International Journal of Medical Microbiology 308 (2018) 358–363

Contents lists available at ScienceDirect

International Journal of Medical Microbiology

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

Molecular characterization, toxin detection and resistance testing of human clinical *Clostridium difficile* isolates from Lebanon

Fabian K. Berger[a,*], Sari S. Rasheed[b,c], George F. Araj[b,d], Rami Mahfouz[b,d], Hussein H. Rimmani[e], Walid R. Karaoui[e], Ala I. Sharara[e], Ghassan Dbaibo[f], Sören L. Becker[a,g,h], Lutz von Müller[a,i], Markus Bischoff[a], Ghassan M. Matar[b,c,1], Barbara Gärtner[a,1]

[a] Institute of Medical Microbiology and Hygiene, National Reference Laboratory for Clostridium difficile, Saarland University, Kirrberger Straße, Building 43, 66421 Homburg/Saar, Germany
[b] Center for Infectious Diseases Research, American University of Beirut Medical Center, Riad El-Solh 1107, 2020, Beirut, Lebanon
[c] Department of Experimental Pathology, Immunology, and Microbiology, American University of Beirut Medical Center, Riad El-Solh 1107, 2020, Beirut, Lebanon
[d] Department of Pathology and Lab Medicine, American University of Beirut Medical Center, Riad El-Solh 1107, 2020, Beirut, Lebanon
[e] Department of Internal Medicine, American University of Beirut Medical Center, Riad El-Solh 1107, 2020, Beirut, Lebanon
[f] Center for Infectious Diseases Research, American University of Beirut Medical Center, Riad El-Solh 1107, 2020 Beirut, Lebanon
[g] Swiss Tropical and Public Health Institute, P.O. Box, CH-4002 Basel, Switzerland
[h] University of Basel, P.O. Box, CH-4003 Basel, Switzerland
[i] Institute for Laboratory Medicine, Microbiology and Hygiene, Christophorus Kliniken, Südwall 22, 48653, Coesfeld, Germany

ARTICLE INFO

Keywords:
Epidemiology
Middle East
Distribution
Prevalence
MLST
BI/NAP1/027

ABSTRACT

Clostridium (*Clostridioides*) *difficile* is the main cause for nosocomial diarrhoea in industrialised nations. Epidemiologic data on the pathogen's occurrence in other world regions are still scarce. In this context we characterized with phenotypic and molecular genetic methods *C. difficile* isolates stemming from hospitalised patients with diarrhoea in Lebanon.

From 129 stool samples of symptomatic patients at a tertiary care University hospital in Lebanon, a total of 107 *C. difficile* strains were cultivated and underwent ribotyping, toxin gene detection and antibiotic resistance testing.

Ribotype 014 (RT014, 16.8%) predominated, followed by RT002 (9.3%), RT106 (8.4%) and RT070 (6.5%). Binary toxin gene-positive isolates (RT023, RT078 and RT126) were rarely detected and RT027 was absent. Interestingly, within one isolate only the toxin A gene (*tcdA*) was detected. Multiple-locus variable-number tandem repeat analysis (MLVA) revealed strong strain diversity in most RTs. The isolates were sensitive to metronidazole and vancomycin, and only a small proportion of strains displayed resistance against moxifloxacin, rifampicin, and clarithromycin (5.6%, 1.9%, and 2.8%), respectively.

The data indicate that the genetic strain composition of Lebanese strains differs markedly from the situation seen in Europe and North America. Especially the epidemic RTs seen in the latter regions were almost absent in Lebanon. Interestingly, most strains showed almost no resistance to commonly used antibiotics that are suspected to play a major role in the development of *C. difficile* infection, despite frequent use of these antibiotics in Lebanon. Thus, the role of antimicrobial resistance as a major driving force for infection development remains uncertain in this area.

1. Introduction

Clostridium difficile (synonymous: *Clostridioides difficile*), is a Gram-positive spore-forming rod-shaped bacterium, which is the main causative agent of nosocomial diarrhoea. Antibiotic treatment is thought to be a main driver for *C. difficile* infection, as *C. difficile* can only successfully colonize the gut if the normal flora of the intestine is disrupted (Rupnik et al., 2009). The main virulence factors produced by this pathogen are the *tcdA*- and *tcdB*-encoded toxins A and B (Gerding et al., 2014). Some strains may express a third toxin termed "binary toxin" which is encoded by *cdtAB* and preferentially found in epidemic clones (Gerding et al., 2014).

* Corresponding author.
E-mail address: fabian.berger@uks.eu (F.K. Berger).
[1] Contributed equally to this work.

https://doi.org/10.1016/j.ijmm.2018.01.004
Received 7 November 2017; Received in revised form 9 January 2018; Accepted 19 January 2018

Several methods have been established to distinguish *C. difficile* isolates, such as ribotyping, surface layer protein A sequence typing (splAST), multiple-locus variable-number tandem repeat analysis (MLVA) and multi locus sequence typing (MLST). Due to the different typing targets of the methods used, certain discrepancies between sequence types and ribotypes (RTs) of a given isolate may sometimes occur (Dingle et al., 2013).

