Pan-European longitudinal surveillance of antibiotic resistance among prevalent *Clostridium difficile* ribotypes

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Abstract

Clostridium difficile infection remains a major healthcare burden. Until the recent introduction of fidaxomicin, antimicrobial treatments were limited to metronidazole and vancomycin. The emergence of epidemic *C. difficile* PCR ribotype 027 and its potential link to decreased antibiotic susceptibility highlight the lack of large-scale antimicrobial susceptibility and epidemiological data available. We report results of epidemiological and antimicrobial susceptibility investigations of *C. difficile* isolates collected prior to fidaxomicin introduction, establishing important baseline data. Thirty-nine sites in 22 countries submitted a total of 953 *C. difficile* isolates for PCR ribotyping, toxin testing, and susceptibility testing to metronidazole, vancomycin, fidaxomicin, rifampicin, moxifloxacin, clindamycin, imipenem, chloramphenicol, and tigecycline. Ninety-nine known ribotypes were identified. Ribotypes 027, 014, 001/072, and 078 were most frequently isolated in line with previous European studies. There was no evidence of resistance to fidaxomicin, and reduced susceptibility to metronidazole and vancomycin was also scarce. Rifampicin, moxifloxacin, and clindamycin resistance (13%, 40%, and 50% of total isolates, respectively) were evident in multiple ribotypes. There was a significant correlation between lack of ribotype diversity and greater antimicrobial resistance (measured by cumulative resistance score). Well-known epidemic ribotypes 027 and 001/072 were associated with multiple antimicrobial resistance, but high levels of resistance were also observed, particularly in 018 and closely related emergent ribotype 356 in Italy. This raises the possibility of antimicrobial exposure as the underlying reason for their appearance, and highlights the need for ongoing epidemiological and antimicrobial resistance surveillance.

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Introduction

Clostridium difficile infection (CDI) is a major concern in healthcare environments, notably associated with excess mortality [1]. CDI represents a significant burden upon healthcare and financial resources. Metronidazole and vancomycin have been the main treatment options for CDI, but high recurrence rates and reports of reduced metronidazole susceptibility among epidemic *C. difficile* ribotypes have highlighted the need for new agents (https://www.gov.uk/government/uploads/system/uploads/attachment_data/file/347169/CDRN_annual_report.pdf) [2,3]. Fidaxomicin is a new macrocyclic antimicrobial with potent anti-*C. difficile* activity, that is non-inferior to vancomycin, with lower rates of CDI recurrence and minimal gut flora disruption [4]. Marketing authorisation for fidaxomicin in 2012 included a commitment to undertake antimicrobial

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resistance surveillance pre- and post-introduction. This affords a welcome opportunity to gather valuable antimicrobial susceptibility, epidemiological, and demographic data across Europe, and so fill a gap in the identification of local, national, and international epidemiological resistance trends.

The *Clos*ER (*Clostridium difficile* European Resistance) study aims to: identify and monitor the longitudinal susceptibility of contemporaneous *C. difficile* clinical isolates to antibiotics used for CDI treatment and those previously implicated in selection pressure; establish a comprehensive susceptibility database baseline for on-going surveillance; and provide data on the geographical distribution of clinical *C. difficile* strain types with analysis by region across Europe. We present here epidemiological and antimicrobial susceptibility data for *C. difficile* isolates collected prior to the introduction of fidaxomicin.

Methods

Study design

*Clos*ER is a 3-year pan-European, multi-centre *in vitro* surveillance study. The study period includes surveillance for 1 year prior to the introduction of fidaxomicin to the European market (July 2011–June 2012). Data will subsequently be available for 2 years post-introduction (2012–2014).

Participating centres were mainly national or regional *C. difficile* referral laboratories, selected using the European Study Group on *Clostridium difficile* (ESGCD) network (ECDIS-net), and with ESGCD approval. The number of sites approached per country was based on population (one site per 15 million population) or reported incidence of CDI (at least two sites for countries with >20 cases per 10,000 patient days per hospital), as in the study by Bauer *et al.* [5]. Sites were recruited on the basis that they:

1) were actively sampling and testing for CDI
2) experienced sufficient numbers of clinical CDI cases in order to reach a target of 25 de-duplicated cases during the 6-month collecting period
3) were willing to submit the required number of samples over 3 years

Fifty-one participating sites from 28 European countries were recruited and asked to submit 25 *C. difficile* isolates or toxin-positive faecal samples from de-duplicated CDI cases during each year. No further stipulations were made.

