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Original Article

Molecular epidemiology and antimicrobial resistance patterns of *Clostridioides difficile* isolates in Algerian hospitals

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Abstract

Introduction*: Clostridioides difficile* is a major pathogen responsible for hospital-associated diarrhoea. This study investigated the molecular epidemiology and antibiotic resistance of *C. difficile* isolates in five Algerian hospitals.

Methodology: Between 2016 and 2019, faecal specimens were collected from in-patients and were cultured for *C. difficile*. Isolates were characterised by toxin genes detection, Polymerase Chain Reaction (PCR)-ribotyping, Multilocus Sequence Typing (MLST), antimicrobial susceptibility testing against a panel of antibiotics, and screened for antimicrobial resistance genes.

Results: Out of 300 patient stools tested, 18 (6%) were positive for *C. difficile* by culture, and were found to belong to 11 different ribotypes (RT) and 12 sequence types (ST): RT 085/ST39, FR 248/ST259, FR 111/ST48, RT 017/ST37, RT 014/ST2, RT 014/ST14, FR 247/new ST, RT 005/ST6, RT 029/ST16, RT 039/ST26, RT 056/ST34 and RT 446/ST58. MLST analysis assigned the isolates to two clades, 1 and 4. Clade 4 was more homogeneous, as it mainly included non-toxigenic isolates. Three toxin gene profiles were detected, two toxigenic, A+B+CDT- (33.3%) and A-B+CDT- (11%); and one non-toxigenic, A-B-CDT- (55.5%). All *C. difficile* isolates were susceptible to metronidazole, vancomycin and moxifloxacin.

Conclusions: Overall prevalence of *C. difficile* in our healthcare settings was 6%. Antibiotic resistance rates ranged from 72.2% (clindamycin) to 16.6% (tetracycline). This study highlighted a relatively high genetic diversity in term of ribotypes, sequence types, toxin and antibiotic resistance patterns, in the *C. difficile* isolates. Further larger studies are needed to assess the true extent of *C. difficile* infections in Algeria.

Key words: *Clostridioides difficile*; Algeria; ribotyping; MLST; antibiotic resistance; toxins.

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Introduction

Clostridioides difficile, formerly known as *Clostridium difficile*, a Gram-positive, anaerobic, spore-forming, toxin producing bacteria, is the leading cause of healthcare-associated diarrhoea [1]. The symptoms of *C. difficile* infections (CDI) can range from mild diarrhoea to pseudomembranous colitis or toxic megacolon, a severe form of the disease [2]. The major risk factors for CDI are advanced age (≥ 65 years old), antibiotic exposure, a prolonged hospital stay, gastro-intestinal surgery as well as chronic conditions such as inflammatory bowel diseases [2].

The main virulence factors of *C. difficile* are the production of two major clostridial toxins: toxin A (TcdA) and toxin B (TcdB), encoded on a 19.6 kb chromosomally-located pathogenicity locus (*PaLoc*), which have cytotoxic and enterotoxic effects, respectively [3]. However, certain strains of *C. difficile* produce a third toxin, called binary toxin (CDT), which acts as an actin-specific ADP-ribosyltransferase, encoded by the *cdtA* and *cdtB* genes, located outside the *PaLoc* [4].

The increase in the incidence of CDI reported worldwide over the last two decades was mainly attributed to the emergence of hypervirulent, multidrug-

resistant strains, such as the epidemic NAP1/BI/027 strain [5]. Compared to Europe and North America, CDI is a largely neglected disease in the developing countries and epidemiological data on *C. difficile* are scarce or lacking. In Africa, the highest prevalence of *C. difficile* was reported in Kenya (93.3%) [6] and the lowest in the Ivory-Coast (2%) [7], whereas in the Middle-East, the highest prevalence was recorded in Lebanon (82.9%) [8], and the lowest in Kuwait (0.5%) [9]. In Algeria only one study was conducted reporting a prevalence of 6.9% [10]. The wide variability in the above prevalence estimates is presumably due to a combination of factors such as study designs, type of population studied and *C. difficile* identification methods.

The aim of this study was to investigate the molecular epidemiology and antibiotic resistance of *C. difficile* in five hospitals located in three different provinces of Algeria.

Methodology

Study design and sample collection

Unformed stool samples were collected from patients admitted to five hospitals in three different provinces, Batna, Ain Defla and Chlef; located in the East, Centre and West of Algeria, respectively, between January 2016 and January 2019. All patients included in the study developed diarrhoea, defined as 3 or more loose or liquid stools per day, or more frequently than is normal for the individual (as defined by the World Health Organization, http://www.who.int/topics/diarrhoea) [11], after three days of admission with or without previous antibiotic treatment. Infants under the age of 2 years old are excluded from this study due to the high asymptomatic carriage of *C. difficile* in this group [12].

C. difficile culture and identification

Following alcohol-treatment of the stool samples to eliminate vegetative cells [13], the remaining spores were cultured on a selective medium (*Clostridium difficile* chromogenic agar; ChromID CDIF, Biomérieux, Marcy l'Etoile, France). The plates were incubated at 37 °C for 48 h under anaerobic conditions (10% H, 2.5% CO_2, 85% N_2) using AnaeroGen 2.5L (Thermo scientific, Tokyo, Japan). Black colonies or suspicious non-black colonies (based on the morphological aspect) were identified using Matrix Assisted Laser Desorption Ionisation-Time Of Flight mass spectrometry (MALDI-TOF MS) (Microflex LT BRUKER, Madison, USA).