Given the impact of previous antimicrobial therapy for the development of *C. difficile* infection (CDI), antibiotic stewardship and surveillance is important to evaluate resistance patterns of *C. difficile* (e.g. against metronidazole, one of the drugs of choice for treating CDI), and to determine antimicrobial factors that might favour the selection of certain *C. difficile* strains.

Epidemiologic data suggest a strong regional diversity of *C. difficile* strains that can be monitored by molecular and antimicrobial surveillance (Becker et al., 2015). Some years ago, epidemic RT027 isolates, which seem to be associated with a more severe clinical course, have spread from North America to Europe and elsewhere (He et al., 2013).

Especially in Europe, North America and Australia, numerous studies concerning the epidemiology and impact on the local health care systems have been conducted for *C. difficile* (Davies et al., 2016; Knight et al., 2015; Tenover et al., 2011). However, only little information is available for *C. difficile* in other parts of the world, particularly regarding low- and middle-income countries. In the Middle East, very few epidemiologic studies have been published and these showed substantial differences to characteristics from other regions (Adler et al., 2015; Jamal and Rotimi, 2016). In Lebanon, only a single study has been conducted so far, which focused on toxinotyping and did not provide in-depth data on the molecular epidemiology and strain relatedness (Moukhaiber et al., 2015).

Here, *C. difficile* isolates originating from symptomatic patients being treated at the American University of the Beirut Medical Center in Beirut, Lebanon, were characterized using ribotyping, toxin gene detection and antimicrobial susceptibility testing. The overarching goal of this study was to comprehensively assess the molecular epidemiology and antimicrobial resistance patterns of *C. difficile* in a Middle Eastern country.

2. Materials and methods

2.1. Specimen collection and initial testing

Stool specimens of symptomatic in-patients with clinical suspicion of CDI treated between December 2015 and January 2017 at the University Hospital (American University) in Beirut, Lebanon were screened for *C. difficile*. Indication for screening was development of diarrhoea during hospitalisation. Diarrheal stool samples were analysed using C.DIFF QUICK CHECK COMPLETE (Techlab, Blacksburg, USA) for glutamate dehydrogenase (GDH) and toxin A/B followed by GeneXpert toxin PCR (Cepheid, Sunnyvale, USA). Samples tested positive for toxin A/B or corresponding toxin genes were frozen at −20 °C and forwarded to the National Reference Laboratory for *Clostridium difficile* in Homburg, Germany.

In 25% of the patients antibiotics were used prior to hospitalisation while in 74% of patients antibiotics were administered during the hospital stay prior to symptom development. Only 1% of the patients received no antibiotics.

2.2. Ribotyping, multiple-locus variable-number tandem repeat analysis (MLVA), toxin profile detection and antimicrobial resistance testing

Upon arrival in Homburg, isolates were cultivated under anaerobic conditions on selective media (CLO-Agar, bioMérieux; Marcy L'Étoile, France). PCR-based ribotyping on isolates was performed as described previously (Färber et al., 2017). Detection of the toxin genes (*tcdA*, *tcdB* and *cdtAB*) was performed using a multiplex PCR in accordance with standard protocols (European harmonized diagnostic procedures ECDIS).

Resistance profiles were determined as described previously (von Müller et al., 2012), i.e. utilizing the E-test method at a McFarland standard of 4.0 for the antibiotics metronidazole, vancomycin, and moxifloxacin (MIC Strip, Liofilchem; Roseto degli Abruzzi, Italy). Minimal inhibitory concentrations (MICs) were interpreted as defined by the European Committee on Antimicrobial Susceptibility Testing (EUCAST; http://www.eucast.org/clinical_breakpoints/version 2017). Clarithromycin and rifampicin susceptibilities were tested by the agar disc diffusion method (Becton Dickinson; Heidelberg, Germany). Resistance was defined by the complete absence of an inhibition zone.

Multiple-locus variable-number tandem repeat analysis (MLVA) was used as described previously (Färber et al., 2017) to exclude possible outbreak settings. Clonality was defined as ≤2 and genetic relatedness by ≤10 repeat differences (Färber et al., 2017).

3. Results

Out of 129 specimens tested positive for toxin or toxin genes, a total of 107 *C. difficile* strains could be grown in culture. Moreover, 10 *Clostridium* spp. strains other than *C. difficile* (*C. aldense, C. clostridioforme, C. symbiosum, C. glycolicum* and *C. perfringens*) were found. In 12 stool samples, no growth of any *Clostridium* spp. was observed. All *C. difficile* isolates underwent ribotyping and were tested for the presence of toxin genes by PCR. Results are shown in Table 1.

Among the 107 *C. difficile* isolates, the most prevalent RTs were RT014 (16.8%), followed by RT002 (9.3%), RT106 (8.4%), and RT070 (6.5%). Of note, in nearly one-fifth of the samples, unclassified and previously undescribed RTs were found (18.7%). Interestingly, no RT027 strains were detected. To exclude a possible overrepresentation of outbreak isolates in our strain set, isolates of the most prevalent RTs (RT002, RT014, RT070, and RT106) were additionally characterized by

Table 1
Ribotype distribution of 107 *C. difficile* isolates obtained from symptomatic patients at a tertiary care University hospital in Beirut, Lebanon (2015–2017) and their characteristic toxin gene profiles.*tcdA*, gene coding for toxin A, *tcdB*, gene coding for toxin B, *cdtAB*, genes coding for binary toxin, RT, ribotype.

