

1 Zoonotic transfer of *Clostridium difficile* harboring antimicrobial
2 resistance between farm animals and humans

3
4 C.W. Knetsch^{1,†}, N. Kumar^{2,†}, S.C. Forster^{2,3,4}, T.R. Connor⁵, H.P. Browne², C. Harmanus¹,
5 I.M. Sanders¹, S.R. Harris⁶, L. Turner⁷, T. Morris⁷, M. Perry⁷, F. Miyajima⁸, P. Roberts⁸, M.
6 Pirmohamed⁸, J.G. Songer⁹, J.S. Weese¹⁰, A. Indra¹¹, J. Corver¹, M. Rupnik^{12,13}, B.W.
7 Wren¹⁴, T.V. Riley^{15,16}, E.J. Kuijper¹ and T.D. Lawley^{2,*}

8
9 ¹Section Experimental Bacteriology, Department of Medical Microbiology, Leiden
10 University Medical Center, Leiden, Netherlands.

11 ²Host-Microbiota Interactions Laboratory, Wellcome Trust Sanger Institute, Hinxton, United
12 Kingdom.

13 ³Centre for Innate Immunity and Infectious Diseases, Hudson Institute of Medical Research,
14 Clayton, Victoria, Australia.

15 ⁴Department of Molecular and Translational Sciences, Monash University, Clayton, Victoria,
16 Australia.

17 ⁵Cardiff School of Biosciences, Sir Martin Evans Building, Cardiff, United Kingdom.

18 ⁶Pathogen Genomics, Wellcome Trust Sanger Institute, Hinxton, United Kingdom.

19 ⁷Public Health Wales, Microbiology, Wales, United Kingdom.

20 ⁸Institute of Translational Medicine, University of Liverpool, Liverpool, United Kingdom.

21 ⁹Department of Veterinary Science and Microbiology, The University of Arizona, Tucson,
22 Arizona, USA.

23 ¹⁰Department of Pathobiology, Canada Veterinary College, University of Guelph, Guelph,
24 Canada.

25 ¹¹Institute of Medical Microbiology and Hygiene, Österreichische Agentur für Gesundheit
26 und Ernährungssicherheit (AGES), Vienna, Austria.

27 ¹²Faculty of Medicine, University of Maribor, Maribor, Slovenia.

28 ¹³National laboratory for Health, Environment and Food, Maribor, Slovenia.

29 ¹⁴Department of Pathogen Molecular Biology, London School of Hygiene and Tropical
30 Medicine, University of London, London, United Kingdom.

31 ¹⁵Department of Microbiology, PathWest Laboratory Medicine, Queen Elizabeth II Medical
32 Centre, Western Australia, Australia.

33 ¹⁶Microbiology & Immunology, School of Pathology & Laboratory Medicine, The University
34 of Western Australia, Western Australia, Australia.

35

36

37 *Correspondence to: Trevor Lawley, tl2@sanger.ac.uk

38

39 †These authors contributed equally to this work

40

41

42 **Running title:** Clonal *C. difficile* infect different hosts globally.

43

44 keywords: *Clostridium difficile*, RT078, intercontinental transmission, inter-host
45 transmission, accessory genome, One Health concept.

46 **Abstract.** The emergence of *Clostridium difficile* as a significant human diarrheal pathogen
47 is associated with the production of highly transmissible spores and the acquisition of
48 antimicrobial resistance genes (ARGs) and virulence factors. Unlike the hospital associated
49 *C. difficile* RT027 lineage, the community associated *C. difficile* RT078 lineage is isolated
50 from both humans and farm animals; however, the geographical population structure and
51 transmission networks remain unknown. Here we applied whole genome phylogenetic
52 analysis of 248 *C. difficile* RT078 strains from 22 countries. Our results demonstrate limited
53 geographical clustering for *C. difficile* RT078 and extensive co-clustering of human and
54 animal strains, thereby revealing a highly-linked, inter-continental transmission network
55 between humans and animals. Comparative whole-genome analysis reveals indistinguishable
56 accessory genomes between human and animal strains, and a variety of antimicrobial
57 resistance genes in the pangenome of *C. difficile* RT078. Thus, bi-directional spread of *C.*
58 *difficile* RT078 between farm animals and humans may represent an unappreciated route
59 disseminating antimicrobial resistance genes between humans and animals. These results
60 highlight the importance of the “One Health” concept to monitor infectious disease
61 emergence and the dissemination of antimicrobial resistance genes.