Molecular identification and toxin genes detection

Genomic DNA was extracted using InstaGene Matrix Kit (Bio-Rad, Hercules, USA), following the manufacturer's instructions. *C. difficile* isolates were stored at -80 °C using Microbank mixed microbial storage vials (Pro-Lab diagnostics, Ontario, Canada).

For molecular characterisation of *C. difficile* isolates and toxin genes, a multiplex PCR assay was carried out according to the protocol of Barbut *et al*., 2019 [14], using seven pairs of primers targeting the following genes: *tpi*, (triose phosphate isomerase), *tcdA* (toxin A), *tcdB* (toxin B) *cdtA* and *cdtB* (binary toxin subunits), the PaLoc and *tcdC* (negative regulator for toxin expression) [15]. *C. difficile* PCR-ribotype (RT) 027, was used as positive control. The amplicons were analysed using a high-resolution capillary electrophoresis detection system (HITACHI ABI 3500 Genetic Analyzer, Applied Biosystems, Massachusetts, USA). The results were visualised using GeneMapper Software version 5.0 (Applied Biosystems, Massachusetts, USA).

Polymerase Chain Reaction (PCR)-ribotyping

PCR-ribotyping was performed according to the protocol recommended by The European Centre for Disease Prevention and Control (ECDC), using primers designed by Bidet *et al* [16]. For the amplification of the 16S-23S rRNA intergenic region, a capillary electrophoresis was performed using a Genetic Analyser (HITACHI ABI 3500, Applied Biosystems, Massachusetts, USA) and electrophoreograms were visualised using GeneMapper Software version 5.0 (Applied Biosystems, Massachusetts, USA). PCR-ribotype (RT) was determined using WEBRIBO database version 2.2 available at: https://webribo.ages.at/. When PCR-ribotyping profiles are unknown, the prefix "FR" was used (French reference laboratory internal nomenclature).

Multilocus sequence typing (MLST)

MLST was performed as described by Griffiths *et al* [17], using PCR primers targeting seven housekeeping genes (*adk*, *atpA*, *dxr*, *glyA*, *recA*, *soda* and *tpi*). The sequence type (ST) and clade were determined by comparing the sequences of strains with the MLST database (http://pubmlst.org/cdifficile).

Antimicrobial susceptibility testing

Antimicrobial susceptibility to clindamycin (CLD), erythromycin (ERY), moxifloxacin (MXF) and tetracycline (TET) was assessed using the disk diffusion method (I2A, France). For metronidazole

(MTZ) and vancomycin (VAN), the minimal inhibitory concentration (MIC) was determined using an E-test (Biomérieux, Marcy l'Etoile, France). Brucella blood agar plates supplemented with 0.5 mg/L hemin, 1mg/L Vitamin K1 and 5% sheep blood (Becton Dickinson GmbH, Heidelberg, Germany) were inoculated with 1.5 MacFarland bacterial suspension. Plates were then incubated for 48 h at 37 °C in an anaerobic atmosphere using AnaeroGen 2.5L (Thermo scientific, Tokyo, Japan). The diameter of inhibition zones was interpreted according to the CA-SFM 2019 recommendations (Antibiotic susceptibility committee of the French society for microbiology) [18]. Breakpoints were set as follows: TET (30 µg) < 19 mm, MXF (5 µg) < 21 mm, CLD (2 UI) < 15 mm, ERY (15 UI) < 22 mm [18]. For MTZ and VAN, MIC breakpoint 2 mg/L was applied as recommended by the European Committee on Antimicrobial Susceptibility Testing (EUCAST) [19]. Multidrug-resistance was considered when the strain showed resistance to 3 or more antimicrobial classes.

Detection of antibiotic-resistance genetic determinants

The presence of the resistance genetic determinants for the macrolide-lincosamide-streptogramin B (MLS_B) family of antibiotics (*ermB*), tetracycline (*tetM*, *tetO*, *tetB[P]*, *tet0/32/0*, *tet40*, *tetA[P]*) [20] and fluoroquinolones (*gyrA*) mutations in the quinolone resistance-determining region (QRDR) was investigated as previously described [20,21].

Statistical analyses

The data were coded using Excel 2013 (Microsoft, Redmond, WA, USA) and analyzed by R software (R Development Core Team, 2016). The distribution of prevalence of *C. difficile* between provinces, ages and sexes of patients was tested by Chi-Square test or Fisher's exact test. A level of *p value* < 0.05 was considered as statistically significant.

Results

Prevalence rates of C. difficile

This multi-center, prospective study included a total of 300 patients admitted to five hospitals in three provinces of Algeria over a period of three years. The majority of patients were from the province of Batna (n = 258, 86%, 1 university hospital), followed by Chlef (n = 31, 10.3%, 3 hospitals) and Ain Defla (n = 11, 3.7%, 1 hospital). There were more females (n = 168, 56%) than males (n = 132, 44%); the majority of patients (n = 237, 79%) were adults (≥ 19 years old), and 21% (n = 63) were ≤ 18 years old.

