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

4

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56 Key Words

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74 **Abstract**

75 The diarrhoeal pathogen *Clostridium difficile* consists of at least six distinct  
76 evolutionary lineages. The RT017 lineage is anomalous as strains only express toxin  
77 B, compared to strains from other lineages that produce toxins A and B and  
78 occasionally binary toxin. Historically, RT017 were initially reported in Asia but have  
79 now been reported worldwide. We used whole genome sequencing and phylogenetic  
80 analysis to investigate the patterns of global spread and population structure of 277  
81 RT017 isolates from animal and human origins from six continents, isolated between  
82 1990 and 2013. We reveal two distinct evenly split sub-lineages (SL1 and SL2) of *C.*  
83 *difficile* RT017 that contain multiple independent clonal expansions. All 24 animal  
84 isolates were contained within SL1 along with human isolates suggesting potential  
85 transmission between animals and humans. Genetic analyses revealed an over  
86 representation of antibiotic resistance genes. Phylogeographic analyses show a North  
87 American origin for RT017 as has been found for the recently emerged epidemic  
88 RT027 lineage. Despite only having one toxin, RT017 strains have evolved in parallel  
89 from at least two independent sources and can readily transmit between continents.

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99 **Introduction**

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

112

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

121

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

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

134

135 Historically, these strains were initially identified in CDI outbreaks in Asia and are  
136 thought to have spread to Europe and other continents. RT017 strains have been  
137 reported in: Canada (35, 42) China (34, 43), Korea (33, 44, 45), Argentina (46),  
138 Australia (47, 48), Israel (49), Japan (50) South Africa (51) and throughout Europe  
139 (36, 39, 41, 52, 53). These strains have also been isolated from non-human sources  
140 including equine, bovines (54) and rabbits (55). We recently performed WGS on 35  
141 human and two hospital environmental isolates of RT017 circulating in London,  
142 United Kingdom and identified three SNP variants (39). One variant was found to be  
143 clonal and had persisted in a London hospital ward for at least five years (39).

144

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

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



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

186

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

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

200

## 201 RESULTS

202 WGS was performed on a global collection of 277 *C. difficile* RT017 isolates.

203 Collectively, these were isolated from human (n=251), bovine (n=9), canine (n=11),

204 equine (n=4) and hospital ward environments (n=2) between 1990 and 2013

205 (Supplementary Information 1). All isolates belonged to multilocus sequence type 37.

206 After sequence quality control and mapping to the M68 RT017 reference genome

207 (GenBank accession number [FN668375](#)), we identified 1288 high quality bi-allelic

208 SNPs with 311 present in greater than 1% of samples and greater than 1 bp from an

209 insertion or deletion. Of these non-rare SNPs, 65.6% (n=204) were non-synonymous,

210 17.7% (n=55) synonymous and 16.7% (n=52) were present in non-coding regions of

211 the genome (non-synonymous SNPs are shown in Supplementary Information 2).

212 Twelve SNPs affected stop-codons; eleven non-synonymous and one synonymous

213 (Table 1).

214

215 SNP data revealed 109 haplotypes containing between 0 and 52 SNPs (with respect to

216 the M68 reference) with 76.5% (212/277) of isolates having between 10 and 35 SNPs

217 (Table 2).

218

219 We generated a maximum-likelihood phylogenetic tree based on the 1288 SNPs,

220 which demonstrates the presence of two genetically diverse sub-lineages; SL1 and

221 SL2 (Figures 1 and 2). Of the 1288 SNPs, 76% (977/1288) had a minor allele

222 frequency (MAF) of  $\leq 1\%$  and/or were within 1 bp of an insertion or deletion. To

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

231

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

245

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

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

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

274

## 275 **DISCUSSION**

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

282

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

288

289 Based on our rifampicin SNP data, we would predict 34.7% (96/277) of isolates in  
290 this study to be resistant to the rifampicin class of antibiotics. Interestingly, 82%  
291 (152/185) of these substitutions were found in SL1. R505K, H502N have previously  
292 been associated with rifampicin resistance in *C. difficile* (72), however, based on our  
293 MIC data, only two (2/8) isolates were sensitive to rifampicin with one of the isolates  
294 containing the R505K and H502N SNP indicating that these alone do not always lead  
295 to phenotypic resistance. Interestingly, S485F was found in three historical isolates  
296 from Wrexham, UK. This resistance conferring SNP has not previously been reported

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

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-

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

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685

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.,  
690 P.M.H., H.K., H.K., E.J.K., T.D.L., H.P., S.R., T.V.R., K.S., P.J.S. and S.J.W.  
691 provided strains and M.D.C. drafted the manuscript with contributions from R.A.S.,  
692 M.D.P. and B.W.W. followed by suggestions and comments from all authors.

