

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.**

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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).

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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).

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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.

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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).

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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.

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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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372 REFERENCES

- 373 1. **Barbut F, Gariazzo B, Bonne L, Lalande V, Burghoffer B, Luiuz R, Petit**
374 **JC.** 2007. Clinical features of Clostridium difficile-associated infections and
375 molecular characterization of strains: results of a retrospective study, 2000-
376 2004. Infection control and hospital epidemiology : the official journal of the
377 Society of Hospital Epidemiologists of America **28**:131-139.
- 378 2. **Voth DE, Ballard JD.** 2005. Clostridium difficile toxins: mechanism of
379 action and role in disease. Clin Microbiol Rev **18**:247-263.
- 380 3. **Knetsch CW, Terveer EM, Lauber C, Gorbalenya AE, Harmanus C,**
381 **Kuijper EJ, Corver J, van Leeuwen HC.** 2012. Comparative analysis of an
382 expanded Clostridium difficile reference strain collection reveals genetic
383 diversity and evolution through six lineages. Infection, genetics and evolution
384 : journal of molecular epidemiology and evolutionary genetics in infectious
385 diseases **12**:1577-1585.
- 386 4. **He M, Miyajima F, Roberts P, Ellison L, Pickard DJ, Martin MJ, Connor**
387 **TR, Harris SR, Fairley D, Bamford KB, D'Arc S, Brazier J, Brown D,**
388 **Coia JE, Douce G, Gerding D, Kim HJ, Koh TH, Kato H, Senoh M, Louie**
389 **T, Michell S, Butt E, Peacock SJ, Brown NM, Riley T, Songer G, Wilcox**
390 **M, Pirmohamed M, Kuijper E, Hawkey P, Wren BW, Dougan G, Parkhill**
391 **J, Lawley TD.** 2013. Emergence and global spread of epidemic healthcare-
392 associated Clostridium difficile. Nature genetics **45**:109-113.
- 393 5. **Camacho-Ortiz A, Lopez-Barrera D, Hernandez-Garcia R, Galvan-De**
394 **Los Santos AM, Flores-Trevino SM, Llaca-Diaz JM, Garza HJ, Bosques-**
395 **Padilla FJ, Garza-Gonzalez E.** 2015. First Report of Clostridium difficile
396 NAP1/027 in a Mexican Hospital. PloS one **10**:e0122627.
- 397 6. **Aguayo C, Flores R, Levesque S, Araya P, Ulloa S, Lagos J, Hormazabal**
398 **JC, Tognarelli J, Ibanez D, Pidal P, Duery O, Olivares B, Fernandez J.**
399 2015. Rapid spread of Clostridium difficile NAP1/027/ST1 in Chile confirms
400 the emergence of the epidemic strain in Latin America. Epidemiology and
401 infection:1-5.
- 402 7. **Hernandez-Rocha C, Barra-Carrasco J, Pizarro-Guajardo M, Ibanez P,**
403 **Bueno SM, Sarker MR, Guzman AM, Alvarez-Lobos M, Paredes-Sabja**
404 **D.** 2012. Epidemic Clostridium difficile ribotype 027 in Chile. Emerging
405 infectious diseases **18**:1370-1372.
- 406 8. **Wang P, Zhou Y, Wang Z, Xie S, Zhang T, Lin M, Li R, Tan J, Chen Y,**
407 **Jiang B.** 2014. Identification of Clostridium difficile ribotype 027 for the first
408 time in Mainland China. Infection control and hospital epidemiology : the
409 official journal of the Society of Hospital Epidemiologists of America **35**:95-
410 98.
- 411 9. **Kato H, Ito Y, van den Berg RJ, Kuijper EJ, Arakawa Y.** 2007. First
412 isolation of Clostridium difficile 027 in Japan. Euro surveillance : bulletin
413 Europeen sur les maladies transmissibles = European communicable disease
414 bulletin **12**:E070111 070113.