Of the 300 patients, a total of 18 *C. difficile* isolates were cultured, giving an overall prevalence of 6% (CI at 95%: 3.3%-8.7%). The highest prevalence was recorded in the province of Ain Defla (18.2%, 2/11), followed by Chlef (9.7%, 3/31) and Batna (5%, 13/258). The prevalence was higher (11.1%, 7/63) in patients who were ≤ 18 years old than those aged ≥ 19 years old (4.6%, 11/237). The prevalence in females (6.5%, 11/168) was slightly higher than in men (5.3%, 7 /132). However, the differences in *C. difficile*

Table 1. Molecular characterisation of *C. difficile* isolates in the study.

Sample	Province	Hospital	Ward	Year	Gender	Age	Genotyping							
							PaLoc	*tcdA*	*tcdB*	*tcdC*	*cdtA*	*cdtB*	Ribotype	Sequence type (Clade)
CD 038	Batna	UH	MIW	2016	F	Ad	+	-	-	-	-	-	RT 085	39 (4)
CD 053	Batna	UH	MIM	2016	M	Ad	+	-	-	-	-	-	RT 039	26 (1)
CD 093	Batna	UH	REA	2016	M	Ad	+	-	-	-	-	-	RT 085	39 (4)
CD 137	Batna	UH	PED	2017	F	Ch	+	-	-	-	-	-	RT 085	39 (4)
CD 144	Batna	UH	PED	2017	M	Ch	+	-	-	-	-	+	FR 111	48 (1)
CD 147	Batna	UH	MIW	2017	F	Ad	+	-	-	-	-	+	FR 111	48 (1)
CD 155	Batna	UH	MIM	2017	M	Ad	-	+	+	$+^{NID}$	-	+	RT 014	2 (1)
CD 181	Batna	UH	PED	2017	F	Ch	-	+	+	$+^{NID}$	-	+	RT 014	14 (1)
CD 190	Batna	UH	MIW	2017	F	Ad	-	+	+	$+^{NID}$	-	+	RT 056	34 (1)
CD 202	Batna	UH	MIW	2017	F	Ad	+	-	-	-	-	-	FR 247	New * (1)
CD 210	Batna	UH	MIW	2017	F	Ad	-	+	+	$+^{NID}$	-	+	RT 446	58 (1)
CD 213	Batna	UH	MIM	2017	M	Ad	-	+	+	$+^{NID}$	-	+	RT 005	6 (1)
CD C05	Chlef	Chettia	MIW	2018	F	Ad	-	+	+	$+^{NID}$	-	+	RT 029	16 (1)
CD C13	Chlef	Chettia	PED	2018	F	Ch	+	-	-	-	-	-	FR 248	259 (4)
CD D04	Ain Defla	S/Bobida	PED	2018	M	Ch	+	-	-	-	-	-	FR 248	259 (4)
CD D07	Ain Defla	S/Bobida	PED	2018	M	Ch	+	-	-	-	-	-	FR 248	259 (4)
CD F02	Chlef	Chorfa	PED	2018	F	Ch	-	$+^{ID}$	+	$+^{NID}$	-	-	RT 017	37 (4)
CD S0	Batna	UH	MIW	2018	F	Ad	-	$+^{ID}$	+	$+^{NID}$	-	-	RT 017	37 (4)

UH: University hospital; MIW: Women's internal medicine; MIM: Men's internal medicine; PED: Paediatric; F: Female; M: Male; Ad: Adult; Ch: Child; ID: internal deletion; NID: no internal deletion; * The closest match to STs: 69; 104 and 596.

prevalence between the three provinces, genders and age groups were not statistically significant (p value > 0.05).

Detection of tcdA, tcdB, cdtA/B and tcdC genes

A PCR multiplex assay for the detection of *tcdA*, *tcdB* and *cdtA/B* genes, revealed the presence of three toxin genes profiles: six *C. difficile* isolates (33%) with intact *tcdA* and *tcdB,* deleted *cdtA* and the *cdtB* as a pseudogene (A+B+CDT-); two *C. difficile* isolates (11%) revealed a deletion in *tcdA* and intact *tcdB*, deleted *cdtA* and *cdtB* (A-B+CDT-); the remaining ten *C. difficile* isolates (55.5%) did not carry any of the toxin genes (A-B-CDT-) (Table 1). The same analysis revealed that the *tcdC* gene was present (without internal deletion) in all the toxigenic isolates and absent in all the non-toxigenic isolates.

PCR ribotyping and multilocus sequence typing

The 18 *C. difficile* isolates were assigned to 11 different ribotypes and 12 sequence types: RT 085/ST39 (n = 3), FR 248/ST259 (n = 3), FR 111/ST48 (n = 2), RT 017/ST37 (n = 2), RT 014/ST2 (n = 1), RT 014/ST14 (n = 1), FR 247/new ST (n = 1), RT 005/ST6 (n = 1), RT 029/ST16 (n = 1), RT 039/ST26 (n = 1) and RT 056/ST34 (n = 1), RT 446/ST58 (n = 1) (Table 1). The three unrecognized isolates, FR 111, FR 247 and FR 248, detected in this study corresponded to ribotypes maintained in the internal database of the French National Reference Laboratory for *C. difficile*.