Isolates or faecal samples were submitted to a central laboratory (Leeds, UK) for PCR ribotyping, determination of toxin status, and susceptibility to metronidazole, vancomycin, rifampicin, fidaxomicin, moxifloxacin, clindamycin, chloramphenicol and tigecycline.

Results were communicated back to the participating laboratories in full.

Culture and toxin testing

Alcohol-shocked faecal specimens/*C. difficile* isolates were inoculated on to cycloserine-cefoxitin-egg-yolk agar (LabM, Heywood, Lancashire, UK) with lysozyme and cultured anaerobically for 48 hours at 37°C. Forty-eight-hour anaerobic brain-heart infusion broth culture supernatants of each test isolate were added to a Vero cell culture cytotoxicity assay with *Clostridium sordellii* antitoxin (ProLab Diagnostics, Bromborough, Merseyside, UK) neutralization.

Ribotyping

PCR ribotyping was performed on each isolate by the *Clostridium difficile* Ribotyping Network Reference Laboratory at Leeds Teaching Hospitals Trust, Leeds, UK (according to Stubbs *et al.* using capillary electrophoresis) [6,7]. Ribotypes were assigned against the Cardiff Anaerobe Reference Unit reference library at Leeds.

Susceptibility testing

Susceptibility of isolates (minimum inhibitory concentrations (MICs)) to metronidazole, vancomycin, rifampicin, chloramphenicol (Sigma, Dorset, UK); moxifloxacin (Bayer, Leverkusen, Germany); clindamycin, tigecycline (Pfizer, New York, NY); imipenem (MSD, Hertfordshire, UK); and fidaxomicin (Astellas, Chertsey, Surrey, UK) were determined by a Wilkins Chalgren agar incorporation method as previously described [2]. Breakpoints are defined in Table 1.

Results

Thirty-nine of the recruited 51 European sites submitted a total of 953 isolates or faecal samples collected during Year 1. Thirty-three (3.5%) submissions did not yield *C. difficile*; 91% of submissions yielded a toxigenic *C. difficile* isolate (n = 866).

PCR ribotyping results

Ninety-nine known ribotypes (RT) and 12 previously unseen profiles were observed. The most common RTs encountered were RTs 027 (12%), 001/072 (9%), 078, and 014 (both 8%) (Fig. 1). RT prevalence differed markedly according to country, with some exhibiting predominant RTs, while others showed a wide diversity of types.

Antimicrobial susceptibility (Tables, 1, 2 and 3)

All isolates were susceptible to fidaxomicin, with MIC_{50} and MIC_{90} well below the epidemiological cut-off value (http://

TABLE 1. Susceptibility of all *Clostridium difficile* isolates to 9 antimicrobials

	Metronidazole	Vancomycin	Fidaxomicin	Rifampicin	Moxifloxacin	Clindamycin	Imipenem	Chloramphenicol	Tigecycline
n =	916	918	918	918	918	917	918	919	919
Range	≤ 0.125–8	≤ 0.125–16	≤ 0.002–0.25	≤ 0.001–>16	0.125–>64	0.125–>64	0.125–>64	≤ 2–256	0.03–1
MIC_{50} (mg/L)	0.25	1	0.06	0.002	2	4	4	8	0.06
MIC_{90} (mg/L)	2	2	0.125	>16	32	>64	8	8	0.06
% S (breakpoint mg/L)	97.82 (≤ 2)	96.84 (≤ 2)	100 (< 1)	79.41 (≤ 0.004)	58.17 (≤ 2)	37.62 (≤ 2)	61.98 (≤ 4)	93.14 (≤ 8)	99.56 (< 0.25)
% I (breakpoint mg/L)	2.07 (4)	2.29 (4)	0[$] (>1)	19.61 (0.004–16)	1.85 (4)	12.76 (4)	30.61 (8)	3.16 (16)	0.44[$] (>0.25)
% R (breakpoint)	0.11 (≥ 8)	0.87 (≥ 8)	–	13.40 (≥ 16)	39.99 (≥ 8)	49.62 (≥ 8)	7.41 (≥ 16)	3.70 (≥ 32)	–