62
63
64
65
66
67
68
69
70
71
72
73

74 **Introduction:** Over the past decade, *Clostridium difficile* has emerged as the primary cause
75 of infectious antibiotic associated diarrhea in hospitalized patients (1). Unlike other common
76 healthcare-associated pathogens, *C. difficile* produces resistant spores that facilitate host-to-
77 host transmission and enable long term survival and dispersal in the healthcare system and
78 the wider environment (2). The emergence of epidemic *C. difficile* ribotype (RT) 027 (NAP1
79 / ST-1), responsible for many large-scale hospital outbreaks worldwide (3, 4), has been
80 linked to environmental spore contamination and the acquisition of fluoroquinolone
81 resistance (5). Enhanced research focus on *C. difficile* in the aftermath of the *C. difficile*
82 RT027 outbreaks has revealed other evolutionarily distinct *C. difficile* lineages, in particular
83 *C. difficile* RT078 (NAP07-08/ST-11), that are now emerging as significant human pathogens
84 for unknown reasons (6).

85 The “One Health” concept, connecting the health of humans to the health of animals
86 and their shared environments, represents a relevant framework for understanding the
87 emergence and spread of pathogens. *C. difficile* RT078 is commonly isolated from both
88 humans and farm animals (7) and increasingly recognized as a causative agent of both
89 healthcare and community-associated *C. difficile* infection (CDI) (8). This lineage typically
90 affects a younger population (9) and results in higher mortality than *C. difficile* RT027 (10).
91 Standard genotyping tools have highlighted genetic similarities between human and animal
92 *C. difficile* RT078 (11-13) strains raising the possibility of zoonotic transmission (14).
93 Nevertheless, the exact evolutionary and epidemiological relationships between human and
94 animal *C. difficile* RT078 strains remain unknown due to the lack of discriminatory power of
95 these typing methods and the clonal nature of *C. difficile* lineages. Recently, using whole
96 genome phylogeny, we reported that asymptomatic farmers and their pigs can be colonized
97 with clonal *C. difficile* RT078 isolates demonstrating evidence for spread between animals
98 and humans (15).

99 **Results and Discussion:** Here we assess the broad genetic diversity of *C. difficile* RT078, by
100 performing whole genome sequence analysis of 247 strains isolated predominantly from
101 humans and animals that were collected from 22 countries across North America, Europe,
102 Australia and Asia between 1996 and 2012 (<https://microreact.org/project/rJs-SYgMe>) (Table
103 S1). We explored the phylogenetic structure of *C. difficile* RT078 by generating a core
104 genome maximum likelihood phylogeny that included the 247 *C. difficile* RT078 strains and
105 the reference genome of *C. difficile* M120 (n=248) (Fig. 1). Superimposing the geographic
106 origin of strains revealed considerable co-clustering of European (dark green) and North
107 American (purple) strains across the phylogeny (Fig. 1). Permutation analysis on randomly
108 generated, equalized subsets of European (dark green) and North American (purple) genomes
109 confirmed co-clustering of geographically diverse strains (Fig. S1). In addition, the absence
110 of a single clade of *C. difficile* RT078 isolated in Australia (light green) is also suggestive of
111 sporadic transmission between Europe and Australia (Fig. 1). Overall, the observed lack of
112 geographic clustering is characteristic of repeated, international transmission.

113 We next examined the phylogenetic distribution of strains isolated from humans
114 (n=184) and animals (n=59) to understand the potential for zoonotic spread. This analysis
115 identified examples of human to human and animal to animal spread and strong evidence of
116 bi-directional spread of *C. difficile* RT078 between animals and humans across the
117 phylogeny. These observations are supported by the extensive co-clustering of human (blue
118 lines) and animal strains (red lines) (Fig. 1). Focused analysis of closely related *C. difficile*
119 RT078 strains identified 6 clusters containing both animal and human isolates with identical
120 core genome and highly similar whole genomes (ANI \geq 99.73%; Table 1). Surprisingly,
121 Cluster 1 consists of an animal strain from Canada and human strains from UK indicating
122 that zoonotic spread of *C. difficile* is not confined to a local population of humans and

123 animals as found previously (15). The existence of highly related human and animal isolates
124 suggests that *C. difficile* RT078 has frequently spread between animals and humans.

125 Next, a detailed analysis of the accessory genome, including mobile genetic
126 elements, was performed to further explore the genomic similarities between human and
127 animal strains. Of the 6,239 unique genes present across our genome collection, 3,368 genes
128 (54.0%) were assigned to the core genome leaving 2,871 genes (46.0%) present in the
129 accessory genome (Fig. S2). Considering only the human and animal isolates, 2,859
130 accessory genes were identified. The vast majority of human and animal specific accessory
131 genes were found at low frequencies in the population (Fig. 2A). We observed no statistically
132 significant difference in the number of strains carrying accessory genes exclusive to either the
133 human or the animal population (χ^2 p-value of 0.39). Considering only those accessory genes
134 present in at least 10% of isolates (n= 465), 461 (99.1%) were identified in both human and
135 animal isolates. The absence of accessory genes unique to either group demonstrates that
136 either *C. difficile* has a stable accessory genome, which is host independent or provides
137 further support for the frequent transmission of *C. difficile* between host populations.

