International Journal of Medical Microbiology 311 (2021) 151507

Contents lists available at ScienceDirect

International Journal of Medical Microbiology

journal homepage: www.elsevier.com/locate/ijmm

Molecular epidemiology and antimicrobial resistance of *Clostridioides difficile* in Germany, 2014–2019

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ARTICLE INFO

Keywords:
Europe
Clostridium difficile
Surveillance
Susceptibility testing
Ribotyping

ABSTRACT

Clostridioides difficile is a Gram positive spore-forming rod and mainly responsible for nosocomial diarrhea in developed nations. Molecular and antimicrobial surveillance is important for monitoring the strain composition including genotypes of high epidemiological importance such as ribotype 027 (RT027) and corresponding resistance patterns.

1535 isolates obtained from samples sent between 2014 and 2019 to the German National Reference Center (NRC) for diagnostic reasons (NRC strain set), and 1143 isolates from a Tertiary Care University Center in Saarland, Germany (non-NRC strain set), were evaluated using antibiotic susceptibility testing and ribotyping.

In the NRC strain set, RT027 overtook RT001, the main RT found in the preceding studies, and dominated with 36.2%, followed by RT001 (13.3%), and RT014 (8.5%). Of note, since 2016 a constant decrease of RT027 could be noticed. In the non-NRC strain set a large strain diversity was present with RT014 (18%) and RT001 (8.9%) being most prevalent. In NRC samples, resistance towards metronidazole, vancomycin, moxifloxacin, clarithromycin and rifampicin was 2.7%, 0%, 57.1%, 53.2% and 19.2%, respectively. Metronidazole resistance was almost exclusively found in RT027 isolates. Rifampicin resistance was also observed predominantly in isolates of RT027, constituting an almost four-fold increase, when compared to preceeding studies in this region.

In conclusion these data demonstrate that RT027 is a driver for rifampicin and metronidazole resistance, underlining the importance of continuous surveillance efforts.

1. Introduction

Clostridioides difficile (formerly *Clostridium difficile*) is a Gram positive rod shaped bacterium that is mainly responsible for nosocomial diarrhea, which can progress to life-threatening disease (e.g. toxic megacolon) contributing to more than 60.000 infections per year in Germany (Lübbert et al., 2016). The majority of clinical strains exhibit two toxins (toxin A and toxin B, respectively, genes: *tcdA, tcdB*), while a third toxin (binary toxin, gene: *cdtAB*) can be detected in so called "hypervirulent" isolates (Gerding et al., 2014). The spread of these "hypervirulent" *C. difficile* strains such as ribotype 027 (RT027) has led to an increase and a higher severity of *C. difficile* infections in North America and Europe in recent years (He et al., 2013). RT027 is also associated with multidrug resistance and the ability to cause outbreaks in the hospital setting. Its tolerance towards fluoroquinolones and macrolides is believed to be a major driving factor for selection of this lineage (Freeman et al., 2010). In Europe, RT027 has a huge impact on the nation-wide molecular epidemiology for several Western- and Eastern-European countries. However, on a local level, larger regional differences may be evident, with RT018 dominating in Italy, and RT176

Abbreviations: CL, Consultant Laboratory; NRC, National Reference Center; RT, Ribotype; slpAST, Surface layer protein A single locus sequence typing.

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https://doi.org/10.1016/j.ijmm.2021.151507
Received 16 December 2020; Received in revised form 12 March 2021; Accepted 15 April 2021
Available online 19 April 2021

playing an important role in Poland and the Czech Republic (Baldan et al., 2015; Krutova et al., 2016). RT027 was first described in Germany causing outbreaks and severe disease in 2007 (Kleinkauf et al., 2007). According to previous epidemiological studies in Germany, RT027 accounted for around 26–27% of all tested *C. difficile* isolates, although larger regional differences were noticed (Arvand and Bettge-Weller, 2016; Davies et al., 2016; von Müller et al., 2015).