CLSI, U.S. Clinical and Laboratory Standards Institute; EUCAST, European Committee on Antimicrobial Susceptibility Testing; MIC, minimum inhibitory concentration. Breakpoints were defined as as sensitive (S), intermediately resistant (I) or resistant (R) with reference to CLSI, EUCAST or published data (see Table 2). For tigecycline and fidaxomicin, MICs were compared to the EUCAST epidemiological cut-off value (1 mg/L) (7) and defined as sensitive or reduced susceptibility.

www.ema.europa.eu/docs/en_GB/document_library/EPAR_-_Public_assessment_report/human/002087/WC500119707.pdf) (Table 1). Metronidazole, vancomycin, and tigecycline were active against 97.82%, 96.84%, and 99.56% of isolates tested, respectively; only a single UK isolate (RT106) had a metronidazole MIC of 8 mg/L. Twenty isolates showed reduced metronidazole susceptibility (from Czech Republic, Denmark, Germany, Italy, Hungary, Poland, Switzerland, and UK), 11 of which were RT027. Geometric mean metronidazole MICs were elevated in RT027 (1.42 mg/L), RT106, RT001/072 (both 0.65 mg/L), and RT356 (0.61mg/L) isolates compared with a range of 0.13–0.41 mg/L for the remaining prevalent RTs. Reduced vancomycin susceptibility was rare (94–100% sensitivity) in the 20 most prevalent RTs. Czech Republic, Ireland, Latvia, and Poland submitted single isolates with vancomycin MICs of 4 mg/L. Italy and Spain submitted multiple RTs displaying reduced vancomycin susceptibility, including resistant RT027, RT126, RT356, and RT001/072 isolates (>8 mg/L). Vancomycin MICs were notably higher among ribotypes 018 and 356, with geometric mean MICs of 2.00 and 2.28 mg/L, respectively, compared with a range of 0.62–0.95 mg/L for the remaining common RTs.

Rifampicin resistance (13.4%) was observed in submissions from 17/22 countries and in multiple RTs. In Italy, only 37.63% of isolates were rifampicin susceptible. All RT018 and 356 isolates (the predominating clones from Italy) were either intermediately or fully resistant to rifampicin. Czech Republic, Denmark, and Hungary also showed high levels of resistant isolates (63.64%, 56.52%, and 58.67% of total isolates, Table 3). In Denmark and Hungary, rifampicin resistance was almost