- 415 10. **Cheng VC, Yam WC, Chan JF, To KK, Ho PL, Yuen KY.** 2009.
416 *Clostridium difficile* ribotype 027 arrives in Hong Kong. *International journal*
417 *of antimicrobial agents* **34**:492-493.
- 418 11. **Kim H, Lee Y, Moon HW, Lim CS, Lee K, Chong Y.** 2011. Emergence of
419 *Clostridium difficile* ribotype 027 in Korea. *Korean J Lab Med* **31**:191-196.
- 420 12. **Tae CH, Jung SA, Song HJ, Kim SE, Choi HJ, Lee M, Hwang Y, Kim H,**
421 **Lee K.** 2009. The first case of antibiotic-associated colitis by *Clostridium*
422 *difficile* PCR ribotype 027 in Korea. *J Korean Med Sci* **24**:520-524.
- 423 13. **Lai MJ, Chiueh TS, Huang ZY, Lin JC.** 2015. The first *Clostridium difficile*
424 ribotype 027 strain isolated in Taiwan. *Journal of the Formosan Medical*
425 *Association = Taiwan yi zhi.*
- 426 14. **Lim PL, Ling ML, Lee HY, Koh TH, Tan AL, Kuijper EJ, Goh SS, Low**
427 **BS, Ang LP, Harmanus C, Lin RT, Krishnan P, James L, Lee CE.** 2011.
428 Isolation of the first three cases of *Clostridium difficile* polymerase chain
429 reaction ribotype 027 in Singapore. *Singapore medical journal* **52**:361-364.
- 430 15. **Riley TV, Thean S, Hool G, Golledge CL.** 2009. First Australian isolation of
431 epidemic *Clostridium difficile* PCR ribotype 027. *The Medical journal of*
432 *Australia* **190**:706-708.
- 433 16. **Richards M, Knox J, Elliott B, Mackin K, Lyras D, Waring LJ, Riley TV.**
434 2011. Severe infection with *Clostridium difficile* PCR ribotype 027 acquired
435 in Melbourne, Australia. *The Medical journal of Australia* **194**:369-371.
- 436 17. **Alzahrani N, Johani SA.** 2013. Emergence of a highly resistant *Clostridium*
437 *difficile* strain (NAP/BI/027) in a tertiary care center in Saudi Arabia. *Annals*
438 *of Saudi medicine* **33**:198-199.
- 439 18. **Wiener-Well Y, Ben-Chetrit E, Abed-Eldaim M, Assous MV, Miller-Roll**
440 **T, Adler A.** 2014. Clinical and molecular characteristics of an outbreak
441 caused by the pandemic (BI/NAP1/027) *Clostridium difficile* clone in a single
442 center in Israel. *Infection control and hospital epidemiology : the official*
443 *journal of the Society of Hospital Epidemiologists of America* **35**:1306-1308.
- 444 19. **Bacci S, St-Martin G, Olesen B, Bruun B, Olsen KE, Nielsen EM, Molbak**
445 **K.** 2009. Outbreak of *Clostridium difficile* 027 in North Zealand, Denmark,
446 2008-2009. *Euro surveillance : bulletin Europeen sur les maladies*
447 *transmissibles = European communicable disease bulletin* **14**.
- 448 20. **Lachowicz D, Szulenska G, Obuch-Woszczatynski P, van Belkum A,**
449 **Pituch H.** 2015. First Polish outbreak of *Clostridium difficile* ribotype 027
450 infections among dialysis patients. *European journal of clinical microbiology*
451 *& infectious diseases : official publication of the European Society of Clinical*
452 *Microbiology* **34**:63-67.
- 453 21. **Orsi GB, Conti C, Mancini C, Giordano A.** 2014. *Clostridium difficile* 027
454 increasing detection in a teaching hospital in Rome, Italy. *Infection* **42**:941-
455 942.
- 456 22. **Arvand M, Vollandt D, Bettge-Weller G, Harmanus C, Kuijper EJ,**
457 **Clostridium difficile study group H.** 2014. Increased incidence of
458 *Clostridium difficile* PCR ribotype 027 in Hesse, Germany, 2011 to 2013.
459 *Euro surveillance : bulletin Europeen sur les maladies transmissibles =*
460 *European communicable disease bulletin* **19**.

