

Imipenem Resistance in *Clostridium difficile* Ribotype 017, Portugal

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We describe imipenem-resistant and imipenem-susceptible clinical isolates of *Clostridium difficile* ribotype 017 in Portugal. All ribotype 017 isolates carried an extra penicillin-binding protein gene, *pbp5*, and the imipenem-resistant isolates had additional substitutions near the transpeptidase active sites of *pbp1* and *pbp3*. These clones could disseminate and contribute to imipenem resistance.

Clostridium difficile, a toxin-producing, spore-forming bacillus, is a main cause of nosocomial antimicrobial drug-associated diarrhea in industrialized countries (1). *C. difficile* infection (CDI) usually develops in previously hospitalized persons with a recent history of antimicrobial drug use and causes illness with symptoms ranging from mild diarrhea to potentially lethal pseudomembranous colitis (2). Antimicrobial drugs disrupt the protective gut microbiota, enabling ingested *C. difficile* spores to germinate in the colon and providing a selective advantage to nonsusceptible strains (3). CDI is mainly mediated by the TcdA and TcdB toxins, though some strains additionally produce a binary toxin. Multiple antimicrobial drugs can promote CDI, and cephalosporins and fluoroquinolones have been associated with a higher risk for CDI (3). Multidrug resistance is frequently found in epidemic *C. difficile* strains; determinants of resistance are often found in horizontally transferable mobile genetic elements (4). In past decades, CDI prominence has increased because of a sudden rise in outbreaks and an increase in disease severity and death (5). This shift was mainly associated with the dissemination of fluoroquinolone-resistant PCR ribotype (RT) 027, which has been responsible for hospital outbreaks worldwide. Strains of other ribotypes, including RT078 and RT017, which have enhanced

virulence, have emerged (6). In particular, RT017, the most common toxin A-negative, toxin B-positive ribotype, is widespread in Asia and is common in Europe (7–9). In a pan-European study of ≈900 *C. difficile* strains, the overall rate of resistance to imipenem, an antimicrobial drug of the carbapenem class, currently widely used as a last-line drug to treat infections by gram-negative bacteria, was found to be 7.41%, and the geometric mean (GM) MIC of imipenem for RT017 strains was 5.91 mg/L (8). In another study, isolates collected in a South Korea hospital during 2000–2009 were analyzed, and a resistance rate to imipenem of 8% (12% among RT017 isolates) was found (10).

The Study

We characterized 191 *C. difficile* isolates collected during September 2012–September 2015 from 15 hospitals in Portugal (online Technical Appendix, <https://wwwnc.cdc.gov/EID/article/24/4/17-0095-Techapp1.pdf>). We found 24 (12.6%) were resistant to imipenem. Of these 24 isolates, 22 were RT017, 1 was RT014, and 1 was RT477. The MIC for imipenem for RT017, the imipenem-resistant isolates, was >32 mg/L (Table 1); the MIC for the 2 non-RT017 isolates was 16 mg/L. The 22 imipenem-resistant RT017 isolates were found at hospital A throughout the study period, suggesting the existence of a persistent clone, a finding supported by whole-genome sequencing data (online Technical Appendix). Among the 25 RT017 isolates, 3 were imipenem-susceptible and from hospital B (MIC range 1.5–3 mg/L) (Table 1).

RT017 *C. difficile* strains are frequently resistant to clindamycin, erythromycin, moxifloxacin, tetracycline, or rifampin (individually or in combination) (8,10). In this study, the 22 RT017 imipenem-resistant isolates were also found to be resistant to all of these antimicrobial drugs and showed higher meropenem and ertapenem MICs than those of the RT017 imipenem-susceptible isolates (Table 1; online Technical Appendix; online Technical Appendix Figure). Multidrug resistance to noncarbapenem antimicrobial drugs correlated with the presence of several genetic determinants, many located in mobile genetic elements (Figure 1; online Technical Appendix), in line with the idea that multidrug-resistant strains have a selective advantage (4) and that horizontal gene transfer plays a major role in the evolution of this pathogen (11).