Our *C. difficile* isolates were classified into two MLST clades, 1 and 4; (Table 1) clade 1 was more heterogeneous and consisted of a diverse set of isolates, RT 005/ST6, RT 14/ST2, RT 14/ST14, RT 029/ST16, RT 039/ST26, RT 056/ST34, FR 111/ST48, RT 446/ST58 and FR 247/New ST; whereas clade 4 included RT 85/ST39, RT 17/ST37 and FR 248/ST259. In addition, clade 4, include mainly non-toxigenic isolates (33.33%), with the exception of 2 isolates belonging to RT 17/ST37, which produce toxin B only.

Detection of antimicrobial susceptibility and antibiotic-resistance genes

Antibiotic-susceptibility data of the 18 *C. difficile* isolates are presented in Table 2. All isolates were susceptible to MTZ and VAN, the first line of antibiotics used for the treatment of CDI, and to MXF, a fourth-generation fluoroquinolone. Sequence analysis showed no substitution in the QRDR of *gyrA* of all our isolates.

Five isolates (27.7%, 5/18) belonging to RT 005 (n = 1), RT 039 (n = 1), RT 085 (n = 1) and FR 111 (n = 2) were resistant to the MLS_B family antimicrobials CLD and ERY, conferred by the presence of the *ermB* gene.

A similar resistance rate against the macrolide antibiotics CLD was found (27.7%, 5/18) in the isolates

Table 2. Antimicrobial resistance patterns of *C. difficile* isolates.

RT Stains		ATB (mm)						MIC (mg/L)			
		TET		CLD	ERY	MXF		MTZ		VAN	
		S	R	S	R	S	R	S	R	S	R
		tetM (+)		*ermB* (+)							
		< 15	≥ 19	< 15	≥ 15	< 18	≥ 21	< 4	≥ 4	< 1	≥ 2
RT 085	038	S		R +	S	S		S		S	
	93	S		R +	R +	S		S		S	
	137	S		S	R +	S		S		S	
FR 248	C13	S		R	S	S		S		S	
	D04	S		R	S	S		S		S	
	D07	S		R	S	S		S		S	
RT 014	155	S		S	S	S		S		S	
	181	R +		R +	R +	S		S		S	
RT 017	F2	R +		R +	R +	S		S		S	
	S0	R +		R +	R +	S		S		S	
FR 111	144	S		R +	R +	S		S		S	
	147	S		R +	R +	S		S		S	
RT 005	213	S		R +	R +	S		S		S	
RT 029	C05	S		R	S	S		S		S	
RT 039	053	S +		R +	R +	S		S		S	
RT 056	190	S		S	S	S		S		S	
RT 446	210	S		S	S	S		S		S	
FR 247	202	S		S	R -	S		S		S	

RT: ribotype; ATB: antibiotic; R: resistant; S: susceptible; TET: tetracycline; MXF: moxifloxacin; ERY: erythromycin; CLD: clindamycin; MTZ: metronidazole; VAN: vancomycin; MIC: minimal inhibition; concentration; +: presence of the gene; -: absence of the gene.

of the ribotypes RT 29 (n = 1), RT 085 (n = 1) and FR 248 (n = 3); one of which (RT 29) was not found to carry the *ermB* gene.

Two RT 085 (n = 1) and FR 247 (n = 1) isolates were resistant to ERY only. The latter isolate did not carry the *ermB* gene. Three isolates belonging to RT 014 (n = 1) and RT 017 (n = 2), which carried the *ermB* and *tetM* genes, were resistant to CLD, ERY and TET.

The remaining three isolates RT 014 (n = 1), RT 056 (n = 1) and RT 446 (n = 1) were susceptible to CLD, ERY and TET, and were not found to carry the *ermB* and the *tetM* genes. Interestingly, one isolate of the RT 039 harbored the *tetM* gene, but was susceptible to TET.

The two isolates of the RT 014, which belonged to two different STs, ST2 and ST14, also exhibited different antibiotic resistance phenotypes and genotypes; one isolate (RT 014/ST14) was resistant to CLD, ERY and TET, and carried the *ermB* and *tetM* genes; whereas the second (RT 014/ST2) was susceptible to these three antibiotics and did not carry the *ermB* and *tetM* genes. Of note, these two isolates were recovered from two different patients admitted to different wards of the same hospital.

Discussion

C. difficile has been identified as a leading nosocomial pathogen worldwide and the main causative agent of antibiotic-associated diarrhoea in humans [1]. Although *C. difficile* infections were generally regarded as primarily healthcare-associated, and community-acquired, *C. difficile* infections have now emerged as a significant public health concern [2]. Algeria is at the crossroads to Europe, Africa, and the Middle-East; and like in many developing countries, CDI is a largely neglected disease, and epidemiological data on *C. difficile* are scarce. There is, however, one previous study, based in two hospitals in one province [10]. Therefore, this study was conducted to investigate the prevalence and the molecular epidemiology of *C. difficile* over a wider geographic region, in five study sites in three provinces of Algeria. Interestingly, the prevalence of *C. difficile* estimated here (6%) was similar to the previous Algerian study (6.9%) [10].