- 461 23. **Lagier JC, Dubourg G, Cassir N, Fournier PE, Colson P, Richet H,**
462 **Brouqui P, Raoult D.** 2013. Clostridium difficile 027 emerging outbreak in
463 Marseille, France. Infection control and hospital epidemiology : the official
464 journal of the Society of Hospital Epidemiologists of America **34**:1339-1341.
- 465 24. **Di Bella S, Paglia MG, Johnson E, Petrosillo N.** 2012. Clostridium difficile
466 027 infection in Central Italy. BMC infectious diseases **12**:370.
- 467 25. **Indra A, Huhulescu S, Fiedler A, Kernbichler S, Blaschitz M, Allerberger**
468 **F.** 2009. Outbreak of Clostridium difficile 027 infection in Vienna, Austria
469 2008-2009. Euro surveillance : bulletin Europeen sur les maladies
470 transmissibles = European communicable disease bulletin **14**.
- 471 26. **Long S, Fenelon L, Fitzgerald S, Nolan N, Burns K, Hannan M, Kyne L,**
472 **Fanning S, Drudy D.** 2007. First isolation and report of clusters of
473 Clostridium difficile PCR 027 cases in Ireland. Euro surveillance : bulletin
474 Europeen sur les maladies transmissibles = European communicable disease
475 bulletin **12**:E070426 070423.
- 476 27. **Drudy D, Goorhuis B, Bakker D, Kyne L, van den Berg R, Fenelon L,**
477 **Fanning S, Kuijper EJ.** 2008. Clindamycin-resistant clone of Clostridium
478 difficile PCR Ribotype 027, Europe. Emerging infectious diseases **14**:1485-
479 1487.
- 480 28. **Kuijper EJ, Coignard B, Tull P, difficile ESGfC, States EUM, European**
481 **Centre for Disease P, Control.** 2006. Emergence of Clostridium difficile-
482 associated disease in North America and Europe. Clinical microbiology and
483 infection : the official publication of the European Society of Clinical
484 Microbiology and Infectious Diseases **12 Suppl 6**:2-18.
- 485 29. **England PH.** 2016. Clostridium difficile Ribotyping Network (CDRN) for
486 England and Northern Ireland, 2013 to 2015. PHE Publication Report.
- 487 30. **Dupuy B, Govind R, Antunes A, Matamouros S.** 2008. Clostridium difficile
488 toxin synthesis is negatively regulated by TcdC. Journal of medical
489 microbiology **57**:685-689.
- 490 31. **Rupnik M, Janezic S.** 2016. An Update on Clostridium difficile
491 Toxinotyping. Journal of clinical microbiology **54**:13-18.
- 492 32. **Shin BM, Kuak EY, Yoo SJ, Shin WC, Yoo HM.** 2008. Emerging toxin A-
493 B+ variant strain of Clostridium difficile responsible for pseudomembranous
494 colitis at a tertiary care hospital in Korea. Diagnostic microbiology and
495 infectious disease **60**:333-337.
- 496 33. **Kim SJ, Kim H, Seo Y, Yong D, Jeong SH, Chong Y, Lee K.** 2010.
497 Molecular characterization of toxin A-negative, toxin B-positive variant
498 strains of Clostridium difficile isolated in Korea. Diagnostic microbiology and
499 infectious disease **67**:198-201.
- 500 34. **Huang H, Weintraub A, Fang H, Wu S, Zhang Y, Nord CE.** 2010.
501 Antimicrobial susceptibility and heteroresistance in Chinese Clostridium
502 difficile strains. Anaerobe **16**:633-635.
- 503 35. **al-Barrak A, Embil J, Dyck B, Olekson K, Nicoll D, Alfa M, Kabani A.**
504 1999. An outbreak of toxin A negative, toxin B positive Clostridium difficile-
505 associated diarrhea in a Canadian tertiary-care hospital. Canada communicable
506 disease report = Releve des maladies transmissibles au Canada **25**:65-69.

