International Journal of Medical Microbiology 309 (2019) 189–193

Contents lists available at ScienceDirect

International Journal of Medical Microbiology

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

Hospital outbreak due to *Clostridium difficile* ribotype 018 (RT018) in Southern Germany

Fabian K. Berger[a,*], Sabine Gfrörer[b], Sören L. Becker[a,c,d], Rossella Baldan[e], Daniela Maria Cirillo[e], Martinique Frentrup[f], Matthias Steglich[f,g], Pit Engling[f], Ulrich Nübel[f,g,h], Alexander Mellmann[i], Markus Bischoff[a], Barbara Gärtner[a], Lutz von Müller[a,j]

[a] *Institute of Medical Microbiology and Hygiene, National Reference Centre for Clostridium difficile, Saarland University, Kirrberger Straße, Building 43, 66421 Homburg, Saar, Germany*
[b] *Regionale Kliniken Holding RKH GmbH, Ludwigsburg, Germany*
[c] *Swiss Tropical and Public Health Institute, P.O. Box, CH-4002 Basel, Switzerland*
[d] *University of Basel, P.O. Box, CH-4003 Basel, Switzerland*
[e] *Emerging Bacterial Pathogens Unit, Division of Immunology, Transplantation and Infectious Diseases, IRCCS San Raffaele Scientific Institute, Milan, Via Olgettina Milano 60, 20132 Italy*
[f] *Leibniz Institute DSMZ, Inhoffenstraße 7B, 38124 Braunschweig, Germany*
[g] *German Centre for Infection Research (DZIF), Partner site Braunschweig-Hannover, Inhoffenstraße 7, 38124 Braunschweig, Germany*
[h] *Braunschweig Integrated Centre of Systems Biology (BRICS), Technical University Braunschweig, Rebenring 56, 38106 Braunschweig, Germany*
[i] *Institute of Hygiene, University Hospital Münster, National Reference Centre for Clostridium difficile, Robert-Koch-Straße 41, 48149 Münster, Germany*
[j] *Institute for Laboratory Medicine, Microbiology and Hygiene, National Reference Centre for Clostridium difficile, Christophorus Kliniken, Südwall 22, 48653 Coesfeld, Germany*

ARTICLE INFO

Keywords:
Epidemiology
Resistance testing
Transmission
Antibiotic susceptibility

ABSTRACT

Clostridium (*Clostridioides*) *difficile* is the main cause of nosocomial diarrhoea. Ribotype 018 (RT018) has been recognized as the predominant strain responsible for *C. difficile* infection (CDI) in Italy, whereas in most other European countries only sporadic RT018 cases occur.

Between August and October 2015, a suspected *C. difficile* outbreak at two associated hospitals in Southern Germany was investigated by comprehensive molecular typing. Surprisingly, RT018 was detected in 9/82 CDI patients, which has never been described before in a German outbreak. Phenotypic analysis revealed fluoroquinolone and macrolide resistance. Genetic subtyping using multiple-locus variable-number tandem-repeat analysis (MLVA) and whole genome sequencing (WGS) was performed and outbreak isolates were directly compared to sporadic German RT018 isolates and to epidemic ones from Milan, Northern Italy. Molecular typing confirmed a hospital outbreak with closely related RT018 isolates. Both, MLVA and WGS revealed high similarity of outbreak strains with epidemic isolates from Italy, but low similarity to other German isolates. Comparison between both typing strategies showed that ribotyping in combination with MLVA was appropriate to identify related isolates and clonal complexes, whereas WGS provided a better discrimination with more detailed information about the phylogenetic relationship of isolates. This is the first hospital outbreak in Germany presumably caused by cross-national transmission of an Italian epidemic RT018 strain.

1. Introduction

Clostridium (*Clostridioides*) *difficile* (Lawson et al., 2016), is a gram-positive spore-forming rod-shaped bacterium causing colitis and diarrhoea (Bartlett et al., 1978) especially following antibiotic treatment. *C. difficile* is the leading cause of nosocomial diarrhoea, but *C. difficile* infections (CDI) can also occur in the community (Bartlett, 2006). Recurrences and severe infections with life-threatening acute complications such as toxic megacolon may arise [3]. Due to its ability to form spores that are largely resistant to many disinfectants, *C. difficile* is predisposed for spreading in healthcare settings and particularly in the hospital environment. Most epidemic strains are characterized by fluoroquinolone and macrolide resistance, which is recognized as a selection advantage for spreading in the hospital setting (Freeman

* Corresponding author at: National Reference Centre for *Clostridium difficile* (Homburg-Münster-Coesfeld), Institute of Medical Microbiology and Hygiene, Saarland University, Kirrberger Straße, Building 43, D-66421 Homburg, Germany.
E-mail address: fabian.berger@uks.eu (F.K. Berger).