Ribotype(s)	n (%)	Detected toxin genes
RT001	3 (2.8%)	*tcdA, tcdB*
RT002	10 (9.3%)	*tcdA, tcdB*
RT005	3 (2.8%)	*tcdA, tcdB*
RT010	1 (0.9%)	no toxin genes detected
RT011	2 (1.9%)	*tcdA, tcdB*
RT012	2 (1.9%)	*tcdA, tcdB*
RT014	18 (16.8%)	*tcdA, tcdB*
RT015	5 (4.7%)	*tcdA, tcdB*
RT020	5 (4.7%)	*tcdA, tcdB*
RT023	1 (0.9%)	*tcdA, tcdB, cdtAB*
RT024	2 (1.8%)	*tcdA, tcdB*
RT029	1 (0.9%)	*tcdA, tcdB*
RT043	1 (0.9%)	*tcdA, tcdB*
RT046	3 (2.8%)	*tcdA, tcdB*
RT054	1 (0.9%)	*tcdA, tcdB*
RT056	4 (3.7%)	*tcdA, tcdB*
RT070	7 (6.5%)	*tcdA, tcdB*
RT078	1 (0.9%)	*tcdA, tcdB, cdtAB*
RT084	1 (0.9%)	no toxin genes detected
RT087	1 (0.9%)	*tcdA, tcdB*
RT090	4 (3.7%)	*tcdA, tcdB*
RT106	9 (8.4%)	*tcdA, tcdB*
RT126	1 (0.9%)	*tcdA, tcdB, cdtAB*
RT140	1 (0.9%)	no toxin genes detected
Unclassified ribotype[a]	14 (13.1%)	*tcdA, tcdB*
	5 (4.7%)	no toxin genes detected
	1 (0.9%)	*tcdA* only

[a] 20 RTs could not be classified (only two samples showed corresponding banding patterns indicating an identical RT).