A recent study demonstrated that the prevalence of RT027 and other predominately nosocomial lineages such as RT001 can be reduced when antibiotic stewardship is applied (Lawes et al., 2017). This is particularly achieved by reducing the utilization of antibiotics thought to induce *C. difficile* infections (CDI) such as cephalosporins, clindamycin, quinolones, and aminopenicillins with beta lactamase inhibitor (Lawes et al., 2017). Of note, specific resistance (*e.g.* against rifampicin) might be of significance concerning certain patient collectives (e.g. orthopedic patients), where this substance is used for antimicrobial therapy that my lead to a higher incidence in CDI (Färber et al., 2017).

In the light of globalization, there is always the risk that epidemic lineages might be introduced from abroad, a scenario that can be exemplified by recent RT018 outbreaks in Germany and France, respectively (Berger et al., 2019a; Gateau et al., 2019). In previous years this has also been the case for RT027 (He et al., 2013; Steglich et al., 2015). Active surveillance is thus important to monitor these hypervirulent RTs, and the level of drug resistance.

In this context, the German National Reference Center (NRC) for *C. difficile* (Homburg-Münster-Coesfeld) evaluated the strain composition and antimicrobial resistance of all *C. difficile* isolates that have been received for diagnostic reasons from all over Germany for the years 2014–2019. Additionally, a strain set isolated from routine samples of a Tertiary Care University Center in the German state of Saarland (where the NRC is partially situated) within the same time frame was analyzed.

2. Materials and methods

2.1. Strain sets

The NRC strain set comprised *C. difficile* isolates that were received from >100 different German diagnostic laboratories (including small to large size commercial laboratories, smaller and larger hospitals, and University Hospitals) between 01.01.2014 and 31.12.2019 in cases of severe disease, recurrent infections or in the context of outbreak investigations. This strain set also included nine RT018 isolates stemming from an outbreak in 2015 (Berger et al., 2019a). As a control group, isolates obtained from routine diagnostic stool samples that were sent to a German Tertiary Care Center (University Hospital of Saarland) in the same time frame, were included (non-NRC strain set). Non-toxigenic isolates and copy strains were excluded from this study.

2.2. Susceptibility testing

Susceptibility testing of metronidazole, vancomycin, moxifloxacin, clarithromycin, and rifampicin was carried out as described before (Berger et al., 2019b, 2018). Briefly, for metronidazole, vancomycin, and moxifloxacin, resistance was determined using epsilometry (gradient testing, Biofilchem, Roseto degli Abruzzi, Italy) on freshly cultured, yet unfrozen samples, and a McFarland of 4.0 as inoculum (Berger et al., 2018). Rifampicin and clarithromycin resistances were determined by agar disk diffusion as described previously (Berger et al., 2018), using the same inoculum. Results were interpreted according to the European Committee on Antimicrobial Susceptibility Testing (EUCAST) clinical breakpoints, version 11.0 2021 (EUCAST, 2021).

2.3. Molecular diagnostics

Ribotyping and toxin gene detection were performed using a standardized European Society of Clinical Microbiology and Infectious Diseases (ESCMID) protocol as applied in previous studies (Berger et al., 2019b, 2018).

2.4. Statistics

Statistical analysis was performed using the Chi square test, except for the determination of a possible metronidazole creep. In this case, a Mann-Whitney *U* test was carried out. Corresponding *P* values <0.05 were considered significant.

3. Results

The NRC strain set comprised in total 1535 *C. difficile* isolates that were received from laboratories all over Germany during the study period. This strain set was dominated by RT027 (n = 556; 36.2%), followed by RT001 (n = 205; 13.3%), and RT014 (n = 131; 8.5%) (Table 1 and Supplementary Table 1). The geographic distribution of the NRC strain set (based on German postal regions) is shown in Fig. 1, and the distribution of prevalent RTs per year is depicted in Fig. 2. Overall resistance towards metronidazole, vancomycin, moxifloxacin, clarithromycin and rifampicin was found in 2.7% (39/1456), 0% (0/1456), 57.1% (831/1456), 53.2% (774/1456) and 19.2% (279/1456) of the NRC isolates, respectively (Table 1, Supplementary Table 1). Antibiotic resistance was not equally distributed among the RTs: When compared to other RTs, increased resistance rates were observed in RT027. The RT027 subset was almost completely resistant against moxifloxacin and clarithromycin with 98.9% (541/547) and 91.8% (502/547), respectively. RT027 also displayed an enhanced resistance rate against rifampicin with 47.9% (262/547) and the highest resistance against metronidazole with 5.9% (32/547; Table 1). Vancomycin resistance was not encountered. Metronidazole MICs remained identical for all years covered by our study (Table 2).