https://doi.org/10.1016/j.ijmm.2019.03.001
Received 11 December 2018; Received in revised form 18 February 2019; Accepted 4 March 2019

et al., 2018). An almost worldwide distribution of very epidemic ribotypes was recorded during recent years, e.g. ribotype 027 (RT027) (He et al., 2013), while other epidemic RTs such as e.g. RT176 (Polivkova et al., 2016) and RT018 (Spigaglia et al., 2010) were reported to cause only regional outbreaks despite being highly transmissible in these regions. RT018 isolates were identified in several countries including Italy, Spain, Slovenia, Austria, Switzerland, Romania, Korea and Japan (Bauer et al., 2011; Collins et al., 2013; Freeman et al., 2018; Kim et al., 2013; Rafila et al., 2014; Spigaglia et al., 2010). In recent years, it turned out that RT018 has become the predominant epidemic outbreak strain in Northern Italy with prevalence rates > 40% (Baldan et al., 2015; De Rosa et al., 2015). Of note, infections with RT018 are associated with a more severe course of clinical disease (Serafino et al., 2018). In Germany, only sporadic cases of RT018 infections have been reported thus far. In the strain collection of the German Reference Centre for *Clostridium difficile* RT018 strains currently account for less than 1%.

Detection of local outbreaks requires *C. difficile* testing of all patients with hospital-associated diarrhoea according to the current diagnostic guidelines. New clinical criteria of the current ESCMID guideline for the endemic and epidemic situation may discriminate wards with baseline or with increased *C. difficile* prevalence (Tschudin-Sutter et al., 2018) and local surveillance data should be correlated to other healthcare settings by the help of national (CDAD-KISS for Germany) or international surveillance networks (e.g. ECDIS-net) (Gastmeier et al., 2009; Krutova et al., 2018). A suspected outbreak should be investigated by molecular typing to discriminate between unrelated cases and clonal hospital transmissions. Until now, ribotyping is the standard method for molecular typing of *C. difficile* and allows discrimination between isolates based on polymorphisms of ribosomal PCR amplification patterns in the 16 S–23 S intergenic spacer region. However, if patients are infected with isolates of the same RT, a second, more discriminative assay is required for more detailed molecular subtyping. Subtyping is available e.g. by multiple locus variable-number of tandem repeat analysis (MLVA) and whole genome sequencing (WGS). Utilization of these subtyping methods revealed that most hospital-associated CDI are not related to direct transmission between patients (Eyre et al., 2013a). However, this general observation should not blind us to the fact that numerous epidemic outbreaks remain undetected due to limited awareness and restricted availability of molecular typing methods.

In the present work we report the first potential cross-national transmission of the Northern Italian epidemic RT018 strain causing a hospital outbreak in two neighbouring hospitals in Southern Germany between August and October 2015. Although this outbreak seemed to be locally restricted, we acknowledge spreading of *C. difficile* in both hospitals, without clear identification of the infection source.

2. Material and methods

Between August and October 2015 a *C. difficile* outbreak was suspected in two 1000 and 80 beds hospitals (hospital A and B, respectively) of the same hospital owner in the State of Baden-Wuerttemberg, Southern Germany, that are located in close proximity (˜10 km). Hospital A is a maximum care centre and hospital B is a rehabilitation clinic. One patient died from complicated CDI in hospital B (no. 1,737,409). At the same time, another patient was transferred from hospital B to the intensive care unit (ICU) of hospital A due to severe CDI (no isolate available). A few days later, four more patients developed diarrhoea at the ICU and were suspected of CDI (nos. 1,737,423 and 1,737,424 of Supplementary Fig. 1). The National Reference Centre for *Clostridium difficile* was contacted due to a suspected outbreak and stool samples from all five patients were provided (retrospective part). Next, all consecutive samples positive for glutamate dehydrogenase (GDH) enzyme immune assay (EIA) in the hospital laboratory were also sent to the Reference Centre (prospective part). Environmental samples were not taken.

All samples were analysed by anaerobic culture, PCR ribotyping and antibiotic susceptibility testing as described previously (Berger et al., 2018; Färber et al., 2017). PCR ribotyping, toxin gene detection and MLVA were performed by capillary gel electrophoresis and automated bioinformatics analysis (Bionumerics, Applied Maths NV, Belgium) according to standard European protocols (ECDIS-net). Antibiotic susceptibility for moxifloxacin and clarithromycin was tested by E-test and for rifampicin by agar diffusion assay (European Committee for Antimicrobial Susceptibility Testing, EUCAST).