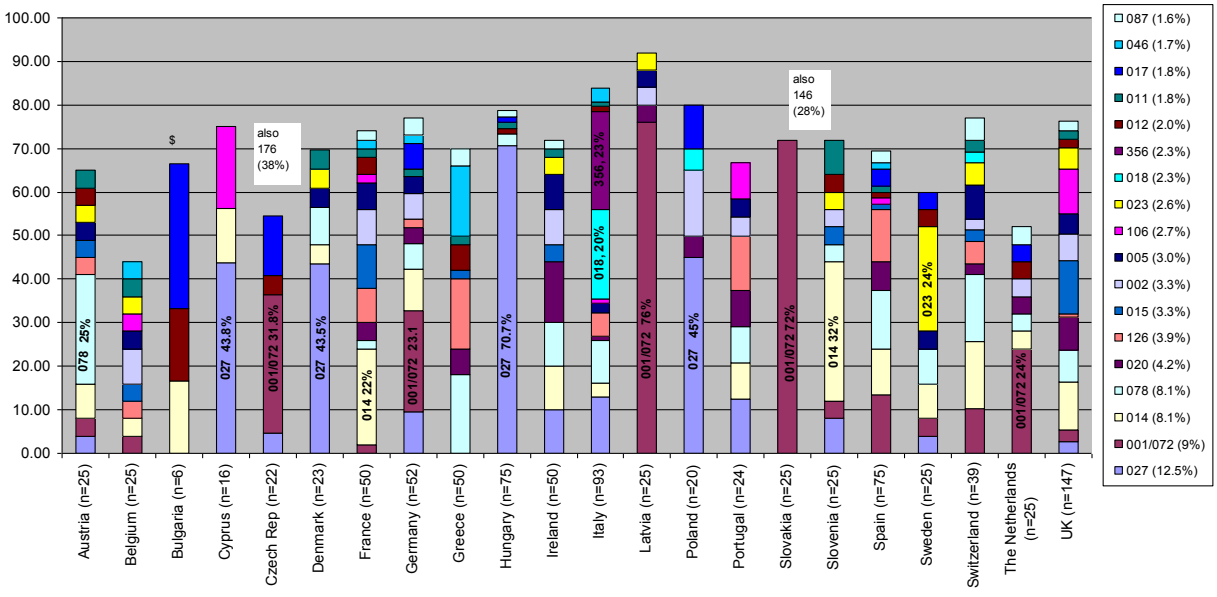

FIG. 1. Distribution of most commonly isolated ribotypes in 22 European countries (expressed as % of total isolates submitted by country). Legend shows the % prevalence among total isolates from all countries. Labelled data points indicate ribotypes of particular prevalence (>20%) in that country. $Only 7 isolates submitted. Number of sites per country: Austria (1); Belgium (1); Bulgaria (1); Cyprus (1); Czech Republic (1); Denmark (1); France (2); Germany (3); Greece (2); Hungary (3); Ireland (2); Italy (4); Latvia (1); Poland (1); Slovakia (1); Slovenia (1); Spain (2); Sweden (1); Switzerland (2); The Netherlands $ (1); and UK (n = 6).

TABLE 2. Geometric mean MICs of the most commonly isolated *Clostridium difficile* ribotypes

RT	027	001/072	014	078	020	126	015	002	005	106	023	018	356	012	011	017	046	087
n =	113	83	73	73	38	35	30	30	27	24	23	21	21	18	16	16	15	14
Metronidazole	1.42[a]	0.65[b]	0.27	0.28	0.31	0.23	0.19	0.25	0.27	0.65[b]	0.19	0.41	0.61[b]	0.13	0.18	0.26	0.23	0.28
Vancomycin	0.68	0.88	0.65	0.62	0.73	0.66	0.62	0.62	0.79	0.82	0.72	2.00[a]	2.28[a]	0.71	0.71	0.65	0.95	0.86
Fidaxomicin	0.03	0.01	0.06	0.04	0.07	0.04	0.04	0.05	0.05	0.07	0.07	0.04	0.04	0.04	0.04	0.02	0.04	0.05
Rifampicin	0.48[b]	0.007	0.002	0.003	0.002	0.002	0.001	0.002	0.003	0.002	0.003	2.07	18.87[b]	0.005	0.001	0.93	0.009	0.002
Moxifloxacin	22.28[a]	12.21	2.42	3.44	2.63	9.37	1.58	2.14	2.59	8.48	1.83	35.33[a]	50.80[a]	2.42	2.00	18.22[a]	4.00	2.00
Clindamycin	3.39	30.40[a]	5.02	2.90	6.20	11.89[a]	3.15	6.35	4.00	7.34	1.06	4.00	9.75[b]	28.51[a]	5.19	14.67[a]	22.11[a]	3.23
Imipenem	7.12[b]	5.27	4.32	3.18	4.80	3.62	5.30	4.19	5.17	6.54	3.65	5.56	5.86	5.44	4.36	5.91	5.28	4.64
Chloramphenicol	4.87	8.70[b]	5.90	5.12	5.66	5.38	5.30	6.20	6.20	6.92	6.48	5.04	4.88	9.70[b]	6.17	7.66[b]	8.38[b]	5.66
Tigecycline	0.04	0.04	0.05	0.05	0.05	0.06	0.05	0.04	0.05	0.04	0.05	0.04	0.04	0.08	0.04	0.04	0.05	0.06

RT, ribotype; MIC, minimum inhibitory concentration.
[a,b] Indicates elevated geometric mean MICs relative to other ribotypes.

exclusively associated with predominating RT027, but was evident in multiple RTs (017, 027, 176, and 001/072) in Czech Republic.