The non-NRC strain set consisted of 1143 isolates that were collected during routine diagnostic efforts of the Tertiary care center served by the same laboratory. In this strain set, RT014 was the most prevalent (206/1143, 18.0%), followed by RT001 (102/1143; 8.9%) and RT020 (65/1143; 5.7%) (Table 1 and Fig. 3), while RT027 (47/1143; 4.1%) was only rarely detected (Supplementary Table 2). Overall resistance towards metronidazole, vancomycin, moxifloxacin, clarithromycin and rifampicin was encountered in this strain set in 0.2% (2/1131), 0% (0/1131), 20.7% (234/1131), 22.0% (249/1131) and 2.7% (30/1131) of isolates, respectively. Metronidazole MICs remained almost identical for all years covered by our study (Table 2). Notably, metronidazole resistance was not detected in RT027 isolates obtained from the non-NRC strain set (Supplementary Table 2). RT027 isolates found in this strain set displayed high resistance rates for moxifloxacin (98%, 46/47) and clarithromycin (89%, 42/47), respectively, while the resistance rate for rifampicin (28%, 13/47) was clearly lower than the rate observed for RT027 within the NRC strain set ($p<0.05$). This was also true for rifampicin resistance in RT027 compared to non-RT027 strains ($p<0.001$) for both strain sets (Table 3). Notably, rather comparable rifampicin resistance rates were observed in the NRC (1.9%, 17/909) and non-NRC strain sets (1.6%, 17/1084), when excluding the RT027 strains from both sets (Table 3), indicating that RT027 is the main driver for rifampicin resistance of *C. difficile* in Germany.

4. Discussion

Epidemiology of *C. difficile* is always in flux and may considerably change within a few years (Lawes et al., 2017; Valiente et al., 2014). This study provides insights into the current molecular epidemiology and antimicrobial resistance of German *C. difficile* isolates accounting for outbreaks and severe courses of disease (NRC strain set), and adds data of strains isolated from routine samples obtained from a single center (non-NRC strain set) as a follow-up to a previous investigation (von Müller et al., 2015).

Table 1
Distribution of antibiotic susceptibility among the two strain sets (NRC and non-NRC) stratified by the most common ribotypes (RT). Susceptibility was determined by epsilometry for available isolates (metronidazole, vancomycin, moxifloxacin) or agar disk diffusion (clarithromycin, rifampicin) on freshly cultured, yet unfrozen isolates.

Ribotype	Resistance Testing*	Metronidazole	Vancomycin	Moxifloxacin	Clarithromycin	Rifampicin
NRC isolates						
RT001 (n = 205)	(n = 176)	0.6%	0%	83.5%	72.2%	1.1%
RT002 (n = 54)	(n = 53)	0%	0%	7.5%	1.9%	0%
RT014 (n = 131)	(n = 125)	0%	0%	12.8%	8.8%	0%
RT020 (n = 43)	(n = 43)	0%	0%	14.0%	7.0%	0%
RT027 (n = 556)	(n = 547)	5.9%	0%	98.9%	91.8%	47.9%
RT078 (n = 61)	(n = 59)	0%	0%	50.8%	44.1%	0%
Other RTs (n = 485)	(n = 453)	1.3%	0%	19.2%	23.0%	3.3%
All (n = 1535)	(n = 1456)	2.7%	0%	57.1%	53.2%	19.2%
Non-NRC Isolates						
RT001 (n = 102)	(n = 102)	0%	0%	52.0%	52.0%	1.0%
RT002 (n = 42)	(n = 42)	0%	0%	2.4%	0%	0%
RT014 (n = 206)	(n = 203)	0%	0%	9.9%	3.0%	1.0%
RT020 (n = 65)	(n = 65)	1.5%	0%	6.2%	12.3%	0%
RT027 (n = 47)	(n = 47)	0%	0%	97.9%	89.4%	27.7%
RT078 (n = 48)	(n = 47)	0%	0%	40.4%	55.3%	12.1%
Other RTs (n = 633)	(n = 625)	0.2%	0%	14.6%	18.2%	2.1%
All (n = 1143)	(n = 1131)	0.2%	0%	20.7%	22.0%	2.7%

* Isolates, which were cultivable for resistance testing.