The repeated isolation of RT018 was the most significant finding. The identified RT018 outbreak isolates were directly compared to sporadic RT018 isolates in other German regions (National Reference Centre strain collection) and also to epidemic Northern Italy RT018 strains from Milan (IRCCS San Raffaele Scientific Institute).

All RT018 isolates derived from the local outbreak, the epidemic Northern Italian strains and the sporadic cases of the German strain collection were subjected to whole genome sequencing (WGS). For WGS, libraries were prepared from bacterial genomic DNA according to a modified Illumina Nextera XT protocol (Baym et al., 2015). Libraries were sequenced on a NextSeq machine (Illumina) to > 50-fold average coverage using Mid Output v2 reagents with 2 × 150 cycles according to the manufacturer's instructions. Consensus genome sequences for individual isolates were determined by mapping sequencing reads to a reference genome sequence (acc. no. FN545816.1) as described previously (Steglich et al., 2015). Polymorphisms that had likely been generated through homologous recombination were identified by using ClonalFrame-ML software (Didelot and Wilson, 2015) and excluded from subsequent phylogenetic analyses, together with mobile genetic elements and repetitive DNA (Steglich et al., 2015). Remaining core-genomic variation was used to construct a maximum-likelihood phylogenetic tree by using RAxML (Stamatakis, 2014).

3. Results

3.1. Ribotyping and toxin gene detection

During the retrospective part of the outbreak investigation, three of five patients with CDI were found to be infected with RT018. During subsequent follow-up (prospectively) conducted six weeks later (77 patients), six CDI were also caused by RT018. Two isolates could not be obtained by anaerobic culture. Among all eighty isolates, the predominant RTs were RT001, RT018, RT027 and RT014 (Table 1). Multiplex PCR for toxin genes demonstrated that all RTs displayed the characteristic genotypic toxin profiles.

3.2. Antibiotic susceptibility

Antimicrobial susceptibility testing revealed that all RT018 outbreak strains and also the tested epidemic Northern Italian RT018 isolates were resistant to fluoroquinolones (moxifloxacin) and macrolides (clarithromycin) (Supplementary Table 1). In contrast, most sporadic German RT018 isolates (strain collection) were susceptible to both compounds (60% and 80%, respectively). Interestingly, rifampicin resistance was detected in the Italian RT018 epidemic strains and in one sporadic CDI case of the German strain collection (no. 1,732,381) but not in the local RT018 outbreak strains.

3.3. MLVA analysis

Subtyping of RT018 isolates was performed by MLVA based on the number of summed tandem-repeat differences (STRDs) in combination with numbers of tested loci with repeat differences. Two of three initial outbreak isolates (nos. 1,737,409, 1,737,423) were assigned to the same clonal complex (≤ 2 STRDs) (Fig. 1) and the third isolate (no. 17,337,424) was closely related to this cluster (3 STRDs). A second clonal cluster with three isolates (nos. 1738375, 1,738,385, and

Table 1
Basic molecular typing of *C. difficile* isolates during hospital outbreak (n = 80). PCR ribotypes (RTs) and characteristic toxin genes profiles are shown (*tcdA* = toxin A gene, *tcdB* = toxin B gene, *cdtA&cdtB*, binary toxin genes). Unclassified RTs showed no match with the institutional data base.

Ribotype(s)	Patients (n = 82)	Toxin genes
RT001	29 (35%)	*tcdA*, *tcdB*
RT014	4 (5%)	*tcdA*, *tcdB*
RT018	9 (11%)	*tcdA*, *tcdB*
RT027	4 (5%)	*tcdA*, *tcdB*, *cdtA&cdtB*
RT078	2 (2%)	*tcdA*, *tcdB*, *cdtA&cdtB*
RT003, RT005, RT012	2 (2%) each	*tcdA*, *tcdB*
RT002, RT011, RT015, RT020, RT057, RT070, RT081, RT097, RT207, RT258	1 (1%) each	*tcdA*, *tcdB*
RT023, RT131	1 (1%) each	*tcdA*, *tcdB*, *cdtA&cdtB*
Unclassified RTs	14 (17%)	*tcdA*, *tcdB*
Culture negative (not evaluated)	2 (2%)	–

1,738,372) was identified in the follow-up study, which was genetically related (≤ 10 STRDs) to the first clonal complex. In the latter cluster, two isolates featured identical repeat patterns (nos. 1738375, 1,738,385). A fourth isolate (no. 1,738,397) showed a close genetic relationship to the second cluster (3 STRDs) and also the remaining RT018 isolates obtained during the follow-up phase displayed differences of five and six STRDs, respectively.