Moxifloxacin (39.99%) and clindamycin (49.62%) resistance were common and present in all participating countries. Rates of moxifloxacin resistance (intermediate or full) varied considerably from 8.00% in France to 100% in Poland (Table 3). Moxifloxacin resistance rates varied across the most common RTs. RT356 was uniformly moxifloxacin resistant (geometric mean MIC = 50.80 mg/L); RTs 018, 027, and 017 also had elevated geometric mean MICs (35.33, 22.28, and 18.22 mg/L, respectively). Clindamycin resistance was seen in all the most common RTs, but most notable among RT001/072, RT012, RT017, RT046, and RT126.

Most isolates were sensitive to imipenem (61.98%). Geometric mean imipenem MICs were highest in RT027 and RT106 (7.12 and 6.54 mg/L, respectively). Intermediate resistance (30.6%) was present in all the common RTs. Most prevalent RTs were largely susceptible (95–100% of isolates) to chloramphenicol, with geometric mean MICs between 4.87 and 9.7 mg/L), but resistance was notable among RT001/072 isolates from Latvia.

Antimicrobial susceptibility according to country (Fig. 2)

MIC results for each isolate were designated susceptible (S), intermediately resistant (I), or fully resistant (R) according to breakpoints (Table 1); each result was assigned a score (S = 0; I = 1, and R = 2). A cumulative resistance score based on susceptibility to each of the nine antimicrobials tested was then generated for each isolate. Thus, an isolate that was fully susceptible to four, intermediately resistant to two, and resistant to three antimicrobials would generate a score of 8 (0 + 0 + 0 + 0 + 1 + 1 + 2 + 2 + 2). Isolates were then grouped according to country, and mean cumulative resistance scores were generated for each country.

Discussion

Epidemiological surveillance of ribotypes

This is the largest pan-European study to date of *C. difficile* RT and antibiotic susceptibility epidemiology. Surveillance of more than 900 isolates submitted by 39 sites across 22 European countries showed a diverse array of known RTs across Europe (*n* = 99), with 12 previously unreported RTs. The most commonly isolated RTs (Fig. 1) were broadly similar to those reported by Bauer *et al.*, who examined 389 *C. difficile* isolates from across Europe [5]. Previously described epidemic or highly prevalent types (014/20, 027, 001/072, and 078)