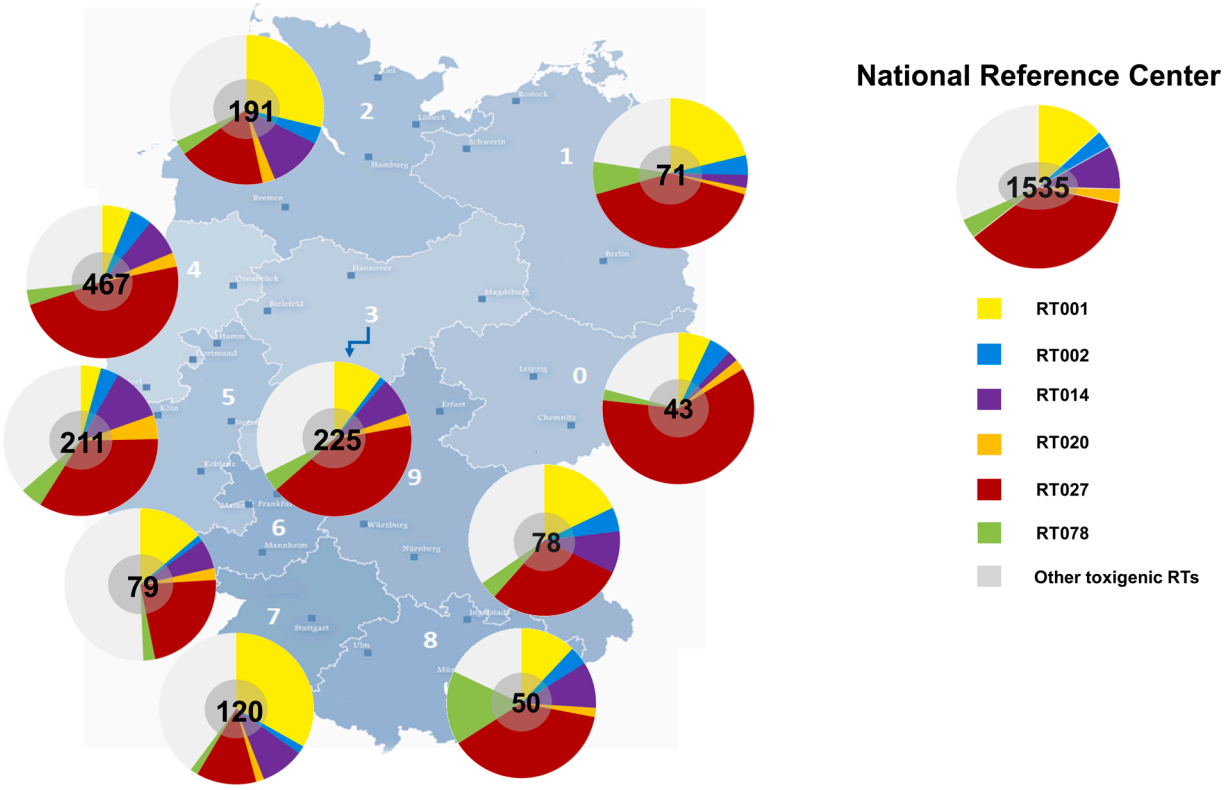

Fig. 1. Distribution of RTs according to the German postal region code of the sending laboratory.

In both strain sets a comparable RT diversity was observed. However, RT027 was encountered in only 4.1% of all cases within the non-NRC strain set, while RT027 was the most abundant RT of the NRC strain set and accounted for more than one-third of all isolates, which underlines its high epidemiological significance for severe CDI and outbreaks in Germany. When compared to the direct precursor study conducted in the same laboratory by von Müller and colleagues (von Müller et al., 2015), who characterized the *C. difficile* strain composition of isolates received from all over Germany sent to the Consultant Laboratory for *Clostridium difficile* of Germany (now NRC) in the years 2011–2013 (CL strain set), an overall increase in RT027 prevalence was noticed, which rose from 26% (von Müller et al., 2015) to 36% in the time-period 2014–2019. In the same time period, the prevalence of RT001 (the predominant RT observed in Germany in 2011–2013) decreased from 35% to 13% in 2014–2019, suggesting that RT027 took over the role of RT001 as predominant RT responsible for CDI in Germany within the latter test era. This trend was also supported by regional comparisons, demonstrating a switch from RT001 to RT027 as predominant RT in the German postal code regions 1 (Berlin/-Brandenburg/Mecklenburg-Western Pomerania), 8 and 9 (Bavaria)