Notably, one of the four epidemic Northern Italian RT018 isolates (no. 1,738,797) displayed a genetic relatedness (8 STRDs) to the isolate obtained from the index patient (no. 1,737,409), showing that German and Northern Italian RT018 isolates might be inherently connected. All historic German RT018 isolates obtained in other German regions showed distinct MLVA repeat patterns (≥ 10 STRDs), indicating that they were not closely related to the clinical outbreak strains.

3.4. Whole genome analysis

For further differentiation of genetic relatedness between RT018 isolates, we performed WGS, and calculated a maximum-likelihood phylogenetic tree based on core-genomic variation (Fig. 2). This investigation revealed that the nine outbreak isolates were closely related to each other forming a tight phylogenetic clade (termed 'Outbreak Clade') with strong (100%) bootstrapping support (Fig. 2). Core

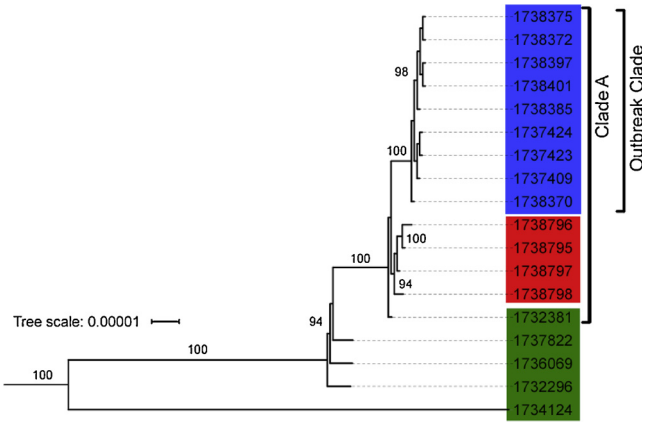

Fig. 2. Maximum-likelihood phylogenetic tree based on sequence variation in the core genome of RT018 isolates. The tree was rooted with a genome from an RT027 isolate (R20291; acc. no. FN545816.1) and bootstrap values > 90% are indicated. Colours correspond to the isolate groups: hospital outbreak (blue), Milan/Northern Italy (red), sporadic sources in Germany (green). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article).

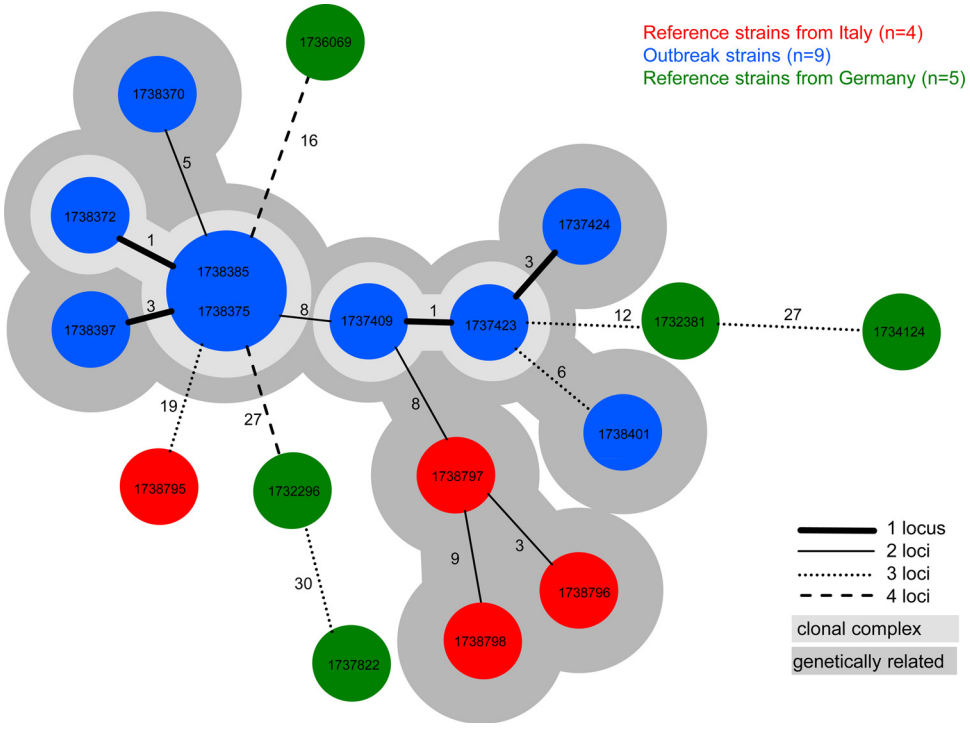

Fig. 1. Results of MLVA analysis investigating an outbreak of *C. difficile* in Southern Germany; isolates from Southern Germany are displayed in blue colour;; reference strains from Italy (SI) in red, reference strains from the German reference laboratory in green. Cross-over stitches: number of different loci; Arabic numbers: amount of different repeats; clonal isolates accompanied by an area in light grey (≤2 repeat difference), genetically related strains in dark grey (≤10 repeat difference). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article).