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DOI: <https://doi.org/10.3201/eid2404.170095>

Table 1. Susceptibility of *Clostridium difficile* RT017 imipenem-resistant isolates from hospital A and imipenem-susceptible isolates from hospital B to 11 antimicrobial drugs, Portugal*

Hospital	Resistance breakpoint†	Antimicrobial drug, MIC breakpoints, mg/L										
		IMP‡	ETP‡	MRP‡	MXF†§	MTZ†§	VAN†§	CLI‡	CHL‡	RIF†	TGC†	TET‡
A, 22 isolates	MIC range	>32	3–16	1.5–4	>32	<0.016–1	0.38–2	>256	2–6	>32	<0.016–0.094	16–32
	GM MIC	32	7.56	2.31	32	0.12	0.73	256	3.29	32	0.025	18.08
	MIC ₉₀	32	12	3	>32	0.38	2	256	4	32	0.032	32
	MIC ₅₀	32	6	2	>32	0.19	0.75	256	3	32	0.023	16
	% Resistant	100	4.5	0	100	0	0	100	0	100	0	100
B, 3 isolates	MIC range	1.5–3	1.5–2	0.5–1.5	1.5	<0.016–0.25	0.38–0.75	>256	3–4	>32	<0.016–0.023	16
	GM MIC	2.08	1.82	0.83	1.5	0.072	0.60	256	3.30	32	0.020	16
	MIC ₉₀	3	2	1.5	1.5	0.25	0.75	256	4	32	0.023	16
	MIC ₅₀	2	2	0.75	1.5	0.094	0.75	256	3	32	0.023	16
	% Resistant	0	0	0	0	0	0	100	0	100	0	100
p value		<0.0001	<0.0001	<0.0001	<0.0001	0.45	0.56	ND	0.98	ND	0.41	0.51

*CHL, chloramphenicol; CLI, clindamycin; ETP, ertapenem; GM, geometric mean; IMP, imipenem; MIC₅₀, minimal inhibitory concentration for 50% of strains; MIC₉₀, minimal inhibitory concentration for 90% of strains; MRP, meropenem; MTZ, metronidazole; MXF, moxifloxacin; ND, not done; RIF, rifampin; TGC, tigecycline; VAN, vancomycin.

†European Committee on Antimicrobial Susceptibility Testing breakpoint.

‡Clinical and Laboratory Standards Institute breakpoint.

§Previously determined (9).

Through whole-genome sequencing, we found 13 single-nucleotide polymorphisms (SNPs) that differentiated the imipenem-resistant and imipenem-susceptible RT017 isolates (Table 2; Figure 1; online Technical Appendix). We found 2 SNPs in the genes coding for 2 high molecular weight (HMW) penicillin-binding proteins (PBPs) (Figure 1). HMW PBPs, which are bifunctional enzymes containing transglycosylase and transpeptidase domains, are categorized into class A, and PBPs lacking the transglycosylase domain are categorized into class B. The transpeptidase domain harbors 3 functional motifs (SXXK, SXN, and KTG[T/S]) that comprise the active site. Carbapenems block cell wall synthesis by inhibiting transpeptidase activity (12). One of the mutations found in the imipenem-resistant isolates affected the gene coding for PBP1, the single class A bifunctional peptidoglycan synthase of *C. difficile*; the mutation resulted in the amino acid substitution Ala555Thr close to the SSN functional motif (Figure 2). The second mutation was found in the gene encoding for the PBP3 class B transpeptidase and caused the amino acid replacement Tyr721Ser between the SXN and KTG motifs (Figure 2). Neither of these changes was found in the 3 imipenem-susceptible RT017 isolates (Figure 1). Moreover, the 2 non-RT017 imipenem-resistant isolates, with a MIC for imipenem lower than that of the RT017 isolates, revealed either the Ala555Thr change or a different substitution (Leu543His), both in PBP1, also close to the functional motif SXN (Table 2). Modified PBPs with reduced affinity for the antimicrobial drug have been associated with resistance to β -lactams and specifically to imipenem in several microorganisms (12). We found no differences between the imipenem-resistant and imipenem-susceptible RT017 isolates in genes encoding other peptidoglycan synthases (Figure 1). It is possible that the substitutions in PBP1 and PBP3 in RT017 confer high-level resistance to

imipenem and reduced susceptibility to other carbapenems, and at least in the RT014 and RT477 isolates studied, the single Ala555Thr substitution (or other substitutions in the vicinity of the SXN motif) is sufficient for an intermediate level of resistance.

However, all RT017 isolates studied herein, as well as the previously annotated strains M68 (GenBank accession no. NC_017175) and BJ08 (accession no. CP003939), have a fifth HMW class B PBP, PBP5, encoded in a mobile element (online Technical Appendix). Whether PBP5 contributes to imipenem resistance remains to be determined. Moreover, in imipenem-resistant isolates, the key sporulation-specific gene *sigK*, which is contiguous to *pbp2*, is interrupted by the 17-kb *skin*^{cd} element (13), and the *pbp5* region is contiguous to a transposon-like element carrying the *ermB* gene (shown as PUBMLST allele 8; https://pubmlst.org/bigsdbs?db=pubmlst_cdiffficile_sqdef&page=alleleInfo&locus=ermB&allele_id=8). It is unknown whether these genetic differences contribute to imipenem resistance.