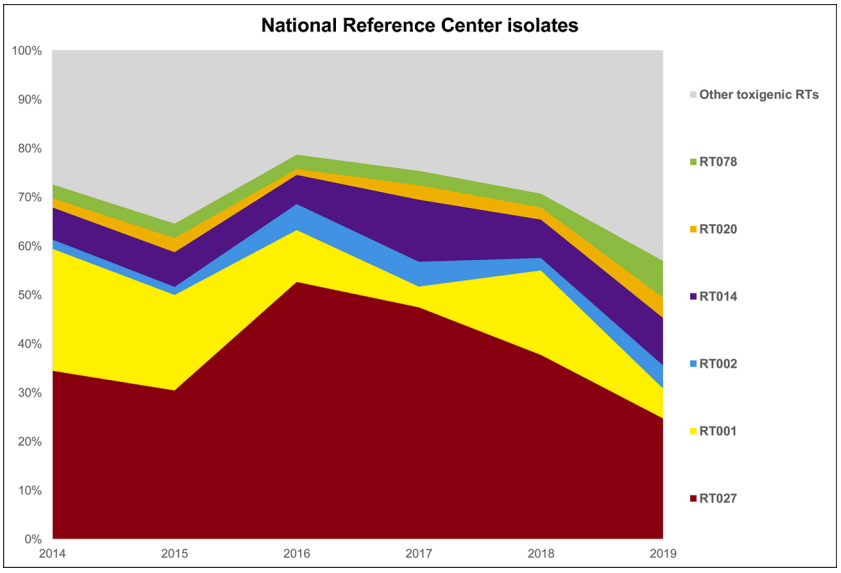

Fig. 2. Annual proportion of National Reference Center (NRC) ribotypes (RTs).

Table 2
Metronidazole MIC 50 per year in the NRC and Non-NRC strain sets.

Metronidazole	2014	2015	2016	2017	2018	2019
NRC isolates						
MIC50	0.75 mg/l	0.75 mg/l	0.75 mg/l	0.75 mg/l	0.75 mg/l	0.75 mg/l
Non-NRC isolates						
MIC50	0.50 mg/l	0.50 mg/l	0.38 mg/l	0.50 mg/l	0.50 mg/l	0.50 mg/l

Mann-Whitney *U* test was carried out between two time points for both strain sets (2014 and 2019) respectively showing no statistical significance. The metronidazole breakpoint was set in accordance with European Committee on Antimicrobial Susceptibility Testing (EUCAST) at 2 mg/l, version 11.0. 2021.

(Fig. 1). In postal code region 2 (Schleswig Holstein/Hamburg/-Bremen/Lower Saxony) RT027 was detected for the first time. However, in the German postal code region 7 (Baden-Wuerttemberg/Bavaria), an opposite trend was observed, as RT027 - which accounted for more than 50% of all strains investigated in 2011–2013 from this region - was replaced by RT001 as predominant RT in the 2014–2019 strain collection of this region [(von Müller et al., 2015) and Fig. 1]. However, since different typing techniques (Surface layer protein A single locus sequence typing [*slpA*ST] and ribotyping, respectively) were used by von Müller and colleagues (von Müller et al., 2015) and the study presented here, direct comparability might be restricted. Notably, when judged on a yearly basis, the overall RT027 prevalence peaked in 2016 and slowly but constantly decreased since then in Germany (Fig. 2). A similar development was observed for RT027 in the USA recently (Tickler et al., 2019), suggesting that the prevalence of this RT might further decline in Germany in the future.