genomes from isolates within the 'Outbreak Clade' differed from each other only by ≤8 single nucleotide polymorphisms (SNPs) as was shown in the distance matrix analysis (Supplementary Fig. 2). Outbreak isolates, epidemic isolates from Italy, and one previously collected isolate from Germany formed a larger clade (termed 'Clade A') within the phylogeny of RT018 (Fig. 2). Overall, relatedness was very similar when comparing the four Italian outbreak strains on the one hand (9–42 SNP differences) and Italian and German outbreak strains on the other hand (16–46 SNP differences). Other sporadic German RT018 isolates in our sample set differed by 91–978 SNPs from the outbreak Clade A (Supplement Fig. 2).

Interestingly, one isolate deriving from the sporadic cases of the German collection (no. 1,732,381) clustered within Clade A, which was dominated by German/Italian outbreak isolates. By MLVA, this isolate also displayed closer relatedness to the current outbreak (12 STRDs) than other historic strains (> 15 STRDs) (Fig. 1). Moreover, antibiotic resistance towards clarithromycin and moxifloxacin was in accordance with the outbreak isolates, but additional rifampicin resistance resembled antibiotic resistance profiles of the Northern Italian RT018 epidemic isolates (Supplementary Table 1).

3.5. Epidemiologic analysis and outcome of the patient with RT018

The outbreak took place in two geographically closely related hospitals owned by the same company. The possible link between the two hospitals was a patient who developed CDI in hospital B and was transferred to the ICU of hospital A (no isolate available). A few days later, two other symptomatic patients were detected at the ICU but were treated in different rooms. Eight weeks later a new cluster of CDI with RT018 occurred in both hospitals on different wards (Supplementary Table 1). None of the patients included in the outbreak analysis had direct relationship to geographic regions with epidemic RT018 outbreaks (e.g. travel history).

When the outbreak was suspected, hygiene management was intensified with strict single room isolation, training of all professional groups, and application of sporicidal cleaning (peracetic acid). The number of CDI decreased in the following weeks. Final examination with extended molecular typing of other isolates in the same region was not performed.

4. Discussion

RT018 is the predominant epidemic *C. difficile* strain in Northern Italy (Baldan et al., 2015). Despite its epidemic presence and transmissibility in Italy, RT018 outbreaks seemed to be regionally restricted without further spread to other European countries (Bauer et al., 2011). However, the present outbreak investigation with molecular typing suggests that RT018 strains related to epidemic isolates from Northern Italy may cause regional outbreaks also in Germany.

Here, RT018 isolates deriving from the local hospital outbreak (Germany), from sporadic CDI cases in other German regions and from epidemics in Northern Italy were subclassified by MLVA and WGS. In clinical decision making these methods show a concordance of 95% for related isolates (Eyre et al., 2013b), which was also confirmed by the present analysis.

Molecular subtyping of the German outbreak strains showed relatedness to epidemic Italian strains by both methods. One sporadic RT018 isolate of the German *C. difficile* strain collection clustered together with the German/Italian outbreak cluster in Clade A and this historic strain also displayed the same rifampicin resistance as the epidemic Italian clone. A direct link between this former isolate of a far distant region in Germany and the epidemic Italian clone is speculative, but not unlikely.

WGS with pairwise comparisons of genomic differences is currently the most comprehensive method for molecular subtyping and phylogenetic analysis. However, technical requirements for MLVA are considerably lower and are more widely distributed in microbiological laboratories.

The observed level of close genomic relatedness (1–8 SNPs) among all nine RT018 isolates from the two investigated hospitals is consistent with an outbreak scenario. Based on genomic diversity among multiple isolates from individual patients and an inferred evolutionary rate, Eyre et al. (Eyre et al., 2013a) recently suggested that isolates from separate patients that differed by ≤2 core-genome SNPs could be related to direct transmission event whereas a direct epidemiologic link was excluded for isolates with > 10 SNPs (Eyre et al., 2013a). A genetic subcluster with direct phylogenetic relationship (≤1 SNP) was found for the initial series of RT018 isolates (nos. 1,737,409, 1,737,423, 1,737,424), whereas RT018 isolates obtained during the follow-up investigation formed a second subcluster (nos. 1738375, 1,738,372, 1,738,397, 1738401, and 1,738,385), except for one isolate (no. 1,738,370). WGS analysis displaying different subclusters in Clade A suggests that the index patient (no. 1,737,409) was diagnosed during an already ongoing outbreak. We assume that the patient was not directly responsible for the initial transnational transmission from Italy to Germany, but he was the first patient detected during the outbreak.