The prevalence estimated in this study was comparable to those reported in certain African and Middle-Eastern countries such as Ghana (4.9%) [22], Tanzania (6.4% and 7.3%) [23,24], Zimbabwe (8.6%) [25], Iran (9%) [26,27], Qatar (7.9%) [28] and Saudi Arabia (8.4%) [29], and also falls within the range reported in a European multi-country surveillance study, from 4% to 39% [30] and in the United States from 6% to 48% [31].

Compared to the previous Algerian study, which reported the detection of only four ribotypes [10], our study revealed a relatively larger diversity of PCR ribotypes. The most prevalent RT were RT 085 and FR 248 (n = 3, 16.7%, each), followed by RT 014/RT 017/FR 111 (n = 2, 11.1%, each) and RT 005/RT 029/RT 039/RT 056/RT 446/ FR 247 (n = 1, 5.6% each). All isolates of the same RT belonged to the same ST, with the exception of RT 014, which was shared by two different STs, ST2 and ST14 (1 isolate each), which is in agreement with a previous study [32]. Among the above ribotypes, only RT 014 was previously reported in Algeria, but in a different hospital [10].

The ten *C. difficile* isolates were non toxigenic and belonged to ribotypes RT 039, RT 085, FR 111, FR 247 and FR 248; with RT 085 and FR 248 as the most prevalent (n = 3, 16.7%, each). The three isolates of RT 085 were detected in three different wards of the same hospital in the eastern province of Algeria (Batna), but were missing in the two other provinces; suggesting a possible distinct geographic distribution of this ribotype in Algeria. Although, the ribotype RT 085 was reported as more common in China [33], it was rarely reported in other countries.

It is also worth mentioning that all the three isolates belonging to the unclassified ribotype FR 248 were recovered from children, that were admitted to two different hospitals from two provinces (Ain Defla and Chlef), and as such, the possibility of an association of this ribotype with children is plausible.

The remaining non toxigenic ribotype isolated with a lesser frequency (n = 1), RT 039, was previously reported as most common in patients with cystic fibrosis in Western Australia [34], and was also detected in health care settings in Iran [35], and Kuwait [36]. Surprisingly, several isolates of RT 039 from Iran were found to carry the toxin genes [35]. In addition, isolates of RT 039 were also recovered from animal samples in the Netherlands [37] and Egypt [38].

Toxigenic isolates accounted for 44.4% (8/18) of the total and were shared between six ribotypes, 005, 014, 017, 029, 056 and 446; among which, those belonging to RTs 014 and 017 were the most frequent (n = 2, 11.1%, each). Isolates of the RT 014 were the most prevalent ribotype in many European countries, where it was reported as responsible for CDI outbreaks in humans, and also commonly associated with animals and different environments [37,39–42]. The RT 014 was also detected in several countries in the Middles-East, Iran [35], Lebanon [8] and Qatar [28]; whereas in the African continent it was reported only in Algeria [10] and South Africa [43].

The other most prevalent ribotype in this study, RT017, which is characterized by a deletion in the *tcdA* gene and the absence of binary toxin genes and, therefore, A-B+CDT- [44], is the predominant ribotype in Asia, and has also caused major outbreaks of CDIs in several countries around the world [45,46]. To date, the only African country that reported the ribotype RT 017 is South Africa [43,47], but it has not been detected so far in any of the Middle-eastern countries.

The toxigenic PCR-ribotype RT 029 was previously reported as one of the most frequent RTs among hospitalized patients in Iran [35], and was also isolated from humans in Egypt [48] as well as from humans and animals in Costa Rica [49].

The toxigenic isolate belonging to the PCR-ribotype RT 056 detected in this study, was commonly isolated from humans, cattle, vegetables and the environment in Australia [42]. Prior studies reported that RT 056 was frequently associated with complicated CDI in hospitalized patients in Europe [39,50]. The only report to date of this PCR-ribotype in Africa comes from Zimbabwe [25], whereas in the Middle-East it was reported in Qatar [28] and Kuwait [9,36].

Although the toxigenic RT 005 identified in this study is among the most common ribotypes in Europe [51], it was isolated with a low frequency in a study from Ghana [52], and has not been documented so far in the Middle-East.

Importantly, both this and the previous Algerian study failed to detect the hypervirulent ribotypes RT 027 or RT 078. It must be noted that the ribotype RT 027 was not reported in the African continent, whereas, in the Middle-East, it was detected, albeit with low frequency, in Iran (n = 14) [26,53,54], Saudi Arabia (n = 4) [55] and Qatar (n = 1) [28]. Similarly, there is very little data on the ribotype RT 078 in Africa and the Middle-East, except for two reports from Egypt (n = 6) and Kuwait (n = 9) [36,48].

It is worth mentioning that the detection of the toxigenic ribotypes RT014, RT017, RT029 and RT 056 in this study is important and interesting from an epidemiological point of view, given that these ribotypes were reported to be either responsible for CDIs in several countries around the world (RT017, RT 014 and RT 056) [50], or commonly associated with animals (RT 014, RT 029 and RT 056) [56], raising concerns about their potential zoonotic transmission.