693

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695 No conflicting interests.

696



697 **FIGURE LEGENDS**

698

699 **Figure 1: Maximum-likelihood Phylogenetic Analysis of 277 global RT017**  
700 **isolates based on core-genome SNPs against the M68 reference.** We used non-rare  
701 (>1% MAF) SNP's that were not in close proximity to insertions or deletions to  
702 determine the phylogenetic tree. The SL1 and SL2 sub-lineages were differentiated by  
703 four SNP's (see table 3) with the reference strain M68 falling into SL2. The coloured  
704 nodes indicate the geographical source of isolates

705

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

711

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

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

726

727 **Figure 5: Global transmission events inferred from Bayesian evolutionary**  
728 **analysis of RT017.** From the geo-temporal analyses we can infer the first movements  
729 into each continent, with the date and originating continent. The analysis indicates a  
730 North American origin with an expansion into Europe in the mid-1980s, followed by  
731 a move into Asia and on to Africa and South America through the 1990s and early  
732 2000s. RT017 was not identified in Oceania (Australia) until the late 2000s, via a  
733 jump from Europe.

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	<i>feoB3</i>	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	<i>msrAB</i>	Peptide methionine sulfoxide reductase	256
1916756	AAT*	GAT*	S	M68_01782	Unknown	3
3304067	TCA*	GCA*	NS	<i>Sigma-54</i>	Controls expression of nitrogen related genes	29
3399853	TTG*	TAA*	NS	M68_03193	Ca <sup>2+</sup> /Na <sup>+</sup> antiporter	13
3402470	CAA	TAA	NS	<i>pljB</i>	Formate acetyltransferase	3
3704987	CCA*	TGA*	NS	<i>steB</i>	Spore-cortex-lytic protein	8
3784055	TTC*	TAA*	NS	M68_03513	Penicillin-binding protein	3
4157880	TTG*	TAA*	NS	M68_03851	PTS system, Ilc component	6

\* = encoded on reverse strand

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
								<i>rpoB</i>	<i>rpoB</i>	<i>rpoB</i>	<i>gyrA</i>	<i>gyrB</i>	<i>gyrB</i>	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

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	<i>dacF</i>	B-lactam resistance
3604289	329	C	A	NS	Hypothetical protein	Unknown

1 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			A, B, C	A		D, E	F, G		
Deletion	Deletion		H	H, I	J	H, J, K	H, J		
Resistant SNPs	<i>rpoB</i> (R505K)		✓	✓	✓		✓	✓	
	<i>rpoB</i> (H502N)	✓	✓	✓			✓	✓	
	<i>rpoB</i> (S485F)				✓				
	<i>gyrA</i> (T82I)	✓	✓	✓			✓		
	<i>gyrB</i> (V426I)					✓			
	<i>gyrB</i> (V426D)	✓	✓	✓	✓	✓	✓	✓	
Antimicrobial Agent	<sup>a</sup> Chloramphenicol	8 (S)	8 (S)	4 (S)	64 (R)	8 (S)	8 (S)	256 (R)	8 (S)
	<sup>a</sup> Rifampicin	0.008 (I)	2 (I)	0.004 (S)	>256 (R)	>256 (R)	0.004 (S)	>256 (R)	>256 (R)
	<sup>b</sup> Tetracycline	32 (R)	32 (R)	0.25 (I)	32 (R)	32 (R)	0.25 (I)	32 (R)	32 (R)
	<sup>b</sup> Erythromycin	>256 (R)	>256 (R)	>256 (R)	>256 (R)	>256 (R)	<2 (S)	>256 (R)	>256 (R)
	<sup>b</sup> Nalidixic acid	256 (R)	256 (R)	256 (R)	256 (R)	256 (R)	256 (R)	256 (R)	256 (R)
	<sup>c</sup> Gentamicin	>256 (R)	>256 (R)	256 (R)	>256 (R)	256 (R)	256 (R)	>256 (R)	>256 (R)
	<sup>c</sup> Teicoplanin	<1 (S)	<1 (S)	<1 (S)	<1 (S)	<1 (S)	<1 (S)	<1 (S)	<1 (S)
	<sup>b</sup> Ampicillin	8 (R)	8 (R)	8 (R)	8 (R)	8 (R)	4 (R)	4 (R)	8 (R)

2  
3 (S) = sensitive, (I) = intermediate resistance (R) = resistant  
4

5 <sup>a</sup> Recommended by the European Committee on Antimicrobial Susceptibility Testing (EUCAST).