138 Given the high percentage of mobile elements including antimicrobial resistance
139 genes harbored by *C. difficile* genomes (5, 6), we next sought to analyze distribution of
140 different ARGs in the pangenome of human and animal strains. In total, 22 different putative
141 ARGs are present in the 243 *C. difficile* RT078 genomes (Fig. 2B). The most common ARG
142 was the chromosome encoded *cdeA*, a well-known multidrug transporter that was detected in
143 all strains; however, other common genes included those encoding resistance to
144 aminoglycosides, tetracycline and erythromycin (Fig. 2B). Importantly, no specific ARGs
145 were statistically enriched in the animal isolates; however, the *ermB* (erythromycin resistance
146 methylase) gene was identified in the human isolates (Fisher's-exact test, q value = 1.25E-
147 07). These results provide further support that a clonal *C. difficile* RT078 population

148 containing a broad array of ARGs is spreading between humans and farm animals except
149 *ermB*, which has signs of unknown selective pressure in the human isolates.

150 *C. difficile* is an ancient, genetically diverse species that has only emerged as a
151 significant human pathogen over the past four decades. It remains to be determined why
152 evolutionary distinct lineages such as *C. difficile* RT027 and RT078 (6) are simultaneously
153 emerging to cause disease in the human population. Previously we have demonstrated that *C.*
154 *difficile* RT027 acquired fluoroquinolone resistance during the 1990s in North America and
155 rapidly spread through the global healthcare system (5). Here we demonstrated that *C.*
156 *difficile* RT078 has spread multiple times between continents, in particular North America
157 and Europe, highlighting that *C. difficile* emergence and spread is a global issue. In contrast
158 to the distinct animal- and human-associated populations observed for the multidrug-resistant
159 enteric pathogen *Salmonella* Typhimurium DT104 (16), we demonstrated that *C. difficile*
160 RT078 is a clonal population moving frequently between livestock and human hosts with no
161 geographical barriers. Although the original reservoir remains unknown, the reciprocal
162 transmission between humans and farm animals emphasizes the importance of a
163 comprehensive One Health perspective in managing and controlling *C. difficile* RT078.

164

165

166

167

168

169

170

171

172

173 **Materials and Methods**

174 **Collection of *C. difficile* strains**

175 *C. difficile* laboratories worldwide were asked to send a diverse representation of their
176 *C. difficile* 078 collections to the Lawley laboratory hosted at the Wellcome Trust Sanger
177 Institute. Sample shipping was coordinated by the Lawley laboratory. After receiving all
178 shipped samples the DNA extraction was performed batch wise by one person using the same
179 protocol and reagents to minimize bias. Phenol-Chloroform was the preferred method for
180 extraction since it provides high DNA yield and intact chromosomal DNA. The genomes of
181 182 strains designated as *C. difficile* RT078 (NAP07-08/ST-11), by PCR ribotyping (17)
182 were sequenced and combined with our previous collection of 65 strains of *C. difficile* RT078
183 (12) making a total of 247 strains analyzed in this study. These 247 strains were collected
184 between 1996 and 2012 and are comprised of representative strains from 4 continents (North
185 America, Europe, Australia and Asia). Of these strains, 183 were derived from humans, 59
186 from animals (pigs, cattle, horses and poultry), 4 foods and 1 environmental sample. Details
187 of all sequenced strains are listed in Table S1, including the European Nucleotide Archive
188 (ENA) sample accession numbers. Metadata of the *C. difficile* RT078 strains has been made
189 freely publicly available through Microreact (18) (<https://microreact.org/project/rJs-SYgMe>).

190 **Bacterial culture and genomic DNA preparation**

191 *C. difficile* strains were cultured on blood agar plates (bioMérieux, the Netherlands)
192 for 48 hours, inoculated into liquid medium (brain–heart infusion (BHI) broth supplemented
193 with yeast extract and cysteine) and grown over night (ca 16 hours) anaerobically at 37 °C.
194 Cells were pelleted, washed with phosphate-buffered saline (PBS), and genomic DNA
195 preparation was performed using a phenol–chloroform extraction as previously described
196 (19).

197 **DNA sequencing, assembly and annotation**

198 Paired-end multiplex libraries were prepared and sequenced using Illumina Hi-Seq
199 platform with fragment size of 200-300bp and a read length of 100bp, as previously
200 described (20, 21). An in-house pipeline developed at the Wellcome Trust Sanger Institute
201 (<https://github.com/sanger-pathogens/Bio-AutomatedAnnotation>) was used for bacterial
202 assembly and annotation. It consisted of *de novo* assembly for each sequenced genome using
203 Velvet v. 1.2.10 (22), SSPACE v. 2.0 (23) and GapFiller v 1.1 (24) followed by annotation
204 using Prokka v. 1.5-1 (25).