- 507 36. **Kuijper EJ, de Weerd J, Kato H, Kato N, van Dam AP, van der Vorm**
508 **ER, Weel J, van Rheenen C, Dankert J.** 2001. Nosocomial outbreak of
509 *Clostridium difficile*-associated diarrhoea due to a clindamycin-resistant
510 enterotoxin A-negative strain. *European journal of clinical microbiology &*
511 *infectious diseases* : official publication of the European Society of Clinical
512 *Microbiology* **20**:528-534.
- 513 37. **Drudy D, Harnedy N, Fanning S, Hannan M, Kyne L.** 2007. Emergence
514 and control of fluoroquinolone-resistant, toxin A-negative, toxin B-positive
515 *Clostridium difficile*. *Infection control and hospital epidemiology* : the official
516 journal of the Society of Hospital Epidemiologists of America **28**:932-940.
- 517 38. **Arvand M, Hauri AM, Zaiss NH, Witte W, Bettge-Weller G.** 2009.
518 *Clostridium difficile* ribotypes 001, 017, and 027 are associated with lethal C.
519 *difficile* infection in Hesse, Germany. *Euro surveillance* : bulletin Europeen
520 sur les maladies transmissibles = *European communicable disease bulletin* **14**.
- 521 39. **Cairns MD, Preston MD, Lawley TD, Clark TG, Stabler RA, Wren BW.**
522 2015. Genomic epidemiology of a protracted hospital outbreak caused by a
523 toxin A negative, *Clostridium difficile* sublineage PCR Ribotype 017 strain in
524 London, England. *Journal of clinical microbiology*.
- 525 40. **Collins DA, Hawkey PM, Riley TV.** 2013. Epidemiology of *Clostridium*
526 *difficile* infection in Asia. *Antimicrobial resistance and infection control* **2**:21.
- 527 41. **Pituch H, Brazier JS, Obuch-Woszczatynski P, Wultanska D, Meisel-**
528 **Mikolajczyk F, Luczak M.** 2006. Prevalence and association of PCR
529 ribotypes of *Clostridium difficile* isolated from symptomatic patients from
530 Warsaw with macrolide-lincosamide-streptogramin B (MLSB) type resistance.
531 *Journal of medical microbiology* **55**:207-213.
- 532 42. **Alfa MJ, Kabani A, Lyerly D, Moncrief S, Neville LM, Al-Barrak A,**
533 **Harding GK, Dyck B, Olekson K, Embil JM.** 2000. Characterization of a
534 toxin A-negative, toxin B-positive strain of *Clostridium difficile* responsible
535 for a nosocomial outbreak of *Clostridium difficile*-associated diarrhea. *Journal*
536 *of clinical microbiology* **38**:2706-2714.
- 537 43. **Hawkey PM, Marriott C, Liu WE, Jian ZJ, Gao Q, Ling TK, Chow V, So**
538 **E, Chan R, Hardy K, Xu L, Manzoor S.** 2013. Molecular epidemiology of
539 *Clostridium difficile* infection in a major chinese hospital: an underrecognized
540 problem in Asia? *Journal of clinical microbiology* **51**:3308-3313.
- 541 44. **Kim H, Riley TV, Kim M, Kim CK, Yong D, Lee K, Chong Y, Park JW.**
542 2008. Increasing prevalence of toxin A-negative, toxin B-positive isolates of
543 *Clostridium difficile* in Korea: impact on laboratory diagnosis. *Journal of*
544 *clinical microbiology* **46**:1116-1117.
- 545 45. **Kim H, Jeong SH, Roh KH, Hong SG, Kim JW, Shin MG, Kim MN, Shin**
546 **HB, Uh Y, Lee H, Lee K.** 2010. Investigation of toxin gene diversity,
547 molecular epidemiology, and antimicrobial resistance of *Clostridium difficile*
548 isolated from 12 hospitals in South Korea. *Korean J Lab Med* **30**:491-497.
- 549 46. **Goorhuis A, Legaria MC, van den Berg RJ, Harmanus C, Klaassen CH,**
550 **Brazier JS, Lumelsky G, Kuijper EJ.** 2009. Application of multiple-locus
551 variable-number tandem-repeat analysis to determine clonal spread of toxin A-
552 negative *Clostridium difficile* in a general hospital in Buenos Aires, Argentina.