Conclusions

Imipenem resistance in *C. difficile* RT017 probably involves the acquisition of mutations in both *pbp1* and *pbp3* that lead to amino acid substitutions close to the functional motifs of their transpeptidase domains. These substitutions might decrease the affinity of PBP1 and PBP3 for imipenem, enabling peptidoglycan synthesis in the presence of the antimicrobial drug. Considering that the presence of an additional PBP (PBP5) is a characteristic of RT017 strains, we suggest that PBP5 facilitates the expression of imipenem resistance through acquisition of mutations in *pbp1* and *pbp3*. In strains of other ribotypes lacking PBP5, such as the RT014 and RT477 isolates herein described, mutations in *pbp1* might only lead to

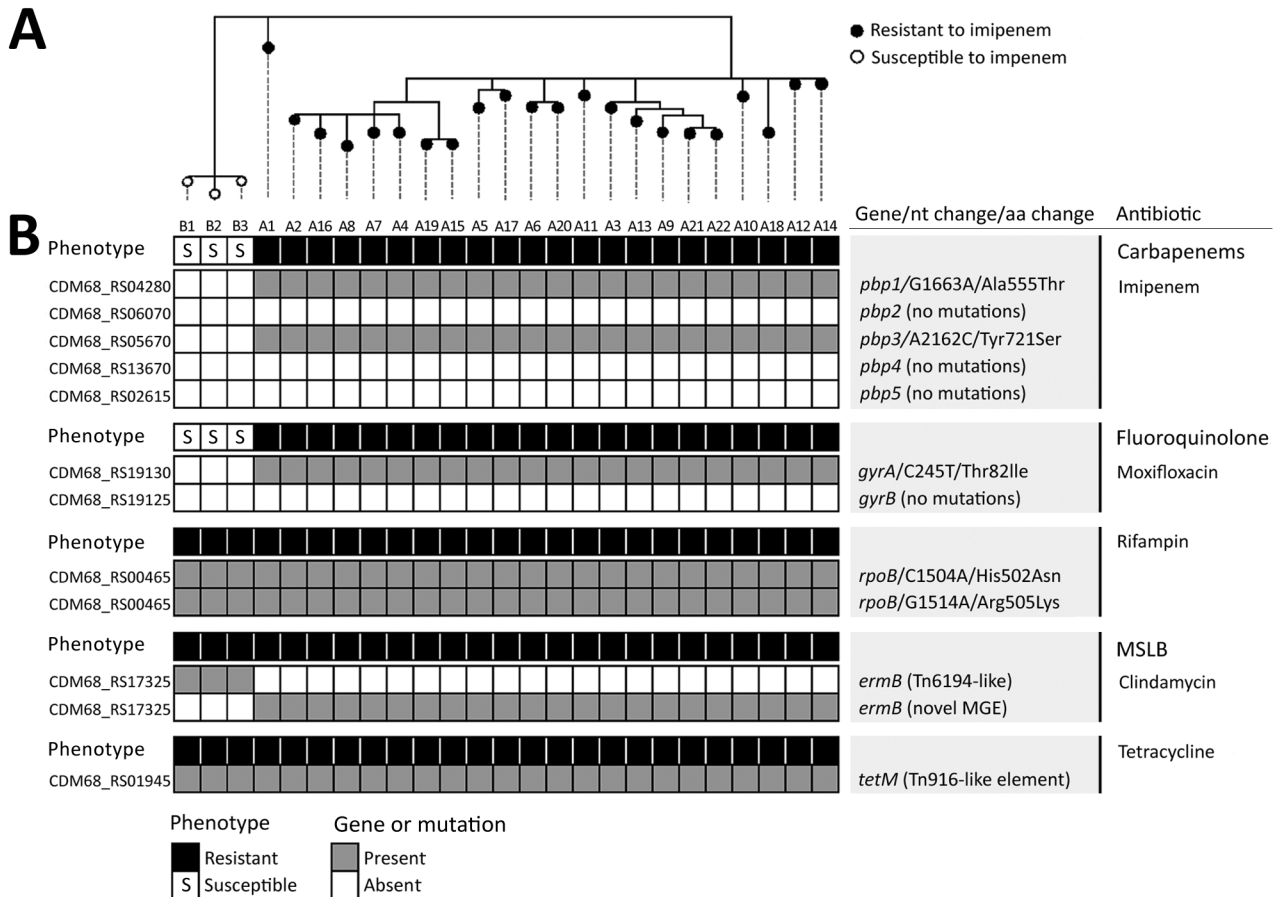


Figure 1. Phylogeny of *Clostridium difficile* RT017 isolates from hospitals A and B and genetic determinants of antimicrobial drug resistance, Portugal. A) Core genome single-nucleotide polymorphism–based neighbor-joining phylogeny of 25 RT017 *C. difficile* clinical isolates reconstructed by using 47 variant sites (outside MGEs) identified when mapping to either the corresponding genomic sequence of close relative *C. difficile* strain M68 (GenBank accession no. NC_017175) or a draft genome sequence of a representative clinical isolate. B) For each isolate, the profile of antimicrobial drug susceptibility is indicated together with respective potential genetic determinants of antimicrobial drug resistance. Only antimicrobial drugs for which a resistant phenotype was observed are displayed. Gene locus tags are relative to the *C. difficile* M68 genome annotation. Both nucleotide and amino acid replacements refer to mutations in the resistant isolates when comparing with susceptible isolates. No mutations means that no mutations are present differentiating resistant isolates of hospital A from