205 **Construction and analysis of the Pan genome**

206 We used the pan genome pipeline Roary (26), to identify the *C. difficile* RT078 pan
207 genome. Roary takes annotated draft assemblies in GFF3 format which were produced by
208 Prokka (25). Predicated coding regions were extracted from the input and converted to
209 protein sequences. Partial sequences (>5% nucleotides unknown or sequence length less than
210 120 nucleotides) were filtered and the remaining sequences were iteratively clustered with
211 CD-HIT beginning with a sequence identity of 100% and matching length of 100% down to a
212 default sequence identity of 98%. One final clustering step was performed again with CD-hit,
213 with a sequence identity of 100% leaving one representative sequence for each cluster in a
214 protein FASTA file. This was followed by a comprehensive, pairwise comparison with
215 BLASTP on the reduced sequences with a default sequence identity percentage of 95% and
216 matching length of 100%. The pan genome embodies the core genome, defined as those
217 genes present in at least 90% of the genomes, and the accessory genome, defined as those
218 genes present in between 10% and 90% of the genomes. Rare variant genes, found in less
219 than 10% of genomes, were discarded.

220 Core genes (n=3,368) alignment, an output from Roary, was used to construct
221 phylogenetic structure of 248 *C. difficile* strains. Single nucleotide polymorphisms (SNPs)
222 were extracted from the core gene alignment using SNP-sites (27). Maximum likelihood tree

223 based on SNPs alignment was constructed using FastTree with $-\text{gamma} -\text{gtr}$ settings (28) and
224 tree was visualized with iTOL (29).

225

226 **Average Nucleotide Identity (ANI) analysis**

227 Using Roary analysis, *C. difficile* RT078 strains isolated from humans and animals
228 with identical core genome were extracted using an in-house R script. ANI was calculated by
229 performing pairwise comparison of genome assemblies of these *C. difficile* RT078 strains
230 using MUMmer (30).

231 **Identification of antimicrobial resistance gene sequence**

232 Antimicrobial resistance genes were identified within the *C. difficile* RT078 genomes through
233 comparison to the CARD database with the ARIBA software ([https://github.com/sanger-](https://github.com/sanger-pathogens/ariba)
234 [pathogens/ariba](https://github.com/sanger-pathogens/ariba)).

235

236

237

238

239

240

241

242

243

244

245

246

247

248

249 **Acknowledgments:**

250 We acknowledge the Wellcome Trust [098051]; the United Kingdom Medical Research
251 Council [PF451 to TL, MR/L015080/1 to TC and MR/K000551/1 to FM, MP, BW and TL];
252 the Australian National Health and Medical Research Council [1091097 to SF] and the
253 Victorian Government's Operational and Infrastructure Support Program [SF] for financial
254 support. CK has been supported by a ZonMw grant (125020004). We are grateful to A.
255 Neville and the Wellcome Trust Sanger Institute Pathogen Informatics team for their input.