When compared to the initial phase of the outbreak analysis (60% RT018), a lower rate of RT018 cases was detected during the 2 months follow-up (8% RT018). There was no more clinical evidence of direct patient-to-patient contact but RT018 remained one of numerous other endemic (e.g. RT001), epidemic (e.g. RT027) and sporadic *C. difficile* RTs in the present hospital setting.

The present analysis allowed direct comparison between MLVA and WGS for molecular subtyping of the outbreak isolates and minor differences between typing methods were expected. MLVA exhibited a higher variety of relatedness in the outbreak isolates. It has been shown earlier that related MLVA subtypes may be present in one faecal specimen, which may differ by > 5 STRDs (Tanner et al., 2010). During the present outbreak, relationship between isolates was confirmed by MLVA, however, the minority of isolates were related as clonal complexes (≤ 2 STRDs). As compared to the potential new diagnostic gold standard for molecular typing (WGS) (Bletz et al., 2018), we demonstrated that absolute numbers of STRD obtained by MLVA can over- or underestimate the direct relatedness between epidemiologic isolates due to method-related reasons, which are based on high variability of the genetic targets. Comprehensive genome-wide comparison of SNP variants in combination with standardized bioinformatics analysis allows identification of potential infection chains based on phylogenetic analysis with higher discriminatory power than MLVA.

Using WGS, the outbreak strains were affiliated to the phylogenetic Clade A within RT018 (Fig. 2). This clade is associated with high-level fluoroquinolone resistance, which is very common for epidemic RTs as multi-resistance might facilitate selection, persistence and spreading in the hospital environment (He et al., 2013).

Fluoroquinolone and macrolide resistant RT018 strains have reached high prevalence rates in Northern Italy in recent years. Despite its transmission capacity, RT018 epidemics remained restricted to Italy until now. Here, we report for the first time that RT018 strains related to the predominant North Italian epidemic were responsible for an outbreak in two hospitals in Southern Germany. It remains unknown why some RTs spread globally (e.g. RT027), whereas others remain restricted to single hospitals, regions and countries. Of note, a recent study indicated that trehalose, which is frequently utilized in food industry, might have facilitated the spread of certain “hypervirulent” strains including RT027, as trehalose can be metabolised by these RTs whereas other RTs cannot make use of this α-glucose based disaccharide (Collins et al., 2018).

Outbreaks caused by transnational spreading of epidemic RTs support the importance of the “one world one health” campaign of the WHO and the need for local and international molecular surveillance activities for risk assessment of global diseases caused by epidemic strains.

Inherently, the present outbreak analysis has several limitations. While it may be tempting to postulate that the RT018 outbreak strains have originated from the Northern Italian epidemic clone, inference of the source and the precise route of spreading into Germany would require a dataset much larger than the one we present here. Therefore, the direct link between Southern German and Northern Italian outbreak strains remained unrevealed and we suppose that unrecognized spreading of an epidemic RT018 precursor isolate might have occurred weeks before the current outbreak detection. Unfortunately, we could not obtain isolates from neighbouring hospitals to investigate the regional transmission, i.e. that information concerning potential regional spreading is missing.

Authors' contributions

Clinical data: FB, SG. Microbiological diagnostics: FB, SG, AN, MC, RB, MF, MS, PE, UN, BG and LvM. Wrote the manuscript: FB, SG, SLB, BG, AM, MB, UN and LvM.

All authors have read and approved the final version of the manuscript.

Ethics approval

For outbreak reports, a separate ethics vote is not required. Despite this fact an independent internal expert committee analysed the manuscript before manuscript submission with regard to privacy, anonymization and data security.

Funding

The German National Reference for *Clostridium difficile* is supported by an unrestricted grant from the Robert Koch Institute, Germany and this study was partially funded by the EU Horizon 2020 programme, grant agreement number 643476, and by the Federal State of Lower Saxony, Niedersächsisches Vorab (VWZN3215/ZN3266; to UN).

Conflict of interest

Dr. Berger reports personal fees from MSD outside the submitted work. Professor Nübel reports grants from EU Horizon 2020, grants from the Federal State of Lower Saxony, Germany, during the conduct of the study. Professor Gärtner reports personal fees from Roche and from Sequirus outside the submitted work.

Acknowledgments

We would like to thank our medical technical assistant Anna Nimmesgern for excellent diagnostic assistance at the German National Reference Centre. We thank Vera Junker and the sequencing unit at the Leibniz Institute DSMZ for excellent technical assistance. An excerpt of this data set has been presented at the Deutsche Gesellschaft für Hygiene und Mikrobiologie (DGHM) meeting in Ulm, Germany in 2016.