Antibiotic susceptibility test results have shown that all 18 *C. difficile* isolates were susceptible to VAN and MTZ, the treatment of choice for CDI [57], as well as to MXF, a fourth generation fluoroquinolone [58], which is in line with the results of several other studies [59].

Thirteen MLS_B-resistant isolates (72.2%, 13/18), carried the *ermB* gene, and were resistant to CLD and/or ERY; whereas two (11.1%, 2/18) MLS_B-resistant isolates were *ermB*-negative but resistant to either CLD or ERY; suggesting that the MLS_B resistance in these isolates might be conferred by other mechanisms; which is in agreement with previous studies [20,60].

We noticed that resistance to TET was always associated with co-resistance to CLD and ERY, as is the case for three isolates belonging to RT 014 (n = 1) and RT 017 (n = 2), which harboured both the *tetM* and the *ermB* gene. The other TET resistance genes investigated in this study (*tetO*, *tetB[P]*, *tet0/32/0*, *tet40*, *tetA[P]*) were not detected in any of our 18 isolates. Surprisingly, one isolate, member of RT 039, was susceptible to TET despite carrying the *tetM* gene. Given that *tetM* is the predominant TET resistance genetic determinant in *C. difficile*, the exact mechanism behind this peculiar phenotype is unclear at this stage; it is possible, however, that the *tetM* gene in this isolate was inactive due to a mutation.

The high rate of resistance of RT 017 to many antimicrobial agents has been largely documented in several studies, and considered as a major contributing factor to the success and dissemination of this ribotype throughout the world [61,62].

There are a number of limitations within this study that needs to be highlighted; first, and most important, our study lacked clinical patient data; second, this is a study based on a small sample size of isolates; third, *C. difficile* isolates were collected in three geographical areas and five hospitals, which may limit the generalization of the *C. difficile* prevalence estimates to the whole country; fourth, lack of antibiotic susceptibility testing against other important antibiotics used for the treatment of CDI such as fidaxomicin and rifaximin. Clearly, larger studies, over wider geographical area and larger number of study sites are merited.

Conclusions

The present study revealed a moderate prevalence of CDI (6%), with a relatively high diversity of *C. difficile* isolates, some of which were toxigenic. All isolates were susceptible to VAN and MTZ; whereas a high proportion of the isolates showed resistance to CLD and/or ERY. Although well-known hypervirulent *C. difficile* strains such as RT 027 and RT 078 were not detected in this study, our findings highlight the significance of this pathogen in a sample of the Algerian

population, and therefore, an active surveillance of CDI is crucial in order to have a more generalized estimation of the burden of this disease in the country.

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Authors' contributions

YB collected the specimens, and isolated, cultured and confirmed the tested isolates. YB, JC, RSZ and FB confirmed the tested isolates with mass spectrometry, carried out the toxin gene profiling and ribotyping; and performed the antibiotic resistance tests. KM performed the MLST and characterized the antibiotic resistance genes. DA helped in the culturing, identification of the isolates and interpretation of the results. SR was involved in the inclusion of one group of patients and provided their specimens. MEB performed the statistical analysis. YB wrote the draft manuscript. FB, KM, DA and MS reviewed the manuscript. MS conceived the study, supervised the research and revised the manuscript.