- 553 Clinical microbiology and infection : the official publication of the European
554 Society of Clinical Microbiology and Infectious Diseases **15**:1080-1086.
- 555 47. **Elliott B, Squire MM, Thean S, Chang BJ, Brazier JS, Rupnik M, Riley**
556 **TV**. 2011. New types of toxin A-negative, toxin B-positive strains among
557 clinical isolates of *Clostridium difficile* in Australia. *Journal of medical*
558 *microbiology* **60**:1108-1111.
- 559 48. **Elliott B, Reed R, Chang BJ, Riley TV**. 2009. Bacteremia with a large
560 clostridial toxin-negative, binary toxin-positive strain of *Clostridium difficile*.
561 *Anaerobe* **15**:249-251.
- 562 49. **Samra Z, Talmor S, Bahar J**. 2002. High prevalence of toxin A-negative
563 toxin B-positive *Clostridium difficile* in hospitalized patients with
564 gastrointestinal disease. *Diagnostic microbiology and infectious disease*
565 **43**:189-192.
- 566 50. **Komatsu M, Kato H, Aihara M, Shimakawa K, Iwasaki M, Nagasaka Y,**
567 **Fukuda S, Matsuo S, Arakawa Y, Watanabe M, Iwatani Y**. 2003. High
568 frequency of antibiotic-associated diarrhea due to toxin A-negative, toxin B-
569 positive *Clostridium difficile* in a hospital in Japan and risk factors for
570 infection. *European journal of clinical microbiology & infectious diseases* :
571 official publication of the European Society of Clinical Microbiology **22**:525-
572 529.
- 573 51. **Rajabally N, Kullin B, Ebrahim K, Brock T, Weintraub A, Whitelaw A,**
574 **Bamford C, Watermeyer G, Thomson S, Abratt V, Reid S**. 2016. A
575 comparison of *Clostridium difficile* diagnostic methods for identification of
576 local strains in a South African centre. *Journal of medical microbiology*.
- 577 52. **Drudy D, Harnedy N, Fanning S, O'Mahony R, Kyne L**. 2007. Isolation
578 and characterisation of toxin A-negative, toxin B-positive *Clostridium difficile*
579 in Dublin, Ireland. *Clinical microbiology and infection* : the official
580 publication of the European Society of Clinical Microbiology and Infectious
581 Diseases **13**:298-304.
- 582 53. **Pituch H, van den Braak N, van Leeuwen W, van Belkum A, Martirosian**
583 **G, Obuch-Woszczatynski P, Luczak M, Meisel-Mikolajczyk F**. 2001.
584 Clonal dissemination of a toxin-A-negative/toxin-B-positive *Clostridium*
585 *difficile* strain from patients with antibiotic-associated diarrhea in Poland.
586 *Clinical microbiology and infection* : the official publication of the European
587 Society of Clinical Microbiology and Infectious Diseases **7**:442-446.
- 588 54. **Rodriguez-Palacios A, Stampfli HR, Duffield T, Peregrine AS, Trotz-**
589 **Williams LA, Arroyo LG, Brazier JS, Weese JS**. 2006. *Clostridium difficile*
590 PCR ribotypes in calves, Canada. *Emerging infectious diseases* **12**:1730-1736.
- 591 55. **Drigo I, Mazzolini E, Bacchin C, Tonon E, Puiatti C, Bano L, Spigaglia P,**
592 **Barbanti F, Agnoletti F**. 2015. Molecular characterization and antimicrobial
593 susceptibility of *Clostridium difficile* isolated from rabbits raised for meat
594 production. *Veterinary microbiology*.
- 595 56. **Stabler RA, He M, Dawson L, Martin M, Valiente E, Corton C, Lawley**
596 **TD, Sebahia M, Quail MA, Rose G, Gerding DN, Gibert M, Popoff MR,**
597 **Parkhill J, Dougan G, Wren BW**. 2009. Comparative genome and