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.2019.03.001.

References

Baldan, R., Trovato, A., Bianchini, V., Biancardi, A., Cichero, P., Mazzotti, M., Nizzero, P., Moro, M., Ossi, C., Scarpellini, P., Cirillo, D.M., 2015. *Clostridium difficile* PCR ribotype 018, a successful epidemic genotype. J. Clin. Microbiol. 53, 2575–2580.

Bartlett, J.G., 2006. Narrative review: the new epidemic of *Clostridium difficile*-associated enteric disease. Ann. Intern. Med. 145, 758–764.

Bartlett, J.G., Moon, N., Chang, T.W., Taylor, N., Onderdonk, A.B., 1978. Role of *Clostridium difficile* in antibiotic-associated pseudomembranous colitis. Gastroenterology 75, 778–782.

Bauer, M.P., Notermans, D.W., van Benthem, B.H., Brazier, J.S., Wilcox, M.H., Rupnik, M., Monnet, D.L., van Dissel, J.T., Kuijper, E.J., Group, E.S., 2011. *Clostridium difficile* infection in Europe: a hospital-based survey. Lancet 377, 63–73.

Baym, M., Kryazhimskiy, S., Lieberman, T.D., Chung, H., Desai, M.M., Kishony, R., 2015. Inexpensive multiplexed library preparation for megabase-sized genomes. PLoS One 10, e0128036.

Berger, F.K., Rasheed, S.S., Araj, G.F., Mahfouz, R., Rimmani, H.H., Karaoui, W.R., Sharara, A.I., Dbaibo, G., Becker, S.L., von Müller, L., Bischoff, M., Matar, G.M., Gärtner, B., 2018. Molecular characterization, toxin detection and resistance testing of human clinical *Clostridium difficile* isolates from Lebanon. Int. J. Med. Microbiol. 308, 358–363.

Bletz, S., Janezic, S., Harmsen, D., Rupnik, M., Mellmann, A., 2018. Defining and evaluating a core genome multilocus sequence typing scheme for genome-wide typing of *Clostridium difficile*. J. Clin. Microbiol. 56, 1–10.

Collins, D.A., Hawkey, P.M., Riley, T.V., 2013. Epidemiology of *Clostridium difficile* infection in Asia. Antimicrob. Resist. Infect. Control 2, 21.

Collins, J., Robinson, C., Danhof, H., Knetsch, C.W., van Leeuwen, H.C., Lawley, T.D., Auchtung, J.M., Britton, R.A., 2018. Dietary trehalose enhances virulence of epidemic Clostridium difficile. Nature 553, 291–294.

De Rosa, F.G., Cavallerio, P., Corcione, S., Parlato, C., Fossati, L., Serra, R., Di Perri, G., Cavallo, R., 2015. Molecular characterization of toxigenic *Clostridium difficile* in a northern Italian hospital. Curr. Microbiol. 70, 154–155.

Didelot, X., Wilson, D.J., 2015. ClonalFrameML: efficient inference of recombination in whole bacterial genomes. PLoS Comput. Biol. 11, e1004041.

Eyre, D.W., Cule, M.L., Wilson, D.J., Griffiths, D., Vaughan, A., O'Connor, L., Ip, C.L., Golubchik, T., Batty, E.M., Finney, J.M., Wyllie, D.H., Didelot, X., Piazza, P., Bowden, R., Dingle, K.E., Harding, R.M., Crook, D.W., Wilcox, M.H., Peto, T.E., Walker, A.S., 2013a. Diverse sources of *C. difficile* infection identified on whole-genome sequencing. N. Engl. J. Med. 369, 1195–1205.

Eyre, D.W., Fawley, W.N., Best, E.L., Griffiths, D., Stoesser, N.E., Crook, D.W., Peto, T.E., Walker, A.S., Wilcox, M.H., 2013b. Comparison of multilocus variable-number tandem-repeat analysis and whole-genome sequencing for investigation of *Clostridium difficile* transmission. J. Clin. Microbiol. 51, 4141–4149.

Färber, J., Illiger, S., Berger, F., Gärtner, B., von Müller, L., Lohmann, C.H., Bauer, K., Grabau, C., Zibolka, S., Schlüter, D., Geginat, G., 2017. Management of a cluster of *Clostridium difficile* infections among patients with osteoarticular infections. Antimicrob. Resist. Infect. Control 6, 22.

Freeman, J., Vernon, J., Pilling, S., Morris, K., Nicholson, S., Shearman, S., Longshaw, C., Wilcox, M.H., Pan-European longitudinal surveillance of antibiotic resistance among prevalent Clostridium difficile ribotypes study, g, 2018. The ClosER study: results from a three-year pan-European longitudinal surveillance of antibiotic resistance among prevalent *Clostridium difficile* ribotypes, 2011-2014. Clin. Microbiol. Infect. 24, 724–731.