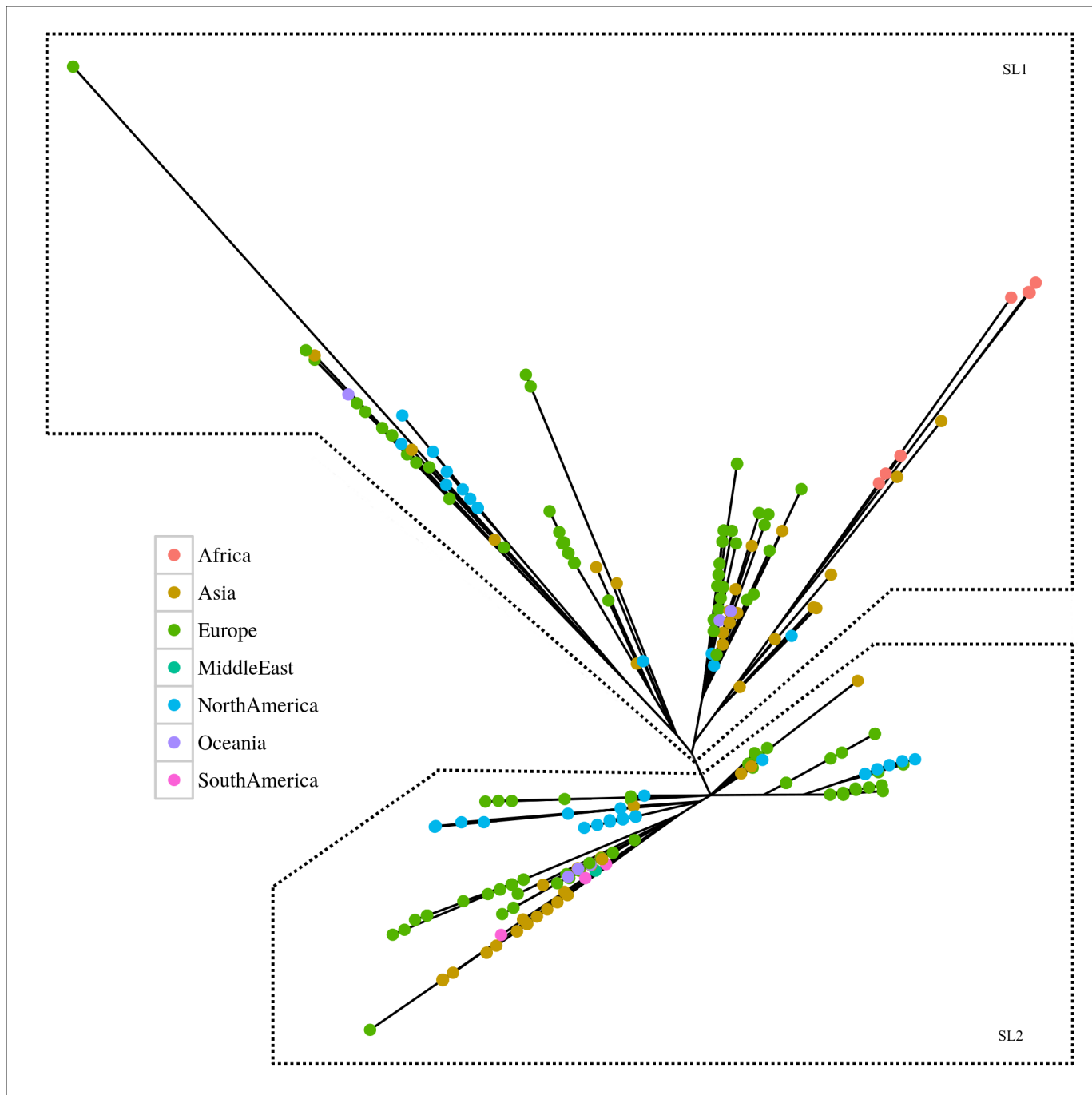
6 <sup>b</sup> Recommended by CLSI (M11-A8, 2012, and M-100-S23, 2013).