- 598 phenotypic analysis of *Clostridium difficile* 027 strains provides insight into
599 the evolution of a hypervirulent bacterium. *Genome biology* **10**:R102.
- 600 57. **Harris SR, Feil EJ, Holden MT, Quail MA, Nickerson EK, Chantratita N,**
601 **Gardete S, Tavares A, Day N, Lindsay JA, Edgeworth JD, de Lencastre**
602 **H, Parkhill J, Peacock SJ, Bentley SD.** 2010. Evolution of MRSA during
603 hospital transmission and intercontinental spread. *Science* **327**:469-474.
- 604 58. **Preston MD, Assefa SA, Ocholla H, Sutherland CJ, Borrmann S, Nzila A,**
605 **Michon P, Hien TT, Bousema T, Drakeley CJ, Zongo I, Ouedraogo JB,**
606 **Djimde AA, Doumbo OK, Nosten F, Fairhurst RM, Conway DJ, Roper C,**
607 **Clark TG.** 2014. PlasmoView: a web-based resource to visualise global
608 *Plasmodium falciparum* genomic variation. *The Journal of infectious diseases*
609 **209**:1808-1815.
- 610 59. **Bolger AM, Lohse M, Usadel B.** 2014. Trimmomatic: a flexible trimmer for
611 Illumina sequence data. *Bioinformatics* **30**:2114-2120.
- 612 60. **Li H, Durbin R.** 2009. Fast and accurate short read alignment with Burrows-
613 Wheeler transform. *Bioinformatics* **25**:1754-1760.
- 614 61. **Li H, Handsaker B, Wysoker A, Fennell T, Ruan J, Homer N, Marth G,**
615 **Abecasis G, Durbin R, Genome Project Data Processing S.** 2009. The
616 Sequence Alignment/Map format and SAMtools. *Bioinformatics* **25**:2078-
617 2079.
- 618 62. **Zerbino DR.** 2010. Using the Velvet de novo assembler for short-read
619 sequencing technologies. *Current protocols in bioinformatics / editorial board,*
620 *Andreas D. Baxeavanis ... [et al.] Chapter 11:Unit 11 15.*
- 621 63. **Seemann SGaT** 2012, posting date. VelvetOptimiser. Victorian
622 Bioinformatics Consortium. [Online.]
- 623 64. **Assefa S, Keane TM, Otto TD, Newbold C, Berriman M.** 2009. ABACAS:
624 algorithm-based automatic contiguation of assembled sequences.
625 *Bioinformatics* **25**:1968-1969.
- 626 65. **Seemann T.** 2014. Prokka: rapid prokaryotic genome annotation.
627 *Bioinformatics* **30**:2068-2069.
- 628 66. **Stamatakis A.** 2014. RAxML version 8: a tool for phylogenetic analysis and
629 post-analysis of large phylogenies. *Bioinformatics* **30**:1312-1313.
- 630 67. **Drummond AJ, Suchard MA, Xie D, Rambaut A.** 2012. Bayesian
631 phylogenetics with BEAUti and the BEAST 1.7. *Molecular biology and*
632 *evolution* **29**:1969-1973.
- 633 68. **Raftery CFAE.** 2002. Model-based clustering, discriminant analysis and
634 density estimation. *Journal of the American Statistical Association* **97**:611.
- 635 69. **Andrews JM.** 2001. Determination of minimum inhibitory concentrations.
636 *The Journal of antimicrobial chemotherapy* **48 Suppl 1**:5-16.
- 637 70. **Lee JH, Lee Y, Lee K, Riley TV, Kim H.** 2014. The changes of PCR
638 ribotype and antimicrobial resistance of *Clostridium difficile* in a tertiary care
639 hospital over 10 years. *Journal of medical microbiology* **63**:819-823.
- 640 71. **Drudy D, Quinn T, O'Mahony R, Kyne L, O'Gaora P, Fanning S.** 2006.
641 High-level resistance to moxifloxacin and gatifloxacin associated with a novel
642 mutation in *gyrB* in toxin-A-negative, toxin-B-positive *Clostridium difficile*.
643 *The Journal of antimicrobial chemotherapy* **58**:1264-1267.