256

257 **Conflict of interests**

258 The authors declare no competing financial interests.

259

260

261

262

263

264

265

266

267

268

269

270

271

272

273

274

275

276

277

278

279

280

281

282

283

284

285

286

287

288

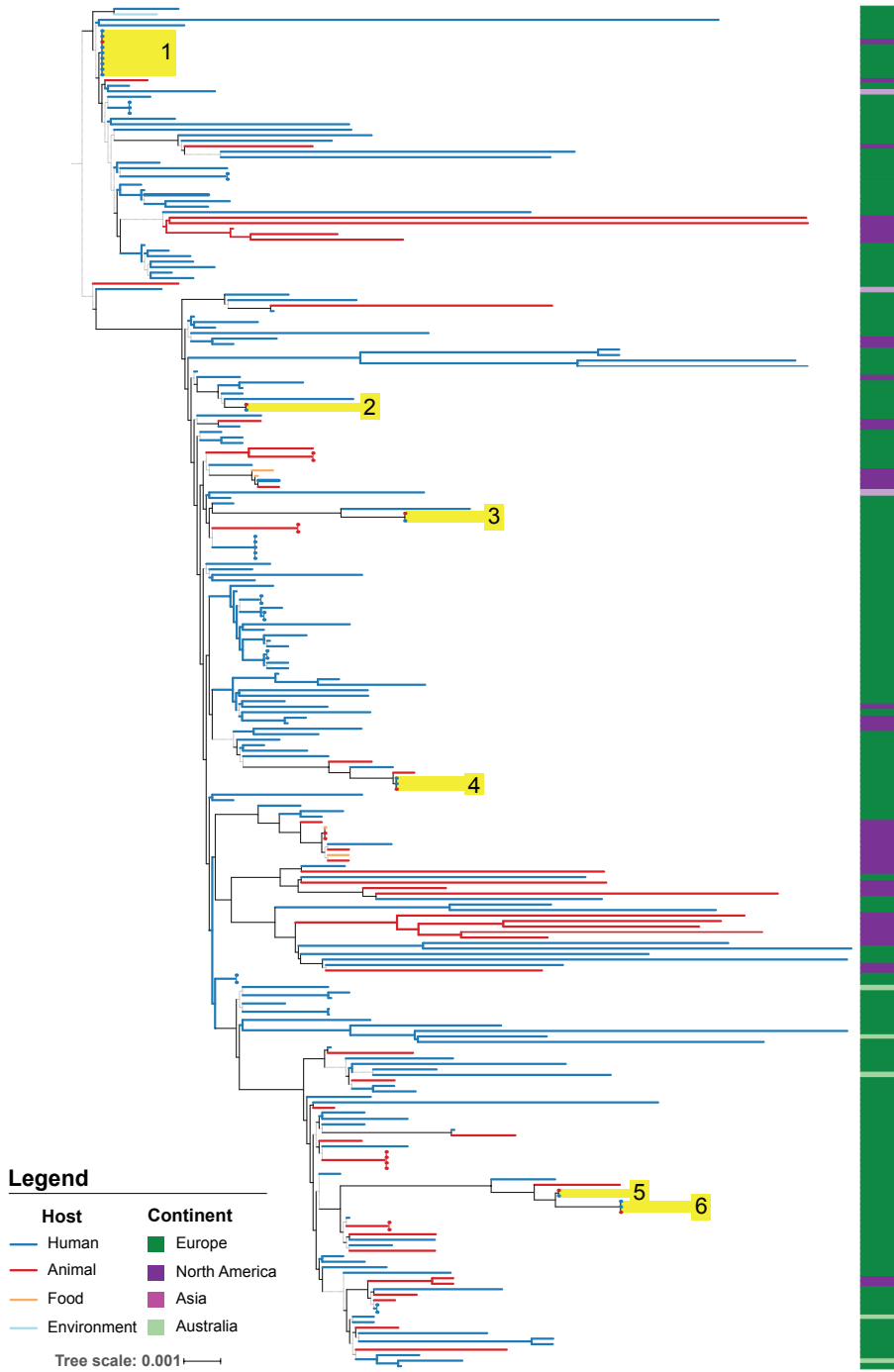
289 **References:**

290

- 291 1. Lessa FC, Winston LG, McDonald LC, Emerging Infections Program CdST. 2015.
292 Burden of *Clostridium difficile* infection in the United States. *N Engl J Med*
293 372:2369-2370.
- 294 2. Leffler DA, Lamont JT. 2015. *Clostridium difficile* Infection. *N Engl J Med* 373:287-
295 288.
- 296 3. Kuijper EJ, Coignard B, Tull P, difficile ESGfC, States EUM, European Centre for
297 Disease P, Control. 2006. Emergence of *Clostridium difficile*-associated disease in
298 North America and Europe. *Clin Microbiol Infect* 12 Suppl 6:2-18.
- 299 4. Loo VG, Poirier L, Miller MA, Oughton M, Libman MD, Michaud S, Bourgault AM,
300 Nguyen T, Frenette C, Kelly M, Vibien A, Brassard P, Fenn S, Dewar K, Hudson TJ,
301 Horn R, Rene P, Monczak Y, Dascal A. 2005. A predominantly clonal multi-
302 institutional outbreak of *Clostridium difficile*-associated diarrhea with high morbidity
303 and mortality. *N Engl J Med* 353:2442-2449.
- 304 5. He M, Miyajima F, Roberts P, Ellison L, Pickard DJ, Martin MJ, Connor TR, Harris
305 SR, Fairley D, Bamford KB, D'Arc S, Brazier J, Brown D, Coia JE, Douce G,
306 Gerding D, Kim HJ, Koh TH, Kato H, Senoh M, Louie T, Michell S, Butt E, Peacock
307 SJ, Brown NM, Riley T, Songer G, Wilcox M, Pirmohamed M, Kuijper E, Hawkey P,
308 Wren BW, Dougan G, Parkhill J, Lawley TD. 2013. Emergence and global spread of
309 epidemic healthcare-associated *Clostridium difficile*. *Nat Genet* 45:109-113.
- 310 6. He M, Sebahia M, Lawley TD, Stabler RA, Dawson LF, Martin MJ, Holt KE, Seth-
311 Smith HM, Quail MA, Rance R, Brooks K, Churcher C, Harris D, Bentley SD,
312 Burrows C, Clark L, Corton C, Murray V, Rose G, Thurston S, van Tonder A, Walker