7 <sup>c</sup> No guidance from CLSI or EUCAST, cut-offs based on data according to the CLSI M-100-S23 (interpretative values for *Staphylococcus aureus*).

8

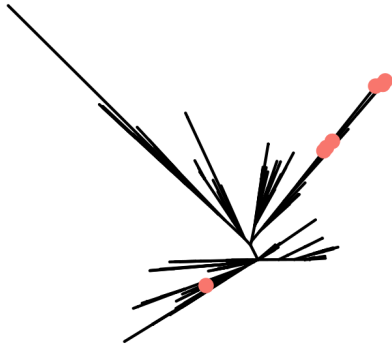
9

- 10 Insertion A: Putative drug/sodium antiporter and radical SAM protein TetR-family transcriptional regulator
- 11 Insertion B: Transcriptional repressor DicA
- 12 Insertion C: Streptogramin A acetyltransferase and multidrug resistance protein
- 13 Insertion D: Putative beta-lactamase repressor
- 14 Insertion E: Putative drug/sodium antiporter
- 15 Insertion F: TetR-family transcriptional regulator
- 16 Insertion G: Chloramphenicol o-acetyltransferase (M68 has one copy of chloramphenicol)
- 17 Deletion H: Dimethyladenosine transferase (ermB)
- 18 Deletion I: Putative teicoplanin resistance protein and putative beta-lactamase repressor
- 19 Deletion J: Aminoglycoside 6-adenylyltransferase
- 20 Deletion K: Putative conjugative transposon FtsK\_SpoIIIE-related protein
- 21

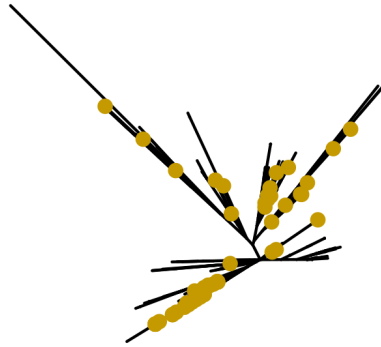




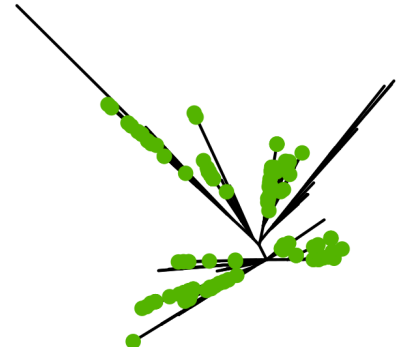
Africa (n = 9)



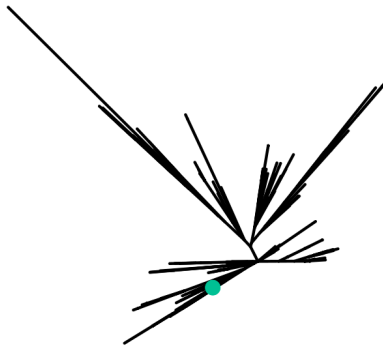
Asia (n = 59)



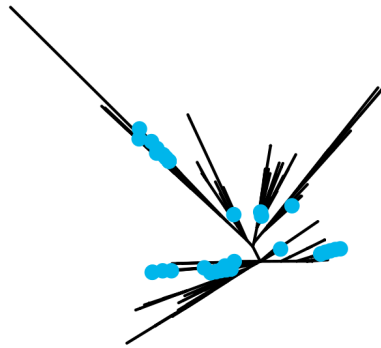
Europe (n = 137)



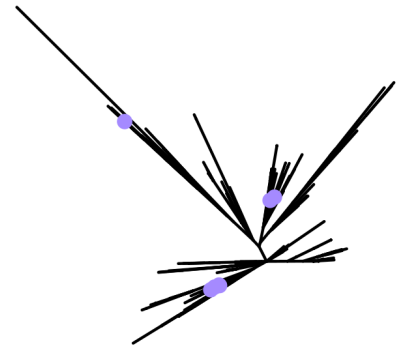
Middle East (n = 2)



North America (n = 59)



Oceania (n = 7)



South America (n = 4)