- 644 72. **Curry SR, Marsh JW, Shutt KA, Muto CA, O'Leary MM, Saul MI,**
645 **Pasculle AW, Harrison LH.** 2009. High frequency of rifampin resistance
646 identified in an epidemic *Clostridium difficile* clone from a large teaching
647 hospital. *Clinical infectious diseases : an official publication of the Infectious*
648 *Diseases Society of America* **48**:425-429.
- 649 73. **Bahrmand AR, Titov LP, Tasbiti AH, Yari S, Graviss EA.** 2009. High-
650 level rifampin resistance correlates with multiple mutations in the *rpoB* gene
651 of pulmonary tuberculosis isolates from the Afghanistan border of Iran.
652 *Journal of clinical microbiology* **47**:2744-2750.

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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

694 **COMPETING FINANCIAL INTERESTS STATEMENT**

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 ²⁺ /Na ⁺ 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	^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)

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

5 ^a Recommended by the European Committee on Antimicrobial Susceptibility Testing (EUCAST).

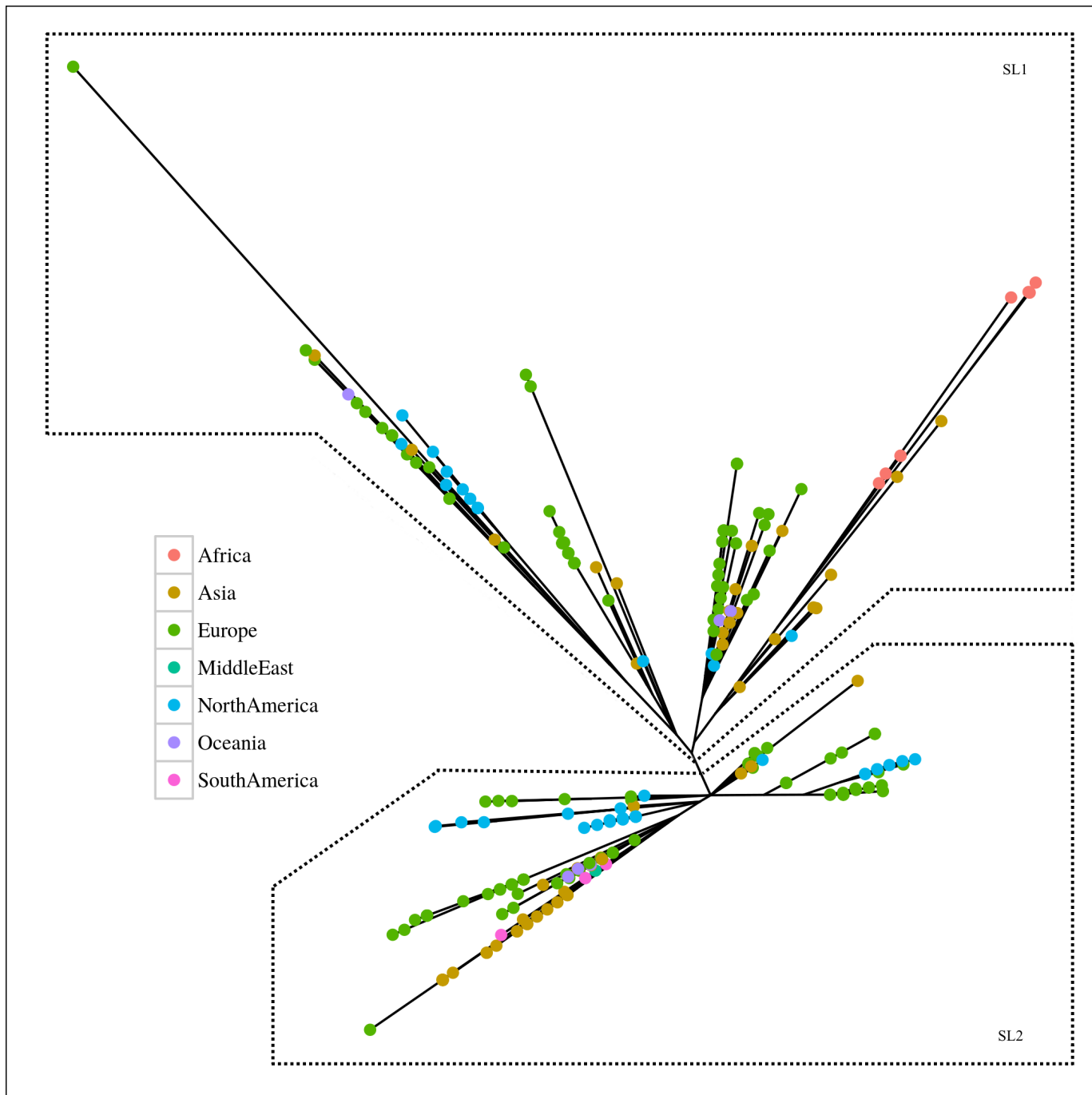
6 ^b Recommended by CLSI (M11-A8, 2012, and M-100-S23, 2013).