susceptible isolates of hospital B, although mutations are present relative to M68. Both the *pbp5*-carrying region and the *ermB* gene (present in all isolates) were found to be inserted in distinct genomic contexts (online Technical Appendix, <https://wwwnc.cdc.gov/EID/article/24/4/17-0095-Techapp1.pdf>). MSLB, macrolide/lincosamide/streptogramin B; MGE, mobile genetic element.

intermediate levels of resistance. We further suggest that the spreading of *pbp5* might contribute to the dissemination of high-level imipenem resistance.

Portugal has a high rate of healthcare-associated infections and is a major consumer of carbapenems (1). Although carbapenem consumption has not been directly linked to *C. difficile* resistance, we speculate that the emergence of resistance and reduced susceptibility to these antimicrobial drugs might recapitulate the scenario observed with fluoroquinolone-resistant RT027 in the United States, where fluoroquinolones were the most prescribed antimicrobial drug (14). Our findings further reinforce the need for the responsible use of antimicrobial

drugs; the emergence of carbapenem resistance in multi-drug-resistant *C. difficile* clones might result in the dissemination of resistant strains.

Acknowledgments

We thank the participating hospitals for providing samples for the laboratory surveillance of CDI.

This work was supported by the National Institute of Health Dr. Ricardo Jorge (grant no. 2016DDI1284). We also acknowledge grant Pest-C/EQB/LA0006/2011 from Fundação para a Ciência e a Tecnologia (FCT; <http://www.fct.pt>) and program Investigador FCT (IF/00268/2013/CP1173/CT0006 to M.S.). A.L.M. was supported by a PhD fellowship (PD/BD/105738/2014) from the FCT.

Table 2. Mutations differentiating *Clostridium difficile* RT017 imipenem-resistant isolates found at hospital A from imipenem-susceptible isolates found at hospital B, Portugal