References

1. Burke KE, Lamont JT (2014) *Clostridium difficile* infection: a worldwide disease. Gut Liver 158: 1-6.
2. Smits WK, Lyras D, Lacy DB, Wilcox MH, Kuijper EJ (2016) *Clostridium difficile* infection. Nat Rev Dis Prim 2: 16020.
3. Pruitt RN, Lacy DB (2012) Toward a structural understanding of *Clostridium difficile* toxins A and B. Front Cell Infect Microbiol 2: 28.
4. Sun X, Savidge T, Feng H (2010) The enterotoxicity of *Clostridium difficile* toxins. Toxins (Basel) 142: 1848-1880.
5. Solanki D, Kichloo A, El-Amir Z, Dahiya DS, Singh J, Wani F, Solanki S (2021) *Clostridium difficile* infection hospitalizations in the United States: insights from the 2017 national inpatient sample. Gastroenterol Res 14: 87-95.
6. Oyaro MO, Plants-Paris K, Bishoff D, Malonza P, Gontier CS, DuPont HL, Darkoh C (2018) High rate of *Clostridium difficile* among young adults presenting with diarrhea at two hospitals in Kenya. Int J Infect Dis 74: 24-28.
7. Becker SL, Chatigre JK, Coulibaly JT, Mertens P, Bonfoh B, Herrmann M, Kuijper EJ, N'Goran EK, 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.
8. Berger FK, Rasheed SS, Araj GF, Mahfouz R, Rimmani HH, Karaoui WR, Sharara AI, Dbaibo G, Becker SL, von Müller L, Bischoff M, Matar GM, 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.
9. Jamal W, Pauline E, Rotimi V (2015) A prospective study of community-associated *Clostridium difficile* infection in Kuwait: epidemiology and ribotypes. Anaerobe 35: 28-32.
10. Djebbar A, Sebaihia M, Kuijper E, Harmanus C, Sanders I, Benbraham N, Hacène H. (2018) First molecular characterisation and PCR ribotyping of *Clostridium difficile* strains isolated in two Algerian Hospitals. J Infect Dev Ctries 12: 15-21. doi: 10.3855/jidc.9580.
11. Debast SB, Bauer MP, Kuijper EJ, (2014) European society of clinical microbiology and infectious diseases: update of the treatment guidance document for *Clostridium difficile* infection. Clin Microbiol Infect 20: 1–26.
12. Schutze GE, Willoughby RE (2013) *Clostridium difficile* infection in infants and children. Pediatrics 131: 196–200.
13. Marler LM, Siders JA, Wolters LC, Pettigrew Y, Skitt BL, Allen SD (1992) Comparison of five cultural procedures for isolation of *Clostridium difficile* from stools. J Clin Microbiol 30: 514–516.
14. Barbut F, Day N, Bouée S, Youssouf A, Grandvoinnet L, Lalande V, Couturier J, Eckert C. (2019) Toxigenic *Clostridium difficile* carriage in general practice: results of a laboratory-based cohort study. Clin Microbiol Infect. 25: 588-594.
15. Persson S, Jensen JN, Olsen KEP (2011) Multiplex PCR method for detection of *Clostridium difficile tcdA*, *tcdB*, *cdtA*, and *cdtB* and internal in-frame deletion of *tcdC*. J Clin Microbiol 49: 4299-4300.
16. Bidet P, Barbut F, Lalande V, Burghoffer B, Petit J-C (1999) Development of a new PCR-ribotyping method for *Clostridium difficile* based on ribosomal RNA gene sequencing. FEMS Microbiol Lett 175: 261-266.
17. Griffiths D, Fawley W, Kachrimanidou M, Bowden R, Crook DW, Fung R, Golubchik T, Harding RM, Jeffery KJ, Jolley KA, Kirton R, Peto TE, Rees G, Stoesser N, Vaughan A, Walker AS, Young BC, Wilcox M, Dingle KE (2010) Multilocus sequence typing of *Clostridium difficile*. J Clin Microbiol 48: 770-778 .
18. CA-SFM Antibiogram Committee of the French Society of Microbiology Ed. 2019 V 2.0 (2019) Available: www.sfm-microbiologie.org. Accessed: 10 Nov 2021.
19. EUCAST The European Committee on Antimicrobial Susceptibility Testing (2020) Breakpoint tables for interpretation of MICs and zone diameters. Available: https://www.eucast.org. Accessed: 10 Nov 2021.
20. Spigaglia P, Mastrantonio P (2004) Comparative analysis of *Clostridium difficile* clinical isolates belonging to different genetic lineages and time periods. J Med Microbiol 53: 1129-1136.
21. Dridi L, Tankovic J, Burghoffer B, Barbut F, Petit J-C (2002) *gyrA* and *gyrB* mutations are implicated in cross-resistance to ciprofloxacin and moxifloxacin in *Clostridium difficile*. Antimicrob Agents Chemother 46: 3418-3421.
22. Janssen I, Cooper P, Gunka K, Rupnik M, Wetzel D, Zimmermann O, Groß U (2016) High prevalence of nontoxigenic *Clostridium difficile* isolated from hospitalized and non-hospitalized individuals in rural Ghana. Int J Med Microbiol 306: 652–656.
23. Seugendo M, Mshana SE, Hokororo A, Okamo B, Mirambo MM, von Müller L, Gunka K, Zimmermann O, Groß U (2015) *Clostridium difficile* infections among adults and children in Mwanza/Tanzania: is it an underappreciated pathogen among immunocompromised patients in sub-Saharan Africa? New Microbes New Infect 8: 99-102.
24. Seugendo M, Hokororo A, Kabyemera R, Msanga DR, Mirambo MM, Silago V, Groß U, Mshana SE (2020) High *Clostridium difficile* infection among HIV-infected children with diarrhea in a tertiary hospital in Mwanza, Tanzania. Int J Pediatr: 3264923.