Gastmeier, P., Weitzel-Kage, D., Behnke, M., Eckmanns, T., 2009. Surveillance of *Clostridium difficile*-associated diarrhoea with the German nosocomial infection surveillance system KISS (CDAD-KISS). Int. J. Antimicrob. Agents 33 (Suppl 1), S19–23.

He, M., Miyajima, F., Roberts, P., Ellison, L., Pickard, D.J., Martin, M.J., Connor, T.R., Harris, S.R., Fairley, D., Bamford, K.B., D'Arc, S., Brazier, J., Brown, D., Coia, J.E., Douce, G., Gerding, D., Kim, H.J., Koh, T.H., Kato, H., Senoh, M., Louie, T., Michell, S., Butt, E., Peacock, S.J., Brown, N.M., Riley, T., Songer, G., Wilcox, M., Pirmohamed, M., Kuijper, E., Hawkey, P., Wren, B.W., Dougan, G., Parkhill, J., Lawley, T.D., 2013. Emergence and global spread of epidemic healthcare-associated *Clostridium difficile*. Nat. Genet. 45, 109–113.

Kim, J., Kang, J.O., Kim, H., Seo, M.R., Choi, T.Y., Pai, H., Kuijper, E.J., Sanders, I., Fawley, W., 2013. Epidemiology of *Clostridium difficile* infections in a tertiary-care hospital in Korea. Clin. Microbiol. Infect. 19, 521–527.

Krutova, M., Kinross, P., Barbut, F., Hajdu, A., Wilcox, M.H., Kuijper, E.J., survey, c, 2018. How to: surveillance of *Clostridium difficile* infections. Clin. Microbiol. Infect. 24, 469–475.

Lawson, P.A., Citron, D.M., Tyrrell, K.L., Finegold, S.M., 2016. Reclassification of *Clostridium difficile* as *Clostridioides difficile* (Hall and O'Toole 1935) Prevot 1938. Anaerobe 40, 95–99.

Polivkova, S., Krutova, M., Petrlova, K., Benes, J., Nyc, O., 2016. *Clostridium difficile* ribotype 176 - a predictor for high mortality and risk of nosocomial spread? Anaerobe 40, 35–40.

Rafila, A., Indra, A., Popescu, G.A., Wewalka, G., Allerberger, F., Benea, S., Badicut, I., Aschbacher, R., Huhulescu, S., 2014. Occurrence of *Clostridium difficile* infections due to PCR ribotype 027 in Bucharest, Romania. J. Infect. Dev. Ctries. 8, 694–698.

Serafino, S., Consonni, D., Migone De Amicis, M., Sisto, F., Domeniconi, G., Formica, S., Zarantonello, M., Maraschini, A., Cappellini, M.D., Spigaglia, P., Barbanti, F., Castaldi, S., Fabio, G., 2018. Clinical outcomes of *Clostridium difficile* infection according to strain type. A prospective study in medical wards. Eur. J. Intern. Med. 54, 21–26.

Spigaglia, P., Barbanti, F., Dionisi, A.M., Mastrantonio, P., 2010. *Clostridium difficile* isolates resistant to fluoroquinolones in Italy: emergence of PCR ribotype 018. J. Clin. Microbiol. 48, 2892–2896.

Stamatakis, A., 2014. RAxML version 8: a tool for phylogenetic analysis and post-analysis of large phylogenies. Bioinformatics 30, 1312–1313.

Steglich, M., Nitsche, A., von Müller, L., Herrmann, M., Kohl, T.A., Niemann, S., Nübel, U., 2015. Tracing the spread of *Clostridium difficile* ribotype 027 in Germany based on bacterial genome sequences. PLoS One 10, e0139811.

Tanner, H.E., Hardy, K.J., Hawkey, P.M., 2010. Coexistence of multiple multilocus variable-number tandem-repeat analysis subtypes of *Clostridium difficile* PCR ribotype 027 strains within fecal specimens. J. Clin. Microbiol. 48, 985–987.

Tschudin-Sutter, S., Kuijper, E.J., Durovic, A., Vehreschild, M., Barbut, F., Eckert, C., Fitzpatrick, F., Hell, M., Noren, T., O'Driscoll, J., Coia, J., Gastmeier, P., von Müller, L., Wilcox, M.H., Widmer, A.F., Committee, 2018. Guidance document for prevention of *Clostridium difficile* infection in acute healthcare settings. Clin. Microbiol. Infect. 24, 1051–1054.