Epidemic genotypes that are particularly successful in certain European countries, such as RT018 in Italy (Davies et al., 2016) or RT176 in Poland and the Czech Republic, respectively (Davies et al., 2016), were only rarely found, except of an RT018 outbreak from 2015 (Berger et al., 2019a). A recent multi-center study from the US demonstrated a decline in RT027 prevalence while RT002, RT056 and RT106 prevalence increased (Tickler et al., 2019). In the NRC strain set presented here, the

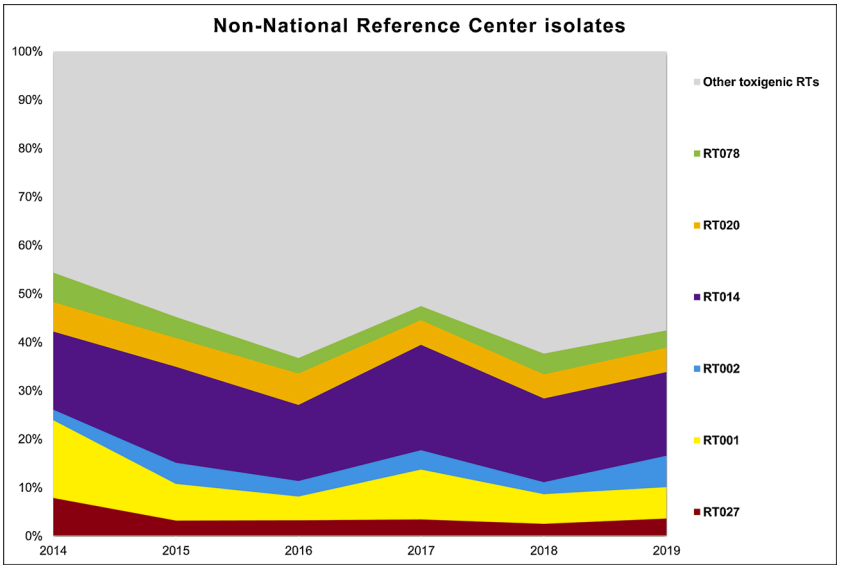

Fig. 3. Annual proportion of Non-National Reference Center ribotypes (RTs).

Table 3
Rifampicin resistance rates of *C. difficile* isolates per year in the NRC and Non-NRC strain sets, NRC National Reference Center, RT ribotype, n.a. not applicable.

NRC strain set						
Year	Isolates received (total)	Isolates received for outbreak investigations	Resistance testing carried out	Resistant isolates	RT027	Non-RT027
2014	212	16	207	42/207 (20%)	36/71 (51%)	6/136 (4%)
2015	240	84	173	34/173 (20%)	32/69 (46%)	2/104 (2%)
2016	169	62	168	38/168 (23%)	37/88 (42%)	1/80 (1%)
2017	236	19	232	30/232 (13%)	28/110 (25%)	2/122 (2%)
2018	318	87	316	82/316 (26%)	78/120 (65%)	4/196 (2%)
2019	360	49	360	53/360 (15%)	51/89 (57%)	2/271 (1%)
All	**1535**	**317**	**1456**	**279/1456** (19%)	**262/547** (48%)	**17/909 (2%)**
Non-NRC strain set						
2014	230	n.a.	225	9/225 (4%)	6/18 (33%)	3/207 (1%)
2015	252	n.a.	247	6/247 (2%)	1/8 (13%)	5/239 (2%)
2016	185	n.a.	185	5/185 (3%)	1/6 (17%)	4/179 (2%)
2017	175	n.a.	175	2/175 (1%)	2/6 (33%)	0/169 (0%)
2018	162	n.a.	160	2/160 (1%)	0/4 (0%)	2/156 (1%)
2019	139	n.a.	139	6/139 (4%)	3/5 (60%)	3/134 (2%)
All	**1143**	**n.a.**	**1131**	**30/1131 (3%)**	**13/47** (28%)	**17/1084 (2%)**

latter RTs were only rarely detected (3.5%, 0.8%, and 0.5%, respectively). Similarly, RT017, being highly prevalent in parts of East Asia (Imwattana et al., 2019) was nearly absent in the NRC strain set (0.3%, 5/1535), confirming earlier findings indicating that larger differences in the *C. difficile* RT composition exist between geographical regions (Collins et al., 2013; Davies et al., 2016).