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

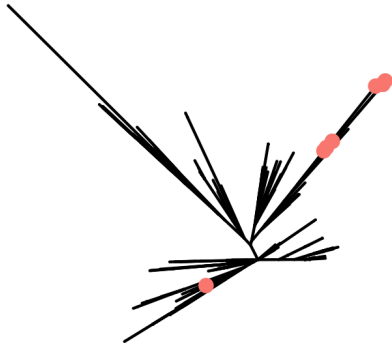
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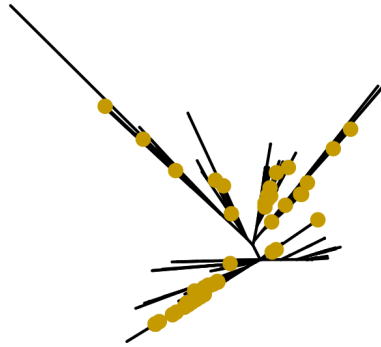
- 10 Insertion A: Putative drug/sodium antiporter and radical SAM protein TetR-family transcriptional regulator
- 11 Insertion B: Transcriptional repressor DicA
- 12 Insertion C: Streptogramin A acetyltransferase and multidrug resistance protein
- 13 Insertion D: Putative beta-lactamase repressor
- 14 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



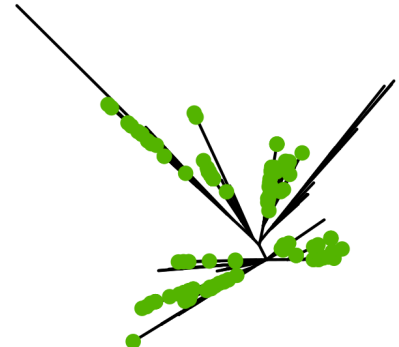
Africa (n = 9)



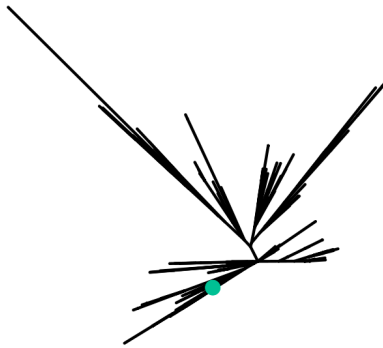
Asia (n = 59)



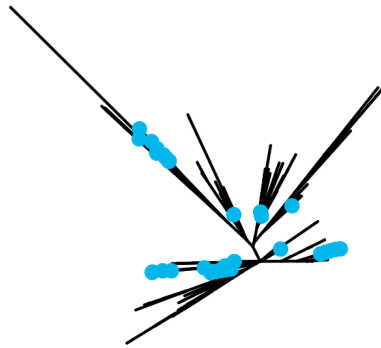
Europe (n = 137)



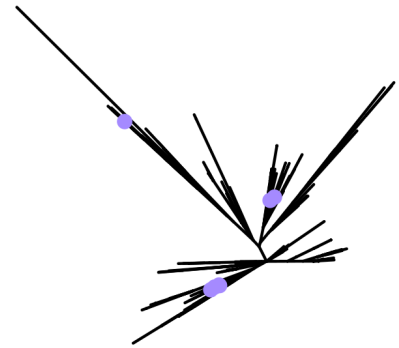
Middle East (n = 2)



North America (n = 59)



Oceania (n = 7)



South America (n = 4)