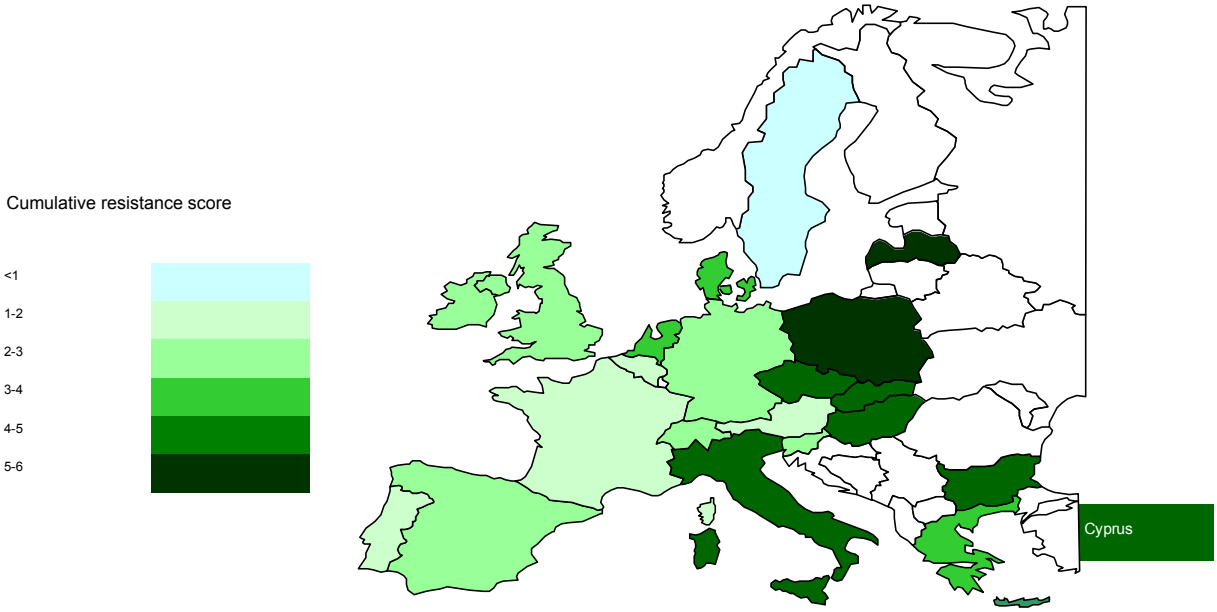

FIG. 2. Distribution of cumulative antimicrobial resistance in *Clostridium difficile* in 22 European countries (% of resistant *C. difficile* isolates per antibiotic).

remained prevalent in this study, but inter-country variations in relative prevalence of particular strains were apparent. This is not unexpected, since both endemic and epidemic spread of *C. difficile* is well documented [5,8–11]. Changes in RT methodology have led to greater discriminatory power and subdivision of types; the present study divides RT014/020 (16% prevalence in an earlier study) into two distinct RTs: 014 and 020 (8.1% and 4.2%, respectively) [5].

RT005, RT087, and RT356 were more prevalent than previously observed [5]. RT005 and RT087 were among the most antibiotic susceptible of the commonly isolated RTs. Contrastingly, RT356 isolates were submitted only from Italy and were among the least antibiotic susceptible of the whole cohort. RT018 accounted for >20% of Italian isolates in this study. The emergence of this type in Italy is documented [11], but RT356 has not been reported previously. Interestingly, the PCR banding profiles of RT018 and RT356 are closely related (94% similarity) and may belong to the same multi-locus sequence (MLST) type (Dr. Warren Fawley, personal communication). RT018 was detected first, indicated by its lower type number and earlier literature citation [11]. Taken together, it is possible that RT356 strains may have evolved from the RT018 lineage. Genome sequencing of these isolates is underway and will potentially add further clarity regarding both strain provenance and resistance development [12].

Antimicrobial susceptibility surveillance

There was no evidence of reduced susceptibility to fidaxomicin, consistent with earlier studies that reported good activity (range 0.006–1 mg/L) [13–18]. Finegold *et al.* reported a single isolate with a fidaxomicin MIC of 2 mg/L [16]. It has been reported that 'hypervirulent' *C. difficile* strains (including RT027) may be less fidaxomicin susceptible than others [13,18]. We observed no clear differences in susceptibility among RTs (geometric mean MICs ranged from 0.01–0.07 mg/L), despite the isolates originating from similar places as in the study by Debast *et al* [18].

These data represent a baseline for future studies of *C. difficile* after the introduction of fidaxomicin. Goldstein *et al.* reported a *C. difficile* strain with a fidaxomicin MIC of 16 mg/L isolated from a patient with recurrent diarrhoea 6 days after the last antibiotic dose [13]. The relatedness of the pre- and post-treatment strains was not determined, and the association of resistance with drug exposure cannot be made definitively. The clinical significance of such a strain is unclear, given that fidaxomicin achieves faecal concentrations in excess of 1000 ug/g [13]. Reduced metronidazole susceptibility was uncommon in our study, but most evident among RT027 and RT106 (geometric mean MIC = 1.42 and 0.65 mg/L, respectively), as previously described (https://www.gov.uk/government/uploads/system/uploads/attachment_data/file/347169/CDRN_annual_report.pdf) [2], and in emergent RT018 and RT356 (geometric means 0.41 and 0.61 mg/L, respectively). Single vancomycin-resistant strains of prevalent RTs were found: 014, 027, 078, 126, 001/072, and three among the emergent RT356 strains. One RT356 isolate had a vancomycin MIC of 16 mg/L; again, the clinical significance of elevated vancomycin MICs is unclear in the light of high gut vancomycin concentrations *in vivo* [19].