Interestingly, in one RT095 sample and one unclassified isolate of the NRC strain set, only *tcdA* was detected. This atypical toxin gene composition was observed before (Monot et al., 2015), but the clinical significance of this toxin gene composition remains unclear yet.

From the therapeutic point of view, resistance data for the resident *C. difficile* strain composition are of major importance to allow for an evidence-based medication. In both strain sets tested here, resistance against metronidazole was scarce (2.7% and 0.2% in NRC samples and non-NRC samples, respectively), and was almost exclusively found in RT027 derivatives, while vancomycin resistance was not detected at all. These findings are in line with another recent multi-center study reporting on the metronidazole resistance of *C. difficile* isolates in Europe (Freeman et al., 2018), which detected an even lower metronidazole resistance incidence (0.2%), and a strong association with RT027. Of note, in studies covering other regions of the world (*e.g.* Israel and China) metronidazole resistance was found in 18% and 23% of the *C. difficile* isolates tested, respectively (Adler et al., 2015; Huang et al., 2010).

Since Piepenbrock and colleagues (Piepenbrock et al., 2019) encountered a MIC (minimal inhibitory concentration) creep for metronidazole in German *C. difficile* isolates in a recent single center study (in which RT027 replaced RT001 in a ten year period), we checked whether such a MIC creep could be also seen in our NRC strain set over time. Unlike observed by Piepenbrock et al. (Piepenbrock et al., 2019), we did not see an increase in metronidazole MICs for *C. difficile* over time, which remained almost identical for all years covered by our study (Table 2). The latter observation might be attributed to the high RT027 prevalence seen in our NRC strain set right from the beginning of the study period (Fig. 2), and taking into account that RT027 is the main contributor to metronidazole resistance in this strain set.

The current Infectious Diseases Society of America (IDSA) guideline does not approve metronidazole as first-line-therapy option against CDI anymore, except as supportive therapy or during non-availability of vancomycin or fidaxomicin (McDonald et al., 2018). Our observations made for the study period 2014–2019 do not ask for an analogous adaptation of the current European (Crobach et al., 2016) and German CDI treatment guidelines (Hagel et al., 2015) at the moment, given the still overall low metronidazole resistance frequency among German CDI isolates (2.7%), and the observation that the RT027 (the main contributor to metronidazole resistance in our NRC strain set) incidence is slowly but constantly decreasing since 2016. However, the main argument against metronidazole usage is the inferiority towards vancomycin in clinical therapy (McDonald et al., 2018). This might be due to lower concentrations of metronidazole in the gut lumen in relation to vancomycin (8 mg/l and >1024 mg/l, respectively) (Baines and Wilcox, 2015). Thus, a similar adaptation of German and European guidelines is likely in the near future.

Resistance against fluoroquinolones and macrolides was detected predominately in epidemic RTs such as RT027 and RT001, which is a common feature of these strains (Wieczorkiewicz et al., 2015). Rifampicin resistance, on the other hand, was mainly driven by RT027, and encountered in 19.2% of the isolates combined in the NRC strain set, and 2.7% of the isolates constituting the non-NRC strain set. These data suggest that the overall rifampicin resistance of *C. difficile* is still comparably low in Germany, especially when taking into account that overall rifampicin resistance ratios are above 40% in other European countries such as Hungary, Poland, Czech Republic, and Italy (Freeman et al., 2018). However, the rifampicin resistance incidence seen in RT027 isolates of our NRC strain set is alarming (48%, Table 3), given that the rifampicin resistance ratio of RT027 was considerably lower with 12% in a previous study in 2012 (von Müller et al., 2012). Our findings also match with recent observations indicating that RT027 is the major RT being associated with rifampicin resistance in Europe (Freeman et al., 2018). When looking at the RT027 incidence and rifampicin resistance rates in the NRC strain set per year, no clear trends were visible: The RT027 incidence rates fluctuated from 53% in 2016 to 25% in 2019 (Fig. 2), while rifampicin resistance rates varied between 26% in 2017 and 65% in 2018 (Table 3). However, as aforementioned, a slight but constant decrease in RT027 prevalence rates was noticed since 2016 (Fig. 2), suggesting a downside trend for this RT in Germany for the future. This trend might be attributed in part to the fact that the usage of antibiotics such as quinolones and macrolides decreased predominately in the outpatient setting in Germany in the years 2012–2014 [-5.3% and -9.4%, respectively (Paul-Ehrlich-Gesellschaft, 2015)]. The prescription of these antibiotics has been described as a possible factor for the selection of the RT027 strain (Freeman et al., 2010).