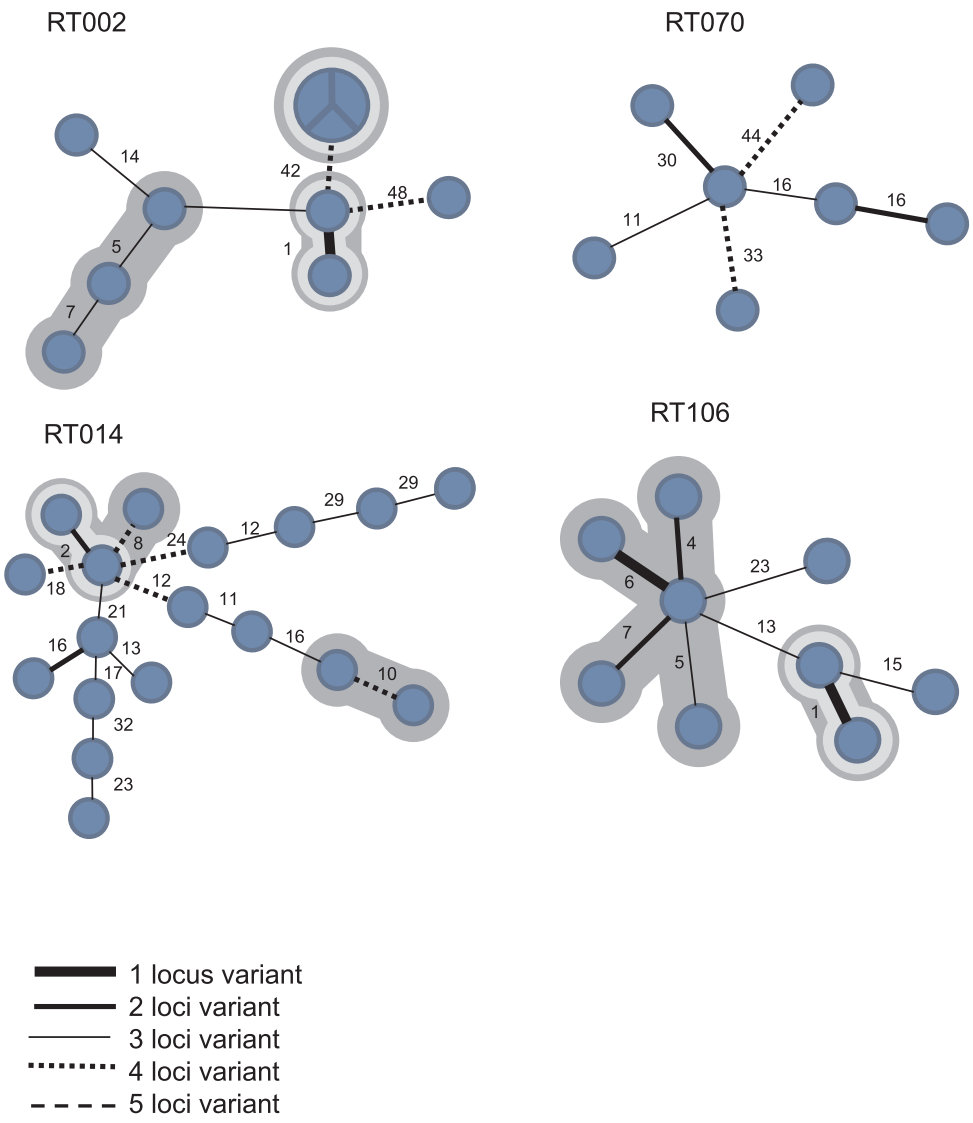

Fig. 1. Minimum spanning trees for predominant ribotypes (RTs) in order to exclude potential outbreak settings using Multiple-locus variable-number tandem repeat analysis (MLVA), isolates in blue; Cross-over stitches: number of different loci; Arabic numbers: amount of different repeats; clonal isolates accompanied by an area in light grey, genetically related strains in dark grey. Relatedness is defined as a repeat difference ≤ 10, clonality as ≤2.

Table 2
Minimal inhibitory concentrations (MICs) of strains possessing a resistance against at least one of the antimicrobial test substances, R, resistant; S, sensitive.

Antibiotic	Metronidazole	Vancomycin	Moxifloxacin	Clarithromycin	Rifampicin
Cut-off values[a]	2 mg/L	2 mg/L	4 mg/L		
Samples with at least one resistance					
RT005[b]	1.00 mg/L (S)	0.38 mg/L (S)	>32 mg/L (R)	S	S
RT012[b]	0.75 mg/L (S)	0.38 mg/L (S)	3.00 mg/L (S)	R	R
RT126[b]	0.38 mg/L (S)	0.50 mg/L (S)	>32 mg/L (R)	R	R
RT078[b]	0.38 mg/L (S)	0.19 mg/L (S)	>32 mg/L (R)	S	S
unclassified RT[b]	0.19 mg/L (S)	0.19 mg/L (S)	>32 mg/L (R)	S	S
unclassified RT[c]	0.25 mg/L (S)	0.38 mg/L (S)	>32 mg/L (R)	S	S
unclassified RT[d]	1.00 mg/L (S)	0.38 mg/L (S)	>32 mg/L (R)	S	S
unclassified RT[d]	0.38 mg/L (S)	0.19 mg/L (S)	0.75 mg/L (S)	R	S

[a] Cut off values according to EUCAST (http://www.eucast.org/clinical_breakpoints/).
[b] strain harbouring *tcdA* and *tcdB*.
[c] strain harbouring *tcdA*.
[d] strain without detected toxin genes.

MLVA (Fig. 1). With the exception of RT106, in which 5 out of the 9 isolates showed a genetic relatedness, only a minor proportion (≤30%) of all RTs displayed clonality and/or genetic relatedness.

Multiplex PCR revealed that most *C. difficile* isolates (91.6%) were positive for *tcdA* and *tcdB*. Three isolates corresponding to RT023, RT078, and RT126, were additionally positive for the binary toxin *cdtAB*. No toxin specific PCR fragments were detected in 8 isolates (7.5%). Importantly, in one unclassified RT isolate, only *tcdA*, but no *tcd*B was found.

The majority of samples (92.5%) were susceptible towards all tested antibiotics, while only eight strains possessed a resistance against at least one of the test substances, most frequently against moxifloxacin

Table 3
Distribution of certain ribotypes (RTs) in different world regions.

Geographic region	Europe	North America	South America	Australia	Far East	Middle East
Study[a]	n = 1196 (Davies et al., 2016)[c]	USA n = 350 (Tenover et al., 2011)[c]	Chile n = 719 (Aguayo et al., 2015)[e]	n = 440 (Knight et al., 2015)[c]	Hong Kong n = 345 (Cheng et al., 2011)[c]; mainland China n = 110 (Huang et al., 2010)[d]; Korea n = 140–408[c,d](Kim et al., 2010, 2013); Taiwan n = 170 (Hung et al., 2016)	Israel n = 208 (Adler et al., 2015)[d]; Kuwait n = 146 (Jamal and Rotimi, 2016) this study n = 107
RT[b]						
RT001/072	11%	3%	nd[f]	nd	< 1% (Hong Kong); 15% (mainland China); ≤ 14% Korea)	14% (Kuwait); 3% (this study)
RT002	4%	5%	nd	16%	10% (Hong Kong); ≤ 4% (Korea)	16% (Kuwait); 9% (this study)
RT014/020	10%	2%	nd	34%	1% (Hong Kong); ≤ 5% (Korea)	6% (Kuwait); 17–22% (this study)
RT018	3%	–	nd	–	≤ 26% (Korea)	–
RT017	nd	4%	nd	2%	< 1% (Hong Kong); 16–26% (Korea); 37% (mainland China); 31% (Taiwan)	–
RT027	19%	26%	79%	nd	≤ 2% Korea	32% (Israel)
RT078	3%	4%	nd	1%	≤ 3% (Korea); < 1% (mainland China); < 1% (Taiwan)	1% (this study)
RT106	nd	5%	nd	nd		8% (this study)
RT126	nd	nd	nd	nd	4% (Taiwan)	10% (Kuwait); 1% (this study)

[a] Only studies using a method comparable to ribotyping with at least 100 *C. difficile* isolates are listed for all regions of the world. Studies with isolates collected prior to the global dissemination of RT027 were also excluded, to better reflect the current situation in RT027 prevalent regions. For Europe and USA, representative multicentre studies covering different representative geographical regions were selected. The indicated studies may also include strains without toxin genes. Origins of the studies, number of isolates tested, and typing methods used are indicated.