TABLE 3. Percentage of *Clostridium difficile* isolates showing intermediate or full resistance to 9 antimicrobials by country

	n	Metronidazole	Vancomycin	Fidaxomicin	Rifampicin	Moxifloxacin	Clindamycin	Imipenem	Chloramphenicol	Tigecycline
Austria	25	0.00	0.00	0.00	0.00	28.00	28.00	16.00	4.00	4.00
Belgium	25	0.00	0.00	0.00	4.00	12.00	56.00	0.00	0.00	0.00
Bulgaria	6	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
Cyprus	16	0.00	0.00	0.00	25.00	68.75	100.00	37.50	0.00	0.00
Czech Rep	22	4.55	4.55	0.00	63.64	77.27	63.64	40.91	18.18	0.00
Denmark	23	8.70	4.35	0.00	56.52	43.48	73.91	39.13	4.35	0.00
France	50	0.00	0.00	0.00	0.00	8.00	58.00	40.00	2.00	0.00
Germany	52	3.85	0.00	0.00	40.38	48.08	36.54	28.85	17.31	0.00
Greece	50	0.00	0.00	0.00	8.00	38.00	70.00	60.00	6.00	0.00
Hungary	75	2.67	0.00	0.00	58.67	77.33	28.00	76.00	1.33	0.00
Ireland	50	0.00	2.00	0.00	0.00	10.00	88.00	40.00	0.00	0.00
Italy	93	3.23	15.05	0.00	62.37	78.49	56.99	43.01	1.08	0.00
Latvia	25	0.00	4.00	0.00	4.00	88.00	76.00	72.00	68.00	0.00
Poland	20	40.00	5.00	0.00	5.00	100.00	100.00	55.00	15.00	0.00
Portugal	24	0.00	0.00	0.00	8.33	20.83	45.83	16.67	0.00	0.00
Slovakia	25	0.00	0.00	0.00	4.00	72.00	100.00	36.00	8.00	8.00
Slovenia	25	0.00	0.00	0.00	0.00	20.00	100.00	12.00	0.00	4.00
Spain	75	0.00	12.00	0.00	13.33	42.67	34.67	29.33	9.33	4.00
Sweden	25	0.00	0.00	0.00	4.00	20.00	20.00	0.00	4.00	0.00
Switzerland	39	2.56	0.00	0.00	2.56	23.08	76.92	23.08	2.56	0.00
The Netherlands	25	0.00	0.00	0.00	4.00	40.00	92.00	12.00	20.00	0.00
UK	147	0.68	0.00	0.00	0.68	18.37	72.79	38.10	0.68	0.00

Rifampicin resistance was relatively common (15 of 22 countries), but was most prevalent in Czech Republic, Denmark, Hungary, and Italy (56.52–63.64%) (Fig. 2). Rifampicin resistance was relatively common in prevalent RTs in some settings; for example, RT027, RT018, and RT356 in Denmark, Hungary, and Italy, respectively. Prevalent RTs exhibited rifampicin resistance related to specific locales; e.g. rifampicin resistance was prevalent in RT001/072 isolates from the Czech Republic, but not in those from Germany, Latvia, and Slovakia. Intermediate rifampicin resistance was found in Italian RT027 isolates, but not in those from Poland.

All RT018 and RT356 isolates from Italy were intermediately or fully rifampicin resistant. Interestingly, the two RT018 isolates from outside of Italy were considerably more susceptible to rifampicin (MIC $\leq$0.002 mg/L) than the Italian RT018 isolates. Notably, the other prevalent clone in Italy, RT027, showed intermediate resistance to rifampicin (geometric mean 1 mg/L). Goldstein *et al.* described markedly higher rifampicin MICs among *C. difficile* isolates from Italy (geometric mean MIC = 8.3 mg/L, MIC_{50} and MIC_{90} >256 mg/L) than most other countries [13]. Miller *et al.* found eightfold higher rifaximin resistance in isolates from Italy than those from Canada. They commented that rifaximin has been in use for over 2 decades in Italy, but remains unlicensed in Canada [20], highlighting the possibility of selection for rifamycin resistance secondary to drug exposure. Associations between rifamycin exposure and selection of resistant *C. difficile* were also made by Curry *et al.* and O'Connor *et al.* [21,22]. Obuch-Woszczatynski *et al.* described emergence of rifampicin resistance in RT046 isolates from patients on long-term rifampicin treatment for tuberculosis [23]. Using WHO data we note that there is a (non-significant, p□ 0.07) correlation between locations harbouring increased rifampicin resistance in tuberculosis of native origin and *Clos*ER study countries showing increased rifampicin resistance in *C. difficile* [24]. It is interesting also that contemporaneous data show that *Staphylococcus aureus* rifampicin resistance in Europe is most prevalent in Italy (14.8%) (http://www.ecdc.europa.eu/en/healthtopics/antimicrobial_resistance/database/Pages/table_reports.aspxf). Thus, there is circumstantial evidence to support the selection and emergence of rifampicin-resistant isolates in Italy. Whole genome sequencing of our study isolates will be helpful to determine the evidence for clonality of RT356 strains.