313 D, Wren BW, Dougan G, Parkhill J. 2010. Evolutionary dynamics of *Clostridium*
314 *difficile* over short and long time scales. *Proc Natl Acad Sci U S A* 107:7527-7532.
- 315 7. Rupnik M, Widmer A, Zimmermann O, Eckert C, Barbut F. 2008. *Clostridium*
316 *difficile* toxinotype V, ribotype 078, in animals and humans. *J Clin Microbiol*
317 46:2146.
- 318 8. Bauer MP, Notermans DW, van Benthem BH, Brazier JS, Wilcox MH, Rupnik M,
319 Monnet DL, van Dissel JT, Kuijper EJ, Group ES. 2011. *Clostridium difficile*
320 infection in Europe: a hospital-based survey. *Lancet* 377:63-73.
- 321 9. Goorhuis A, Bakker D, Corver J, Debast SB, Harmanus C, Notermans DW,
322 Bergwerff AA, Dekker FW, Kuijper EJ. 2008. Emergence of *Clostridium difficile*
323 infection due to a new hypervirulent strain, polymerase chain reaction ribotype 078.
324 *Clin Infect Dis* 47:1162-1170.
- 325 10. Walker AS, Eyre DW, Wyllie DH, Dingle KE, Griffiths D, Shine B, Oakley S,
326 O'Connor L, Finney J, Vaughan A, Crook DW, Wilcox MH, Peto TE, Infections in
327 Oxfordshire Research D. 2013. Relationship between bacterial strain type, host
328 biomarkers, and mortality in *Clostridium difficile* infection. *Clin Infect Dis* 56:1589-
329 1600.
- 330 11. Bakker D, Corver J, Harmanus C, Goorhuis A, Keessen EC, Fawley WN, Wilcox
331 MH, Kuijper EJ. 2010. Relatedness of human and animal *Clostridium difficile* PCR
332 ribotype 078 isolates determined on the basis of multilocus variable-number tandem-
333 repeat analysis and tetracycline resistance. *J Clin Microbiol* 48:3744-3749.
- 334 12. Keel K, Brazier JS, Post KW, Weese S, Songer JG. 2007. Prevalence of PCR
335 ribotypes among *Clostridium difficile* isolates from pigs, calves, and other species. *J*
336 *Clin Microbiol* 45:1963-1964.
- 337 13. Keessen EC, Gaastra W, Lipman LJ. 2011. *Clostridium difficile* infection in humans
338 and animals, differences and similarities. *Vet Microbiol* 153:205-217.