[b] Frequency of a RT in this study (%); nd, not deducable.

[c] ribotyping.

[d] splAST.

[e] PFGE/MLST.

[f] not detected.

(Table 2). For comparison to the global situation, epidemiologic data were allocated and are presented in Tables 3 and 4.

4. Discussion

A comparison of our findings with those made by others indicates that some RTs seem to be distributed almost worldwide with a relevant prevalence such as RT001 (except Australia), RT002 and RT014 (Table 3). Other RTs occur epidemically, i.e., being highly prevalent in only some regions of the world but largely missing in others. One example for the latter is RT027, which is frequently found in Europe, North America, and Israel but seems to have only a minor impact in the Arab world, Asia, and Australia.

Data pertaining to the most prevalent *C. difficile* strains in the Middle East are scarce. Only few studies have been conducted with a meaningful number of isolates (i.e. n ≥ 100). A recent study conducted in Kuwait reported a high prevalence for RT001, RT002, RT003, RT014, RT126, and RT139 (Jamal and Rotimi, 2016). This is quite similar to the situation seen with the Lebanese isolates, except for RT003 and RT139 which were not found in our strain collection. However, in sharp contrast, in neighbouring Israel, RT027 was the most prevalent RT which amounted for 32% of all isolates (Adler et al., 2015), while this RT was completely absent in our study. The lack of RT027 suggests only minor exchanges of supposed *C. difficile* reservoirs (e.g. patients, animals, food etc.) between these two countries and also between Lebanon and North America or Europe, respectively. Other results of the study from Israel could not be directly compared with our ribotyping data since splAST was used as an alternative typing method. Therefore a comparison to the ribotyping nomenclature for most of the other RTs remains restricted.

RT078, another epidemic RT that recently emerged in Europe (Goorhuis et al., 2008) and North America (Mulvey et al., 2010), was only isolated once in this study. Hence, it appears that this zoonotic RT is still of minor importance in Lebanon presumably due to limited livestock farming and predominance of crop farming (http://www.fao.org/ag/agp/agpc/doc/counprof/lebanon/lebanon.html#4). Nevertheless with the presence of this outbreak strain and the closely related RT126 both being of proposed zoonotic origin (Stabler et al., 2012), there harbours a risk of potential further spreading in the future.

Notably, rather similar distributions for RT014 (16.8%) and RT002 (9.3%) were observed in our study when compared to Europe [RT002 4%, RT014/RT020 10% (Davies et al., 2016)], Kuwait [RT002 16%, RT014 6% (Jamal and Rotimi, 2016)] and Australia [RT002 16% RT014/020 34% (Knight et al., 2015)]. RT002 was also found relatively often in the USA with 5% (Tenover et al., 2011). RT001 is an endemic strain of high prevalence in most regions including Europe (11%) and Kuwait (14%) but was only rarely found in our isolate set from Lebanon (2.8%).

In one strain only *tcdA* was detected. This finding within a clinical isolate has been reported very rarely in literature and its clinical relevance has not yet been fully understood (Monot et al., 2015).

MLVA of the most prominent RTs demonstrated a comparably low degree of genetically related isolates within these RTs, with the exception of RT106 in which more than half of the isolates displayed a genetic relatedness, possibly indicative for a local transmission within the hospital. In conclusion this strain set seems not to be relevantly influenced by local outbreaks indicating that it represents the true epidemiology in that region.

Interestingly, in the Lebanese strain set, resistance to antibiotics was extremely low. This is quite different to the situation seen in most regions of the world (Table 4). Elevated or resistant MICs for vancomycin and metronidazole were absent in all tested samples from Lebanon. This is in sharp contrast to an Israeli study in which 47% of all isolates were found to be resistant to vancomycin. This might be caused by the fact that two epidemic Israeli RTs/splASTs, RT027 and cr-02 (>85% resistance each) dominated and accounted predominately for this high

Table 4
Resistance patterns of *C. difficile* isolates, relative amount of strains classified as non-susceptible.

Geographic region	Europe	North America	Australia	Far East	Middle East
Study[a]	n = 953 (Freeman et al., 2017)[b]	USA n = 316 (Tenover et al., 2012)[c]	n = 440[c] (Knight et al., 2015)	Taiwan n = 113 (Lin et al., 2011)[c]; Korea n = 120[c]; China n = 110 (Huang et al., 2010)[c]	Israel n = 208 (Adler et al., 2015)[d]; Kuwait n = 146 (Jamal and Rotimi, 2016)[b]; this study n = 107[d]
Substance					
Metronidazole	2%	0%	0%	23% (China)	18% (Israel); 0–3%[f] (Kuwait); 0% (this study)
Vancomycin	1%[g]	–[e]	0%	0%	47% (Israel); 0% (Kuwait); 0% (this study)
Moxifloxacin	37%	38%	3%	16% (Taiwan); 62% (China); 42% (Korea)	60% (Israel); 5.6% this study
Macrolides	–[h]	42%	84%	46% (Taiwan); 85% (China); 80% (Korea)	14–48%[f] (Kuwait); 2.8% (this study)
Rifampicin	17%	8%	0%	29% (China)	0–16%f (Kuwait); 1.9% (this study)