The present study confirmed previously reported associations between prevalent types, such as RT027 and RT001/072, and resistance to moxifloxacin, clindamycin, and chloramphenicol [8,11,15,25]. Clusters of chloramphenicol-resistant 001/072 isolates were found in Germany, The Netherlands, and Latvia. Contrastingly, in Slovakia, from which 72% of submissions were RT001/072, resistance was observed in only two isolates. This may indicate local expansion of chloramphenicol-resistant strains following acquisition of mobile resistance determinants [26].

Imipenem resistance is not well documented in *C. difficile*, but we found evidence of intermediate and full resistance (38%). Geometric mean imipenem MICs were highest among RT027 (7.12 mg/L), but high-level resistance (>64 mg/L) was observed in single RT014 and RT050 isolates from the same institution. Higher rates of imipenem resistance among human vs. animal *C. difficile* isolates (55.6 vs. 28.1%) were attributed to absent veterinary carbapenem use in one study [27]. Our data suggest that resistance to imipenem in *C. difficile* could be emerging as a result of carbapenem prescribing.

There was a significant inverse correlation between number of RTs identified in a locality and mean cumulative resistance score (Pearson correlation efficient, r = −0.64; p=0.0017), indicating lower antimicrobial resistance levels among

countries with a greater diversity of *C. difficile* RTs (largely Northern and Western Europe) (Fig. 2). This may be due to the introduction of mandatory reporting programmes, with consequent increase in awareness, antimicrobial stewardship, and infection control interventions driving down the rates of endemic RTs. There are few published data describing the epidemiology and antimicrobial susceptibility of circulating RTs across Europe. The emergence of hypervirulent RT027 in the early 2000s had a significant impact upon healthcare systems [28]. The rapid emergence of RT027, and recent reports of reduced metronidazole susceptibility (https://www.gov.uk/government/uploads/system/uploads/attachment_data/file/347169/CDRN_annual_report.pdf) [2] highlight the need for large-scale surveillance schemes to identify emerging RTs and potential resistance development. To date, surveillance schemes have tended to be local or national, rather than international, in scope, and this may limit their sensitivity to identify potentially problematic strains. Large-scale studies are invariably limited by sample numbers per country, and subject to potential selection bias [5]. We requested 25 patient de-duplicated, toxin-positive faecal samples (or *C. difficile* isolates) collected during the 12 months from July 2011 onwards, but made no further stipulations. Participating centres were, in the main, national or regional *C. difficile* reference facilities. Thus, some submissions likely included outbreak strains, possibly influencing the data. However, the similarity of the predominant RTs between this study and that of Bauer *et al.* indicates a degree of confidence [5].

It is clear that certain prevalent RTs are associated with multiple antimicrobial resistance determinants. This study underlines the association of well-known epidemic RT027 and RT001/072 with multiple antimicrobial resistance, but also highlights the association of other RTs with high levels of resistance (017, 018, and 356). The potential relatedness of RT356 and RT018 is also intriguing and requires further analysis. Similarly, the relatedness between highly resistant RT284 (rifampicin, moxifloxacin, clindamycin, imipenem, and chloramphenicol) and RT046 (both found in Italy) may also indicate strain evolution concurrent with increasing antimicrobial resistance. Geographic associations are consistent with local antimicrobial prescribing as a selection pressure. The potential emergence of these highly resistant RTs warrants further monitoring and investigation, which will be undertaken as part of the *Clos*ER program over the next 2 years.

Transparency declaration

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