Canada, China, Czech Republic, Greece, Hong Kong, Japan, Korea, Kuwait, Poland, Portugal, Romania, Singapore, Slovenia, South Africa, The Netherlands, UK, USA	1994 to 2013	55 (50.5%)	0 to 35	49 (30.1%)	44 (30%)	73 44.8%	79 48.5%	0 0%	124 76.1%	134 82.2%	4 2.5%	
2	114	Australia, Hong Kong, Indonesia, Ireland, Korea, Poland, Singapore, South Africa, Taiwan, The Netherlands, UK, USA	1990 to 2013	54 (49.5%)	17 to 52	65 (57%)	109 (96%)	17 15%	13 11.4%	3 2.6%	55 48.2%	114 100%	9 7.9%	

* Reference residue/amino acid/ alternative residue

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Table 3: Lineage defining SNPs

Position	Amino Acid	Reference Base	Alternative Base	Non-Synonymous / Synonymous / Non-Coding	Gene	Predicted Function and/or Potential Impact
650374	19	A	G	NS	MerR	Altered response to environmental stimuli
900866	.	C	T	NC	.	.
2914248	257	A	G	NS	*dacF*	B-lactam resistance
3604289	329	C	A	NS	Hypothetical protein	Unknown

Table 4: Antimicrobial susceptibility data and their genotypic characteristics

	Strain	M68	S- 017.72	WA 1514	S- 017.92	S- 017.27	S- 017.74	I 6	01-116
	Location	Ireland	Walsall	Australia	China	Wrexham	Walsall	Indonesia	Korea
	Date Isolated	2006	2011	2012	2009	1996	2011	2011	2001
	Insertion	Insertion		A, B, C	A		D, E	F, G	
	Deletion	Deletion		H	H, I	J	H, J, K	H, J	
Resistant SNPs	*rpoB* (R505K)			✓	✓	✓		✓	✓
	rpoB (H502N)		✓	✓	✓			✓	✓
	rpoB (S485F)					✓			
	gyrA (T82I)		✓	✓	✓			✓	
	gyrB (V426I)					✓			
	gyrB (V426D)		✓	✓	✓	✓	✓	✓	✓
Antimicrobial Agent	[a]Chloramphenicol	8 (S)	8 (S)	4 (S)	64 (R)	8 (S)	8 (S)	256 (R)	8 (S)
	[a]Rifampicin	0.008 (I)	2 (I)	0.004 (S)	>256 (R)	>256 (R)	0.004 (S)	>256 (R)	>256 (R)
	[b]Tetracycline	32 (R)	32 (R)	0.25 (I)	32 (R)	32 (R)	0.25 (I)	32 (R)	32 (R)
	[b]Erythromycin	>256 (R)	>256 (R)	>256 (R)	>256 (R)	>256 (R)	<2 (S)	>256 (R)	>256 (R)
	[b]Nalidixic acid	256 (R)	256 (R)	256 (R)	256 (R)	256 (R)	256 (R)	256 (R)	256 (R)
	[c]Gentamicin	>256 (R)	>256 (R)	256 (R)	>256 (R)	256 (R)	256 (R)	>256 (R)	>256 (R)
	[c]Teicoplanin	<1 (S)	<1 (S)	<1 (S)	<1 (S)	<1 (S)	<1 (S)	<1 (S)	<1 (S)
	[b]Ampicillin	8 (R)	8 (R)	8 (R)	8 (R)	8 (R)	4 (R)	4 (R)	8 (R)

(S) = sensitive, (I) = intermediate resistance (R) = resistant

[a] Recommended by the European Committee on Antimicrobial Susceptibility Testing (EUCAST).
[b] Recommended by CLSI (M11-A8, 2012, and M-100-S23, 2013).
[c] No guidance from CLSI or EUCAST, cut-offs based on data according to the CLSI M-100-S23 (interpretative values for *Staphylococcus aureus*).

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Insertion A: Putative drug/sodium antiporter and radical SAM protein TetR-family trancscriptional regulator
Insertion B: Transcriptional repressor DicA
Insertion C: Streptogramin A acetyltransferase and multidrug resistance protein
Insertion D: Putative beta-lactamase repressor
Insertion E: Putative drug/sodium antiporter
Insertion F: TetR-family transcriptional regulator
Insertion G: Chloramphenicol o-acetyltransferase (M68 has one copy of chloramphenicol)
Deletion H: Dimethyladenosine transferase (ermB)
Deletion I: Putative teicoplanin resistance protein and putative beta-lactamase repressor
Deletion J: Aminoglycoside 6-adenylyltransferase
Deletion K: Putative conjugative transposon FtsK_SpoIIIE-related protein