[a] Only studies with at least 100 samples are listed for all available world regions. Studies with isolates collected prior to the global dissemination of RT027 were also excluded, to better reflect the current situation in RT027 prevalent regions. For Europe and USA, representative multicentre studies covering different representative geographical regions were selected.
[b] interpreted according to EUCAST/CLSI.
[c] interpreted according to CLSI.
[d] interpreted according to EUCAST.
[e] substance not tested.
[f] depending on nosocomial and community acquired infection.
[g] since the vancomycin concentration in human stools is regularly >1.000 mg/L (Baines and Wilcox, 2015) a clinical significance of elevated MICs >4 mg/L or even "resistant" isolates >8 mg/L remains unclear.
[h] for clindamycin (lincosamide) an overall non-full susceptibility was 71%.

rate (Adler et al., 2015). Similarly, these strains in Israel were frequently resistant to metronidazole (RT027: 45%, cr-02: 18%).

Moxifloxacin resistance was the most common resistance type seen in our study (6/107, 5.6%), however, rather low compared to other regions of the world (Table 4). For the Middle Eastern country Kuwait, resistance was common for macrolides and rifampicin [up to 48% and 16%, respectively (Jamal and Rotimi, 2016)], while in our strain set resistance against these substances was rather low (2.8 and 1.9%, respectively).

The comparably low level of antimicrobial resistance in these Lebanon-derived *C. difficile* isolates is in sharp contrast to the antibiotic use in this region (Alhomoud et al., 2017). According to statistics from the Center for Disease Dynamics, Economics & Policy (CDDEP, https://resistancemap.cddep.org/AntibioticUse.php), the overall antibiotic use for the year 2014 in Lebanon was 19.363 standard units per 1.000 population (SUP), which is comparable to the rate seen in the USA (19.551 SUP). For fluoroquinolones and macrolides (2.013 SUP and 2990 SUP, respectively), the consumption rates in Lebanon even exceeded the numbers seen in the USA (1.696 SUP and 2.379 SUP, respectively). However, resistance rates for these antibiotic classes were much higher in the USA with 38% for fluoroquinolones and 42% for macrolides (Tenover et al., 2012). In Kuwait, on the other hand, consumption rates for the two agents (485 SUP and 894 SUP, respectively) were much lower, albeit of the fact that *C. difficile* isolates obtained in this country exhibited much higher resistance rates against these antibiotic classes (Table 4). An association between epidemic isolates, lack of strain diversity and high rates of antibiotic resistance within certain RTs such as RT001 and RT027 has been described e.g. in Europe (Freeman et al., 2015; Freeman et al., 2017). These RTs were only of minor importance in our strain set where a broader strain diversity was present.

Taken together, our data might suggest that use of antibiotics is not the major factor for selection of *C. difficile* strains in this region. Limitations of the study are the monocentric approach and that fidaxomicin as an important antibiotic used for therapy could not be tested due to the non-availability of the test substance.

In 12 stool samples no *Clostridium* isolates could be cultivated. In 8 other specimens only non-toxigenic *C. difficile* isolates could be obtained and in further 10 samples *Clostridium* spp. other than *C. difficile* were detected. This might be attributed to the shipping and freezing process to some extent and that patients might have been colonized by more than one *Clostridium* species. This might suggest that in 18 patients the cause of diarrhoea remains unclear.

The epidemiologic data show that the genetic makeup of Lebanese strains differs significantly from the situation seen in other world regions (e.g. between Israel and Kuwait but also North America and Europe), since well-known epidemic strains such as RT027 were not found despite the presence of other RTs with high prevalence in industrialised nations. Moreover, quite different to the situation seen in many other countries, the Lebanese strains developed almost no resistance to commonly used antibiotics such as macrolides and fluoroquinolones that are suspected to be a major driving factor for selection of nosocomial *C. difficile* strains and hospital-associated disease (Freeman et al., 2010). Based on molecular typing results with high variety of unrelated strains and the predominance of supposed wild-type isolates without antibiotic resistance, we hypothesize that most cases of CDI in this Lebanese hospital were not directly transmitted between patients. Until now, the prevalence of nosocomial strains characterized by epidemic spreading and fluoroquinolone resistance is low. It might be speculated that based on generally high antibiotic consumption a potential introduction and spreading of resistant and virulent ribotypes such as RT027 might challenge the health-care system in the future. Hence, ongoing epidemiologic surveillance is warranted.

Authors' contributions

FKB, SR, LVM, SLB, RM, GD, HR, WK, AC, MB and GM were involved in the conception and design of the study. FKB, SR, RM, GD, HR, WK, AC took part in the acquisition of data. FB, GM, MB, SR, BG, SLB performed the data analysis and interpretation. All authors contributed in drafting and revising the article. All authors approved the final version of the manuscript.

Ethics approval

The study was approved by the institutional review board (IRB) of the American University, Beirut Lebanon (AUB IRB IM.AS1.37).