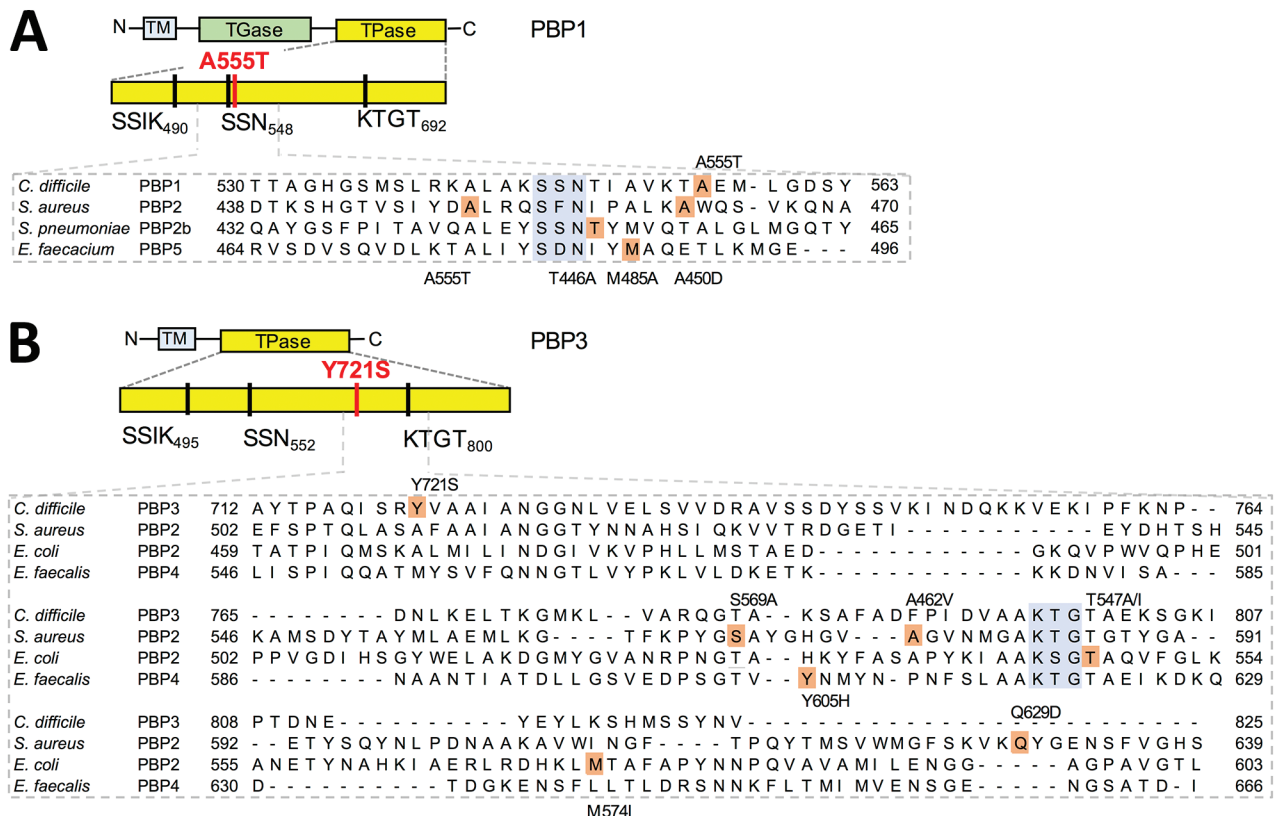
Gene in M68 genome*	Genome position*	Nucleotide in M68	Nucleotide change†	Amino acid change‡	Gene product
RS02665	512416	C	C578T	Ala193Val‡	Multidrug ATP-binding cassette transporter permease, associated with antimicrobial drug resistance
RS04280/ <i>pbp1</i>	905394	G	G1663A	Ala555Thr‡	Penicillin-binding transpeptidase
RS04935	1048151	C	T1010C	Ile337Thr‡	3-Isopropylmalate dehydratase large subunit
RS05670/ <i>pbp3</i>	1221182	G	A2162C	Tyr721Ser‡	Penicillin-binding protein
RS07765	1666351	G	G214T	Gly72§	Hypothetical protein
RS07795/ <i>hisB</i>	1671129	T	T209C	Ile70Thr‡	Imidazoleglycerol-phosphate dehydratase
RS07810	1673280	T	C474T	Ala158Ala	Imidazoleglycerol-phosphate synthase cyclase subunit
RS08415	1792079	G	A241G	Lys81Glu‡	Hypothetical protein (domain of MerR-like transcriptional regulators)
RS08810	1882950	C	C420T	Asp140Asp	Flavodoxin
RS14235	3083548	G	G421T	Gly141§	Haloacid dehalogenase
RS18530	4054525	C	C220T	Gln74§	S-adenosyl methionine-dependent methyltransferase
RS19130/ <i>gyrA</i>	4174650	C	C245T	Thr82Ile‡	DNA gyrase subunit A
RS19545	4255124	C	C400T	His134Tyr‡	Phage portal, SPP1 Gp6-like family protein

*Relative to the annotation of the *C. difficile* M68 genome (GenBank accession no. NC_017175).

†Changes observed between imipenem-resistant and imipenem-susceptible isolates.

‡Nonsynonymous mutations.

§Mutations leading to putative protein truncation.



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Technical Appendix

Materials and Methods

Clostridium difficile Strains

A group of 191 strains isolated during 2012–2015 from 15 Portuguese hospitals (*I*) were tested for imipenem susceptibility. The group included strains from the ribotypes most commonly found in Portugal, including ribotype (RT) 027 (n = 33), RT017 (n = 25), RT014 (n = 16), RT203 (n = 9), RT126 (n = 8), RT078 (n = 5), and RT020 (n = 3). The remaining 92 isolates comprised 51 less common ribotypes (*I*).

Antimicrobial Susceptibility Testing

MIC of imipenem was determined with Etest strips (bioMérieux, Marcy l'Etoile, France) on brucella blood agar with hemin and vitamin K1 (Becton Dickinson, Heidelberg, Germany), according to the manufacturer's instructions. Considering that microcolonies were found inside the inhibition ellipse (MIC >32 mg/L) at 48 h of growth but not at 24 h for most susceptible strains, MICs of imipenem were read at 24 h to avoid false resistance. Subculturing of these microcolonies yielded the same susceptible profile, which was further confirmed by the agar dilution method. For control purposes, the agar dilution method was also performed for 10 resistant strains (8 RT017, 1 RT014, and 1 RT477 isolates) and 45 susceptible strains (multiple RTs) to confirm resistant and susceptible phenotypes.