- 339 14. Squire MM, Riley TV. 2013. *Clostridium difficile* infection in humans and piglets: a
340 'One Health' opportunity. *Curr Top Microbiol Immunol* 365:299-314.
- 341 15. Knetsch CW, Connor TR, Mutreja A, van Dorp SM, Sanders IM, Browne HP, Harris
342 D, Lipman L, Keessen EC, Corver J, Kuijper EJ, Lawley TD. 2014. Whole genome
343 sequencing reveals potential spread of *Clostridium difficile* between humans and farm
344 animals in the Netherlands, 2002 to 2011. *Euro Surveill* 19:20954.
- 345 16. Mather AE, Reid SW, Maskell DJ, Parkhill J, Fookes MC, Harris SR, Brown DJ,
346 Coia JE, Mulvey MR, Gilmour MW, Petrovska L, de Pinna E, Kuroda M, Akiba M,
347 Izumiya H, Connor TR, Suchard MA, Lemey P, Mellor DJ, Haydon DT, Thomson
348 NR. 2013. Distinguishable epidemics of multidrug-resistant *Salmonella* Typhimurium
349 DT104 in different hosts. *Science* 341:1514-1517.
- 350 17. Stubbs SL, Brazier JS, O'Neill GL, Duerden BI. 1999. PCR targeted to the 16S-23S
351 rRNA gene intergenic spacer region of *Clostridium difficile* and construction of a
352 library consisting of 116 different PCR ribotypes. *J Clin Microbiol* 37:461-463.
- 353 18. Argimon S, Abudahab K, Goater RJ, Fedosejev A, Bhai J, Glasner C, Feil EJ, Holden
354 MT, Yeats CA, Grundmann H, Spratt BG, Aanensen DM. 2016. Microreact:
355 visualizing and sharing data for genomic epidemiology and phylogeography. *Microb
356 Genom* 2:e000093.
- 357 19. Croucher NJ, Harris SR, Fraser C, Quail MA, Burton J, van der Linden M, McGee L,
358 von Gottberg A, Song JH, Ko KS, Pichon B, Baker S, Parry CM, Lambertsen LM,
359 Shahinas D, Pillai DR, Mitchell TJ, Dougan G, Tomasz A, Klugman KP, Parkhill J,
360 Hanage WP, Bentley SD. 2011. Rapid pneumococcal evolution in response to clinical
361 interventions. *Science* 331:430-434.
- 362 20. Harris SR, Feil EJ, Holden MT, Quail MA, Nickerson EK, Chantratita N, Gardete S,
363 Tavares A, Day N, Lindsay JA, Edgeworth JD, de Lencastre H, Parkhill J, Peacock
364 SJ, Bentley SD. 2010. Evolution of MRSA during hospital transmission and
365 intercontinental spread. *Science* 327:469-474.
- 366 21. Quail MA, Kozarewa I, Smith F, Scally A, Stephens PJ, Durbin R, Swerdlow H,
367 Turner DJ. 2008. A large genome center's improvements to the Illumina sequencing
368 system. *Nat Methods* 5:1005-1010.
- 369 22. Zerbino DR, Birney E. 2008. Velvet: algorithms for de novo short read assembly
370 using de Bruijn graphs. *Genome Res* 18:821-829.
- 371 23. Boetzer M, Henkel CV, Jansen HJ, Butler D, Pirovano W. 2011. Scaffolding pre-
372 assembled contigs using SSPACE. *Bioinformatics* 27:578-579.
- 373 24. Boetzer M, Pirovano W. 2012. Toward almost closed genomes with GapFiller.
374 *Genome Biol* 13:R56.
- 375 25. Seemann T. 2014. Prokka: rapid prokaryotic genome annotation. *Bioinformatics*
376 30:2068-2069.
- 377 26. Page AJ, Cummins CA, Hunt M, Wong VK, Reuter S, Holden MT, Fookes M, Falush
378 D, Keane JA, Parkhill J. 2015. Roary: rapid large-scale prokaryote pan genome
379 analysis. *Bioinformatics* 31:3691-3693.
- 380 27. Page AJ, Taylor B, Delaney AJ, Soares J, Seemann T, Keane JA, Harris SR. 2016.
381 SNP-sites: rapid efficient extraction of SNPs from multi-FASTA alignments.
382 *Microbial Genomics* 2.
- 383 28. Price MN, Dehal PS, Arkin AP. 2009. FastTree: computing large minimum evolution
384 trees with profiles instead of a distance matrix. *Mol Biol Evol* 26:1641-1650.
- 385 29. Letunic I, Bork P. 2011. Interactive Tree Of Life v2: online annotation and display of
386 phylogenetic trees made easy. *Nucleic Acids Res* 39:W475-478.
- 387 30. Delcher AL, Phillippy A, Carlton J, Salzberg SL. 2002. Fast algorithms for large-
388 scale genome alignment and comparison. *Nucleic Acids Res* 30:2478-2483.