Conflict of interest

Dr. Berger has received consultant fees from MSD, outside the submitted work.

Funding

The *C. difficile* diagnostic of stool samples (GDH and toxin testing) and molecular testing (GeneXpert toxin PCR), performed at the American University of Beirut Medical Center, was funded by Astellas Pharma (Tokyo, Japan). The company had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript.

Acknowledgments

We would like to thank our medical technical assistant Anna Nimmesgern for support during the microbiological diagnostics in Homburg. We are grateful to the Center for Disease Dynamics, Economics & Policy (CDDEP) for sharing their data on global antibiotic consumption under Creative Commons Attribution-NonCommercial-NoDerivs 3.0 Unported License.

References

Adler, A., Miller-Roll, T., Bradenstein, R., Block, C., Mendelson, B., Parizade, M., Paitan, Y., Schwartz, D., Peled, N., Carmeli, Y., Schwaber, M.J., 2015. A national survey of the molecular epidemiology of *Clostridium difficile* in Israel: the dissemination of the ribotype 027 strain with reduced susceptibility to vancomycin and metronidazole. Diagn. Microbiol. Infect. Dis. 83 (1), 21–24.

Aguayo, C., Flores, R., Levesque, S., Araya, P., Ulloa, S., Lagos, J., Hormazabal, J.C., Tognarelli, J., Ibanez, D., Pidal, P., Duery, O., Olivares, B., Fernandez, J., 2015. Rapid spread of *Clostridium difficile* NAP1/027/ST1 in Chile confirms the emergence of the epidemic strain in Latin America. Epidemiol. Infect. 143 (14), 3069–3073.

Alhomoud, F., Aljamea, Z., Almahasnah, R., Alkhalifah, K., Basalelah, L., Alhomoud, F.K., 2017. Self-medication and self-prescription with antibiotics in the Middle East-do they really happen? A systematic review of the prevalence, possible reasons, and outcomes. Int. J. Infect. Dis. 57, 3–12.

Baines, S.D., Wilcox, M.H., 2015. Antimicrobial resistance and reduced susceptibility in *Clostridium difficile*: potential consequences for induction treatment, and recurrence of *C. difficile* infection. Antibiotics (Basel) 4, 267–298.

Becker, S.L., Chatigre, J.K., Coulibaly, J.T., Mertens, P., Bonfoh, B., Herrmann, M., Kuijper, E.J., N'Goran, E.K., Utzinger, J., von Müller, L., 2015. Molecular and culture-based diagnosis of *Clostridium difficile* isolates from Côte d'Ivoire after prolonged storage at disrupted cold chain conditions. Trans. R. Soc. Trop. Med. Hyg. 109, 660–668.

Cheng, V.C., Yam, W.C., Lam, O.T., Tsang, J.L., Tse, E.Y., Siu, G.K., Chan, J.F., Tse, H., To, K.K., Tai, J.W., Ho, P.L., Yuen, K.Y., 2011. *Clostridium difficile* isolates with increased sporulation: emergence of PCR ribotype 002 in Hong Kong. Eur. J. Clin. Microbiol. Infect. Dis. 30 (11), 1371–1381.

Davies, K.A., Ashwin, H., Longshaw, C.M., Burns, D.A., Davis, G.L., Wilcox, M.H., E.s. group, 2016. Diversity of Clostridium difficile PCR ribotypes in Europe: results from the European, multicentre, prospective, biannual, point-prevalence study of *Clostridium difficile* infection in hospitalised patients with diarrhoea (EUCLID), 2012 and 2013. Euro Surveill. 21 (29).

Dingle, K.E., Didelot, X., Ansari, M.A., Eyre, D.W., Vaughan, A., Griffiths, D., Ip, C.L., Batty, E.M., Golubchik, T., Bowden, R., Jolley, K.A., Hood, D.W., Fawley, W.N., Walker, A.S., Peto, T.E., Wilcox, M.H., Crook, D.W., 2013. Recombinational switching of the *Clostridium difficile* S-layer and a novel glycosylation gene cluster revealed by large-scale whole-genome sequencing. J. Infect. Dis. 207, 675–686.

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. Resistance Infect. Control 6, 22.

Freeman, J., Bauer, M.P., Baines, S.D., Corver, J., Fawley, W.N., Goorhuis, B., Kuijper, E.J., Wilcox, M.H., 2010. The changing epidemiology of *Clostridium difficile* infections. Clin. Microbiol. Rev. 23, 529–549.

Freeman, J., Vernon, J., Morris, K., Nicholson, S., Todhunter, S., Longshaw, C., Wilcox, M.H., Pan-European Longitudinal Surveillance of antibiotic resistance among prevalent Clostridium difficile ribotypes' Study, G, 2015. Pan-European longitudinal surveillance of antibiotic resistance among prevalent *Clostridium difficile* ribotypes. Clin. Microbiol. Infect. 21 (248), e248–e249 (e216).

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, Group, 2017. 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. http://dx.doi.org/10.1016/j.cmi.2017.10.008.

Gerding, D.N., Johnson, S., Rupnik, M., Aktories, K., 2014. *Clostridium difficile* binary toxin CDT: mechanism, epidemiology, and potential clinical importance. Gut Microbes 5, 15–27.