The imipenem resistant isolates identified were subsequently tested against other carbapenems (meropenem and ertapenem) and also against rifampin, clindamycin, chloramphenicol, tetracycline, and tigecycline. Resistance to imipenem was confirmed by the agar dilution method on Wilkins-Chalgren (Oxoid, Basingstoke, UK) agar, as described by Freeman et al., with imipenem at 8 mg/L, 16 mg/L, and 32 mg/L, plus drug-free control plates (2). The EUCAST (European Committee on Antimicrobial Susceptibility Testing) and CLSI

(Clinical and Laboratory Standards Institute) breakpoints used are presented in Table 1 of main text.

Whole-Genome Sequencing and Data Analysis

Genomic DNA was extracted from pure cultures of 25 *Clostridium difficile* strains by using the Isolate II Genomic DNA kit (Bioline, London, UK). For each strain, whole-genome sequencing (WGS) was carried out as previously described (3). For each strain, draft genome sequences were de novo assembled by using Velvet (version 1.2.10) (4) with runs optimized taking advantage of VelvetOptimiser script version 2.2.5 (<http://www.vicbioinformatics.com/software.velvetoptimiser.shtml>). Draft genome sequences were analyzed to do the following: a) perform in silico multilocus sequence typing (MLST) and allele determination of well-known virulence-associated genes by using the online platform available at PUBMLST (<http://pubmlst.org/>); b) search for the presence of putative antimicrobial resistance (AMR) genes by using both CARD (<https://card.mcmaster.ca/>) and ResFinder (<http://www.genomicepidemiology.org/>); c) identify potential dissimilarities enrolling AMR genes; and d) verify the genomic context of potential horizontally transferable AMR genes. To identify mutations likely associated with imipenem resistance, 2 core genome SNP-based approaches were followed: a de novo assembly strategy with Harvest (5) and an assembly-free strategy with Snippy v3.1 (<https://github.com/tseemann/snippy>). To potentiate the use of high-quality SNPs, only variant calls passing default criteria (5) were considered in the Harvest approach, while for Snippy, only variant sites with minimum mapping quality of 60, minimum number of reads covering the variant position of 10, and minimum proportion of reads differing from the reference of 90% were considered for downstream analysis. For the Snippy approach, reads were initially mapped against the most closely related *C. difficile* genome available at GenBank (strain M68; accession no. NC_017175). To maximize the number of core genome sites available for SNP comparison, core genome SNP-based analyses were repeated by using a draft assembled genome of 1 representative clinical strain (isolate B2) as a reference sequence. MEGA5 software (<http://www.megasoftware.net>) was applied to calculate matrices of nucleotide distances and perform phylogenetic reconstructions over the obtained core genome SNP alignment by using the neighbor-joining method with bootstrapping (1,000 replicates). Non-RT017 imipenem-resistant strains were subjected to PCR and Sanger sequencing (Technical Appendix Table) as a matter of scrutiny for *pbp*-associated mutations found for the RT017

isolates. Raw sequence reads of the 25 *C. difficile* isolates subjected to WGS were deposited in Sequence Read Archive under the accession nos. SRR4199259, SRR4199346, SRR4199859, SRR4201714, SRR4205841, SRR4205953, SRR4205954, SRR4213076, SRR4213123, SRR4237569, SRR4237571, SRR4237665, SRR4237666, SRR4237667, SRR4238391, SRR4238392, SRR4238569, SRR4240476, SRR4240477, SRR4240494, SRR4240495, SRR4240496, SRR4240497, SRR4240498, and SRR4240499.

Results and Discussion

Antimicrobial Drug Resistance Profiles

Among the 191 *C. difficile* studied, 24 (12.6%) were resistant to imipenem (at 24 h of growth), of which 22 were RT017 strains (MIC >32 mg/L), 1 was RT014, and 1 was RT477 (MIC of 16 mg/L) (Table 1 in main text). Intermediary resistance was observed for 27 strains (14.1%; MIC range 6–12 mg/L), most of which (24 isolates) were of RT027. RT017 strains here identified to be imipenem-resistant had been previously found to be resistant to moxifloxacin, with a MIC of >32 mg/L, while the 3 selected imipenem-susceptible strains were found to be susceptible to moxifloxacin (Table 1 main text). Both groups had also been found to be susceptible to metronidazole and vancomycin (*I*).

In the present study, further susceptibility testing against other carbapenems (Technical Appendix Figure) showed that imipenem-resistant strains exhibited a geometric mean (GM) MIC of 7.56 mg/L for ertapenem, with 17 presenting an intermediate MIC of 4–16 mg/L and 1 being resistant (MIC of 16 mg/L) (Technical Appendix Figure). The imipenem-susceptible strains were susceptible to ertapenem (GM MIC of 1.82 mg/L). All strains were susceptible to meropenem, although the imipenem-resistant strains had a significantly higher GM MIC (2.31 mg/L) when compared with the imipenem-susceptible strains (GM MIC of 0.83 mg/L) (Figure 1 in main text; Table 1 in main text; Technical Appendix Figure). Both groups of strains were highly resistant to rifampin (MIC₅₀ of 32 mg/L) and clindamycin (MIC₅₀ of 256 mg/L), while resistance to tetracycline was borderline (MIC₅₀ of 16 mg/L). All strains were susceptible to chloramphenicol and tigecycline.