389 **Figures**



390

391 **Figure 1. Phylogeography of human and animal *Clostridium difficile* RT078.** Maximum
392 likelihood, midpoint rooted phylogenetic tree of 248 genomes, representing strains isolated from
393 human (dark blue), animal (red), food (orange) and environment (light blue) and collected from
394 Europe (dark green), North America (purple), Asia (pink) and Australia (light green). Branches with
395 bootstrap confidence values above 0.7 are shown as solid lines. The phylogeny demonstrates clear
396 mixing of European and North American strain indicating multiple transmission events between
397 continents and mixing of human and animal strains indicating multiple transmissions events between
398 these hosts. Closely related clusters (see Table 1) containing both human and animal isolates are
399 labeled 1 – 6 and highlighted in yellow.

400

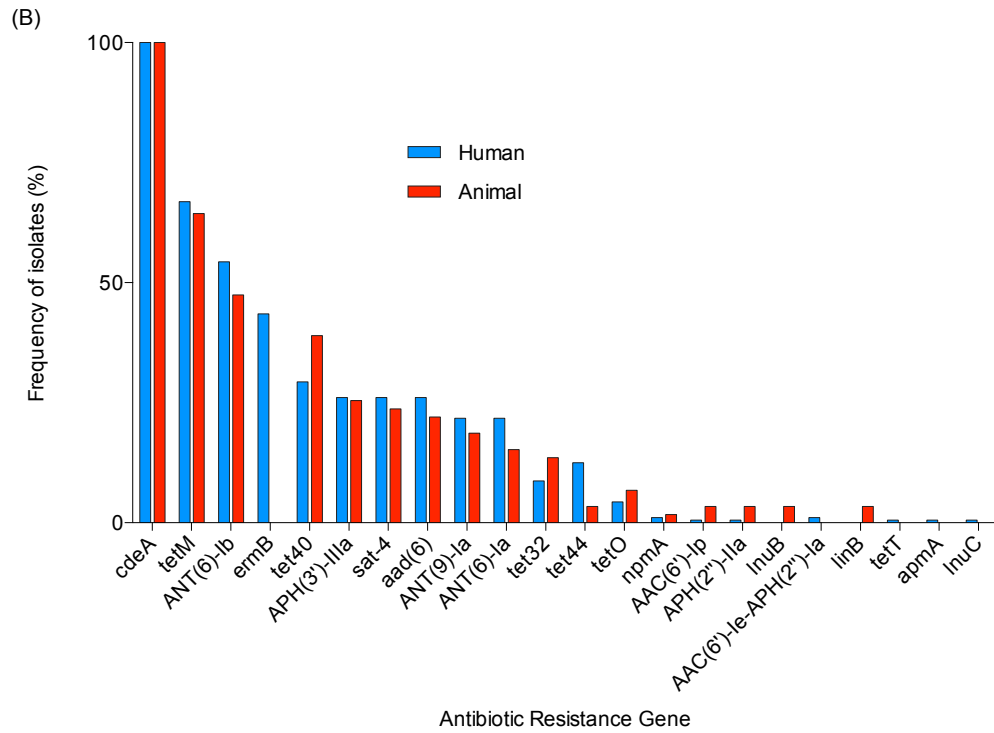
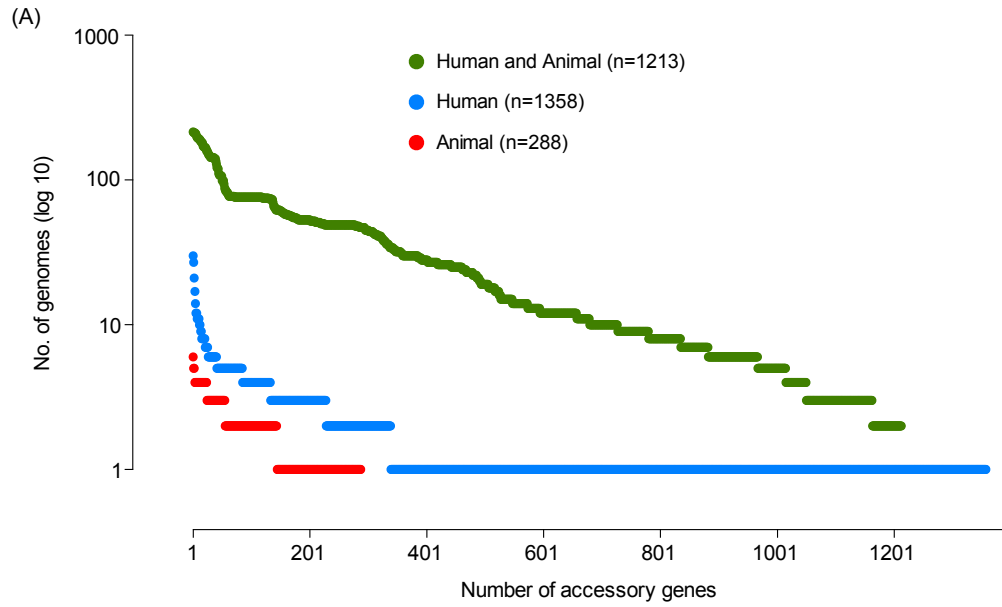
401

402

403

404

405



406

407

408

409 **Figure 2. Indistinguishable accessory genome of *C. difficile* RT078 harbours a variety of**
410 **antimicrobial resistance genes.**

411 A. The accessory genes (n=2,859) categorized according to host origin. The number of accessory
412 genes (x-axis) only found in human genomes (dark blue), only found in animal genomes (red) or
413 found in both human and animal genomes (green) is plotted against the number of genomes in which
414 these genes are present (y-axis).

415 B. The frequency of predicted antimicrobial resistances genes (ARGs) within the 243 *C. difficile*
416 RT078 strains. Human (dark blue) and animal (red) isolation sources are shown by color.

417

418

419

420

421

422

423

424

425

426

427

428

429

430

431 **Table 1.** Table of 6 highly similar *C. difficile* RT078 clusters identified as identical through core
 432 genome analysis, where isolates from both human and animal are present. Average Nucleotide
 433 Identity (ANI) for human isolate compared to the animal isolate is also shown.

434

Cluster	ENA ID	Year	Continent	Country	Host	ANI (%)
1	ERR171209	2004	North America	Canada	Animal	-
	ERR171230	2010	Europe	UK	Human	99.93
	ERR256911	2011	Europe	UK	Human	99.91
	ERR171303	2008	Europe	UK	Human	99.90
	ERR256986	2012	Europe	UK	Human	99.84
	ERR256910	2011	Europe	UK	Human	99.83
	ERR1910469	1997	Europe	UK	Human	99.82
	ERR1910468	1997	Europe	UK	Human	99.80
	ERR256981	2008	Europe	UK	Human	99.75
2	ERR257071	2011	Europe	Netherlands	Animal	-
	ERR257072	2011	Europe	Netherlands	Human	99.94
3	ERR257053	2011	Europe	Netherlands	Animal	-
	ERR257057	2011	Europe	Netherlands	Human	99.77
4	ERR257067	2011	Europe	Netherlands	Animal	-
	ERR171352	2011	Europe	Netherlands	Human	99.97
	ERR257052	2011	Europe	Netherlands	Human	99.91
5	ERR257046	2011	Europe	Netherlands	Animal	-
	ERR257061	2011	Europe	Netherlands	Human	99.82
6	ERR257065	2011	Europe	Netherlands	Animal	-
	ERR257044	2011	Europe	Netherlands	Human	99.80
	ERR257050	2011	Europe	Netherlands	Human	99.73

435