Goorhuis, A., Bakker, D., Corver, J., Debast, S.B., Harmanus, C., Notermans, D.W., Bergwerff, A.A., Dekker, F.W., Kuijper, E.J., 2008. Emergence of *Clostridium difficile* infection due to a new hypervirulent strain, polymerase chain reaction ribotype 078. Clin. Infect. Dis. 47, 1162–1170.

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.

Huang, H., Weintraub, A., Fang, H., Wu, S., Zhang, Y., Nord, C.E., 2010. Antimicrobial susceptibility and heteroresistance in Chinese *Clostridium difficile* strains. Anaerobe 16 (6), 633–635.

Hung, Y.P., Huang, I.H., Lin, H.J., Tsai, B.Y., Liu, H.C., Liu, H.C., Lee, J.C., Wu, Y.H., Tsai, P.J., Ko, W.C., 2016. Predominance of *Clostridium difficile* ribotypes 017 and 078 among toxigenic clinical isolates in Southern Taiwan. PLoS One 11 (11), e0166159.

Jamal, W.Y., Rotimi, V.O., 2016. Surveillance of antibiotic resistance among hospital- and community-acquired toxigenic *Clostridium difficile* isolates over 5-year period in Kuwait. PLoS One 11 (8), e0161411.

Kim, H., Jeong, S.H., Roh, K.H., Hong, S.G., Kim, J.W., Shin, M.G., Kim, M.N., Shin, H.B., Uh, Y., Lee, H., Lee, K., 2010. Investigation of toxin gene diversity, molecular epidemiology, and antimicrobial resistance of *Clostridium difficile* isolated from 12 hospitals in South Korea. Korean J. Lab. Med. 30 (5), 491–497.

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 (6), 521–527.

Knight, D.R., Giglio, S., Huntington, P.G., Korman, T.M., Kotsanas, D., Moore, C.V., Paterson, D.L., Prendergast, L., Huber, C.A., Robson, J., Waring, L., Wehrhahn, M.C., Weldhagen, G.F., Wilson, R.M., Riley, T.V., 2015. Surveillance for antimicrobial resistance in Australian isolates of *Clostridium difficile*, 2013–2014. J. Antimicrob. Chemother. 70 (11), 2992–2999.

Lin, Y.C., Huang, Y.T., Tsai, P.J., Lee, T.F., Lee, N.Y., Liao, C.H., Lin, S.Y., Ko, W.C., Hsueh, P.R., 2011. Antimicrobial susceptibilities and molecular epidemiology of clinical isolates of *Clostridium difficile* in Taiwan. Antimicrob. Agents Chemother. 55, 1701–1705.

Monot, M., Eckert, C., Lemire, A., Hamiot, A., Dubois, T., Tessier, C., Dumoulard, B., Hamel, B., Petit, A., Lalande, V., Ma, L., Bouchier, C., Barbut, F., Dupuy, B., 2015. *Clostridium difficile*: new insights into the evolution of the pathogenicity locus. Sci. Rep. 5, 15023.

Moukhaiber, R., Araj, G.F., Kissoyan, K.A., Cheaito, K.A., Matar, G.M., 2015. Prevalence of *Clostridium difficile* toxinotypes in infected patients at a tertiary care center in Lebanon. J. Infect. Dev. Countries 9, 732–735.

Mulvey, M.R., Boyd, D.A., Gravel, D., Hutchinson, J., Kelly, S., McGeer, A., Moore, D., Simor, A., Suh, K.N., Taylor, G., Weese, J.S., Miller, M., Canadian nosocomial infection surveillance, P., 2010. Hypervirulent *Clostridium difficile* strains in hospitalized patients, Canada. Emerg. Infect. Dis. 16, 678–681.

Rupnik, M., Wilcox, M.H., Gerding, D.N., 2009. *Clostridium difficile* infection: new developments in epidemiology and pathogenesis. Nat. Rev. Microbiol. 7, 526–536.

Stabler, R.A., Dawson, L.F., Valiente, E., Cairns, M.D., Martin, M.J., Donahue, E.H., Riley, T.V., Songer, J.G., Kuijper, E.J., Dingle, K.E., Wren, B.W., 2012. Macro and micro diversity of *Clostridium difficile* isolates from diverse sources and geographical locations. PLoS One 7, e31559.

Tenover, F.C., Akerlund, T., Gerding, D.N., Goering, R.V., Bostrom, T., Jonsson, A.M., Wong, E., Wortman, A.T., Persing, D.H., 2011. Comparison of strain typing results for *Clostridium difficile* isolates from North America. J. Clin. Microbiol. 49 (5), 1831–1837.

Tenover, F.C., Tickler, I.A., Persing, D.H., 2012. Antimicrobial-resistant strains of *Clostridium difficile* from North America. Antimicrob. Agents Chemother. 56, 2929–2932.

von Müller, L., Halfmann, A., Herrmann, M., 2012. Current data and trends on the development of antibiotic resistance of *Clostridium difficile*. Bundesgesundheitsblatt Gesundheitsforschung Gesundheitsschutz 55, 1410–1417 in German.

Further reading

Yalçin, S., Songül, Yalçin, Suzan, Blood boron levels and anthropometric measurements in prepubertal children. Int. J.