Potential Genetic Determinants of Imipenem Resistance

WGS data was first subjected to in silico MLST analysis and evaluation of well-known *C. difficile* virulence-associated genes. All 25 RT017 strains belonged to sequence type 37 (MLST clade 4). The pathogenicity locus (PaLoc) showed a complete *tcdB* gene (PubMLST allele 9) but a disrupted *tcdA* gene, characteristic of this ribotype (6). No mutations were found in the putative toxin-negative regulatory gene *tcdC* (PubMLST allele 7, https://pubmlst.org/bigsub?db=pubmlst_cdiffficile_seqdef&page=alleleInfo&locus=tcdC_comple te&allele_id=7) or in the *tcdE* gene, encoding holin-like protein. The transcriptional regulator *tcdR* is predicted to be functional, contrarily to the closest related strain (M68). Genes *cdtA* and *cdtB* were absent from all the genomes.

Core genome SNP-based analysis confirmed that the 3 RT017 susceptible strains from hospital B were separated in a single cluster apart from the 22 imipenem-resistant strains from hospital A (Figure 1 in main text). All strains revealed a large core genome sequence identity, with only 13 variant sites perfectly discriminating the imipenem-resistant from the imipenem-susceptible strains (Table 2 in main text). The imipenem-resistant strains had a G to A nucleotide substitution at position 1,663 (relative to the start codon) of *pbp1*, a homologue of the CDM68_RS04280, that results in the amino acid substitution Ala555Thr near the conserved SSN motif. They also showed an A to C mutation at position 2,162 in *pbp3*, a homologue of CDM68_RS05670, leading to the amino acid replacement Tyr721Ser located between the conserved SCN and KTGT transpeptidase motifs (Figure 2 in main text). These 2 *pbp* mutations are likely genetic determinants of imipenem resistance in *C. difficile* (see main text). Among the other 11 group-specific mutations, we highlight the well-known substitution Thr82Ile in *gyrA* (PubMLST allele 35, https://pubmlst.org/bigsub?db=pubmlst_cdiffficile_seqdef&page=alleleInfo&locus=gyrA&allele_id=35), which could be associated with the resistance to fluoroquinolones displayed by the 22 hospital A strains. The remaining 10 SNPs (8 nonsynonymous mutations) differentiating the 2 groups (Table 2 in main text) affected genes belonging to different functional categories, such as metabolic enzymes, a transcriptional regulator, a hypothetical protein, and a multidrug ATP-binding cassette transporter permease. Other penicillin-binding protein (PBP)-encoding genes revealed no differences among the 25 isolates of RT017 (Figure 1 in main text).

Additional Differences between Imipenem-Resistant and Susceptible Strains

The genomic architecture surrounding CDM68_RS06070 (here designated as *pbp2*) was found to differ between imipenem-resistant and imipenem-susceptible isolates. In the resistant isolates, *sigK* (contiguous to *pbp2*) is interrupted by the 17-kb *skin*^{cd} element, a previously described prophage-like insertion (7) highly similar in both genetic content and organization to that of the M68 strain. Of note, this element includes the *vanZ* gene, which is related to teicoplanin resistance. We note, however, that although important for the timely activity of the regulatory mother cell-specific sigma factor σ^K , *skin*^{cd} is dispensable for sporulation and has not been associated with β -lactam resistance (8).

On the other hand, CDM68_RS02615, herein referred to as *pbp5*, which has not been found in ribotypes other than RT017 (*C. difficile* usually has genes coding for only 4 high molecular weight PBP genes), is located inside a large region that displays traces of horizontal gene transfer (flanked by multiple repeat regions and containing genes coding for recombinases, integrases, and other phage-related proteins). Although all RT017 isolates carry the *pbp5* gene, in imipenem-resistant isolates (but not in the imipenem-susceptible isolates), the *pbp5* region is contiguous to a transposon-like element carrying the *ermB* gene (PUBMLST allele 8), which confers resistance to the MLS_B (macrolide, lincosamide, streptogramin B) class of antimicrobials. This *ermB*-containing element is likely rare, considering it has only been found in 1 of the available *C. difficile* genomes (strain F253; accession nos. NZ_AVKO01000052.1 and AVKO01000420.1). A single copy of *ermB* was also identified in the imipenem-susceptible isolates (PUBMLST allele 1) but located in a previously described Tn6194-like mobile element, similarly to the genome of strain M68 (genome position 3,779,408–3,806,743).