Schindler and colleagues (Schindler et al., 2013) suggested in an earlier report that rifampicin usage might exert a protective effect towards CDI in patients treated for osteoarticular infections. This positive correlation might be attributed to the fact that most of the *C. difficile* isolates were rifampicin sensitive at the study side (Schindler et al., 2013). However, given the high rates of rifampicin resistance in RT027 isolates encountered in our study, we suggest that presence of RT027 should be considered in patients intended to receive antibiotic treatment with this agent. The main indications for rifampicin use are infections including a biofilm such as prosthetic valve endocarditis and orthopedic

device infection (Zimmerli and Sendi, 2019). Besides therapy in humans, rifampicin is used as antimicrobial treatment option in a variety of infections in veterinary medicine (De Briyne et al., 2014), including pneumonia due to *Rhodococcus equi* (Giguere et al., 2017), and invasive disease by *Staphylococcus aureus* (De Lucia et al., 2017). However, data about rifampicin usage in Germany and worldwide are currently not available.

Overall, frequencies for metronidazole and rifampicin resistance differed clearly between the NRC and non-NRC strain sets, an observation that is likely attributed to the fact that the prevalence of RT027 (the main contributor to metronidazole and rifampicin resistance in our NRC strain set) was much lower in the non-NRC strain set.

In conclusion, an overall increase in RT027 prevalence was found for Germany in the observation period 2014–2019 in patients suffering from more severe courses of CDI and in outbreaks when compared to data obtained from 2011–2013 (von Müller et al., 2015). Consistent with the overall increase in RT027 prevalence, a high proportion of rifampicin resistance was noticed among the NRC strain set, suggesting that RT027 is the major driver for rifampicin resistance among *C. difficile* isolates that caused severe cases of CDI in Germany within the study period. However, since 2016, a constant decrease in RT027 incidence was noticed, suggesting that the prevalence of this RT might further decline in Germany in the future, and thus maybe the incidence of rifampicin resistant *C. difficile* as well.

It should not be left out that our study has one major limitation: the data is not population based. Samples are sent from several laboratories from whole Germany without standardization for different reasons (e.g. severe clinical case, outbreak investigations), which is however, a common denominator for most surveillance studies. In order to assess the current strain composition in Germany, more precisely multi-centric studies are needed which cover all regions of Germany and targeting all patient groups (in-patients and out-patients) regardless of disease severity. Rising antimicrobial resistance and the thread of introduction of foreign hypervirulent RTs into Germany emphasizes the need for ongoing surveillance.

Funding

The German National Reference Center for *Clostridioides* (*Clostridium*) *difficile* is supported by an unrestricted grant from the Robert Koch Institute, Germany. Ahmed Mohamed Mostafa Abdrabou was funded by the DAAD-GERLS Program (Deutscher Akademischer Austauschdienst - German Egyptian Research Long-Term Scholarship). We acknowledge support by the Deutsche Forschungsgemeinschaft (DFG, German Research Foundation) and Saarland University within the funding programme Open Access Publishing. The sponsors did not have any involvement in the study design, collection, analysis and interpretation of the data; writing the report or the decision to submit this article for publication.

Authors' contributions

Data collection: ZB, AMMA, FKB, AH, Statistical data: AMMA, ZB, AH, data verification AMMA, ZB, Microbiological diagnostics: AN, LM, AMMA, FKB, Wrote the manuscript: FKB, AMMA, MB, ZB, AM, LvM, and BG.

The material has not been published elsewhere.

Declaration of Competing Interest

None.

Acknowledgements

We like to thank the microbiological laboratories throughout Germany for sending specimens. The map of the German postal codes was received from https://www.openstreetmap.org, © OpenStreetMap (OSM) contributors, under the Creative Commons Attribution-Share Alike 2.0 license (CC BY-SA 2.0, https://www.openstreetmap.org/copyright). We thank OSM foundation for sharing these data.

Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:https://doi.org/10.1016/j.ijmm.2021.151507.

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