Genotype–phenotype associations were also found for other antimicrobial drugs tested (Figure 1 in main text). Indeed, all strains displayed 2 nucleotide mutations in *rpoB* (PubMLST allele 20, https://pubmlst.org/bigsub?db=pubmlst_cdiffficile_seqdef&page=alleleInfo&locus=rpoB&allele_id=20) conferring resistance to rifampin. The *tetM* gene (PUBMLST allele 15, https://pubmlst.org/bigsub?db=pubmlst_cdiffficile_seqdef&page=alleleInfo&locus=tetM&allele_id=15), which confers resistance to tetracycline, was found to be present in all 25 strains, integrated in a Tn916-like element. Additional in silico screening for antimicrobial resistance genes revealed the presence of a gene encoding a chloramphenicol acetyltransferase

(CDM68_02605 homologue) in all strains. However, its presence was not associated with resistance to chloramphenicol because all strains were susceptible to this antimicrobial drug.

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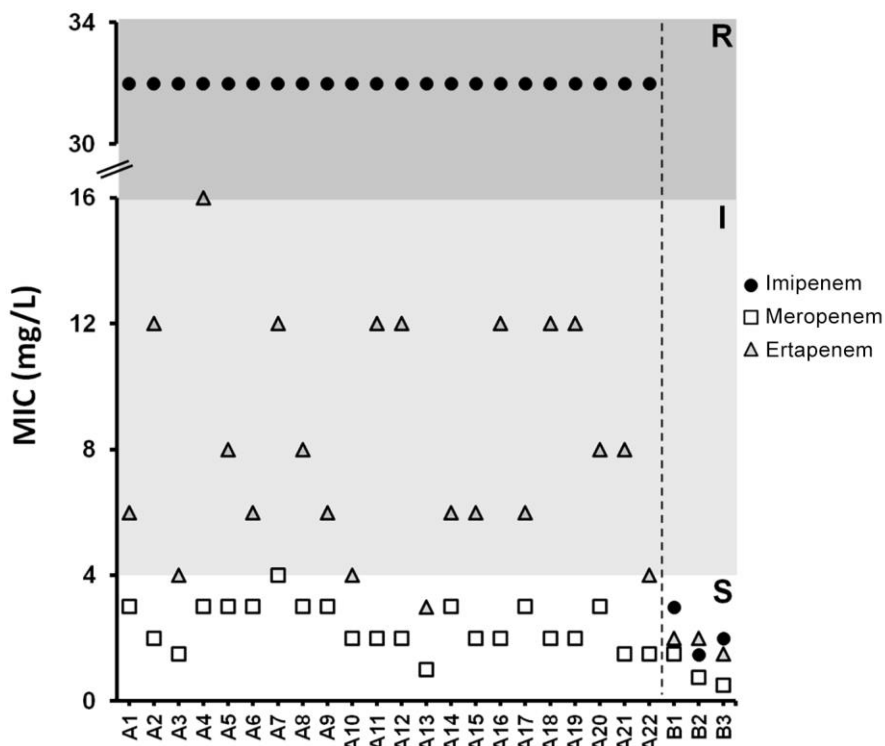
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Technical Appendix Table. Primers designed for amplification and sequencing of the transpeptidase region of the 2 *Clostridium difficile* penicillin-binding proteins genes mutated in the imipenem-resistant isolates, Portugal*

Gene†	Primer	Sequence	Product size, bp	Annealing temperature, °C
CDM68_RS04280 and CD630_07810	PBP1-F	5'-TTAGATGACCCTACTCAAGTGGAC-3'	1,482	53
	PBP1-R	5'-AGAGCCAGAAGATTGCTTTCCT-3'		
CDM68_RS05670 and CD630_11480	PBP1-SEQ†	5'-TGGTGGTCTAATTGTAACACAAC-3'	NA	NA
	PBP3-F	5'-CTGAGTGCCAAAGAAGCGTT-3'	2,134	52
	PBP3-R	5'-CTGCTGGAGCAAAAGCAACA-3'		
	PBP3-SEQ†	5'-GCAGGAAAAGCACAGTCAGC-3'	NA	NA

*NA, not application; PBP, penicillin-binding protein.

†Primers used only for sequencing.



Technical Appendix Figure. Carbapenem susceptibility profile of the *Clostridium difficile* ribotype 017 isolates from hospitals A and B, Portugal. The graph shows the MIC values (mg/L) of each carbapenem tested (imipenem, meropenem, and ertapenem) for the 25 ribotype 017 isolates. MIC ranges, according to CLSI (Clinical and Laboratory Standards Institute) breakpoints, are also shown. Imipenem-resistant and imipenem-susceptible isolates are separated by a vertical dashed line. I, intermediate; R, resistant; S, susceptible.