25. Berger FK, Mellmann A, Bischoff M, von Müller L, Becker SL, Simango C, Gärtner B (2020) Molecular epidemiology and antimicrobial resistance of *Clostridioides difficile* detected in chicken, soil and human samples from Zimbabwe. Int J Infect Dis 96: 82-87.
26. Kouzegaran S, Ganjifard M, Tanha AS (2016) Detection, ribotyping and antimicrobial resistance properties of *Clostridium difficile* strains isolated from the cases of diarrhea. Mater Socio Medica 28: 324-328.
27. Kouhsari E, Douraghi M, Fakhre Yaseri H, Talebi M, Ahmadi A, Sholeh M, Amirmozafari N (2019) Molecular typing of *Clostridioides difficile* isolates from clinical and non-clinical samples in Iran. APMIS. 127: 222–227.
28. Al-Thani AA, Hamdi WS, Al-Ansari NA, Doiphode SH (2014) Polymerase chain reaction ribotyping of *Clostridium difficile* isolates in Qatar: a hospital-based study. BMC Infect Dis 14: 502.
29. Aljafel NA, Al-shaikhy HH, Alnahdi MA, Thabit AK (2020) Incidence of *Clostridioides difficile* infection at a Saudi Tertiary Academic Medical Center and compliance with IDSA/SHEA, ACG, and ESCMID guidelines for treatment over a 10-year period. J Infect Public Health13: 1156–1160.
30. Couturier J, Davies K, Gateau C, Barbut F. (2018) Ribotypes and new virulent strains across Europe. Adv Exp Med Biol 1050: 45-58.
31. Crobach MJT, Planche T, Eckert C, Barbut F, Terveer EM, Dekkers OM, Wilcox MH, Kuijper EJ (2016) European Society of Clinical Microbiology and Infectious Diseases: update of the diagnostic guidance document for *Clostridium difficile* infection. Clin Microbiol Infect Suppl 4: S63-81.
32. Knetsch CW, Terveer EM, Lauber C, Gorbalenya AE, Harmanus C, Kuijper EJ, Corver J, van Leeuwen HC (2012) Comparative analysis of an expanded *Clostridium difficile* reference strain collection reveals genetic diversity and evolution through six lineages. Infect Genet Evol 12: 1577-1585.
33. Dai W, Yang T, Yan L, Niu S, Zhang C, Sun J, Wang Z, Xia Y (2020) Characteristics of *Clostridium difficile* isolates and the burden of hospital-acquired *Clostridium difficile* infection in a tertiary teaching hospital in Chongqing, Southwest China. BMC Infect Dis 20: 277.
34. Tai AS, Putsathit P, Eng L, Imwattana K, Collins DA, Mulrennan S, Riley TV (2021) *Clostridioides difficile* colonization and infection in a cohort of Australian adults with cystic fibrosis. J Hosp Infect 113: 44-51.
35. Azimirad M, Krutova M, Yadegar A, Shahrokh S, Olfatifar M, Aghdaei HA, Fawley WN, Wilcox MH, Zali MR (2018) *Clostridioides difficile* ribotypes 001 and 126 were predominant in Tehran healthcare settings from 2004 to 2018: a 14-year-long cross-sectional study. Emerg Microbes Infect 9: 1432-1443.
36. Rotimi VO, Jamal WY, Mokaddas EM, Brazier JS, Johny M, Duerden BI (2003) Prevalent PCR ribotypes of clinical and environmental strains of *Clostridium difficile* isolated from intensive-therapy unit patients in Kuwait. J Med Microbiol 52: 705–709.
37. Koene MGJ, Mevius D, Wagenaar JA, Harmanus C, Hensgens MPM, Meetsma AM, Putirulan FF, van Bergen MA, Kuijper EJ. (2012) *Clostridium difficile* in Dutch animals: their presence, characteristics and similarities with human isolates. Clin Microbiol Infect18: 778-784.
38. Abdel-Glil MY, Thomas P, Schmoock G, Abou-El-Azm K, Wieler LH, Neubauer H, Seyboldt C (2018) Presence of *Clostridium difficile* in poultry and poultry meat in Egypt. Anaerobe 51: 21-25.
39. Bauer MP, Notermans DW, Van Benthem BH, Brazier JS, Wilcox MH, Rupnik M, Monnet DL, van Dissel JT, Kuijper EJ (2011) *Clostridium difficile* infection in Europe: a hospital-based survey. Lancet 377: 63–73.
40. Tkalec V, Janezic S, Skok B, Simonic T, Mesaric S, Vrabic T, Rupnik M. High (2019) High *Clostridium difficile* contamination rates of domestic and imported potatoes compared to some other vegetables in Slovenia. Food Microbiol 78: 194-200.
41. Janezic S, Zidaric V, Pardon B, Indra A, Kokotovic B, Blanco JL, Seyboldt C, Diaz CR, Poxton IR, Perreten V, Drigo I, Jiraskova A, Ocepek M, Weese JS, Songer JG, Wilcox MH, Rupnik M (2014) International *Clostridium difficile* animal strain collection and large diversity of animal associated strains. BMC Microbiol 14: 173.
42. Moono P, Lim SC, Riley T V (2017) High prevalence of toxigenic *Clostridium difficile* in public space lawns in Western Australia. Sci Rep 7: 41196.
43. Kullin B, Wojno J, Abratt V, Reid SJ (2017) Toxin A-negative toxin B-positive ribotype 017 *Clostridium difficile* is the dominant strain type in patients with diarrhoea attending tuberculosis hospitals in Cape Town, South Africa. Eur J Clin Microbiol Infect Dis 36: 163-175.
44. Imwattana K, Knight DR, Kullin B, Collins DA, Putsathit P, Kiratisin P, Riley TV (2019) *Clostridiu difficile* ribotype 017 – characterization, evolution and epidemiology of the dominant strain in Asia. Emerg Microbes Infect 8: 796-807.
45. Cairns MD, Preston MD, Lawley TD, Clark TG, Stabler RA, Wren BW (2015) Genomic epidemiology of a protracted hospital outbreak caused by a toxin A-negative *Clostridium difficile* sublineage PCR ribotype 017 strain in London, England. J Clin Microbiol 53: 3141-3147.
46. Kuijper E, Weerdt J, Kato H, Kato N, Dam A, Vorm E, Weel J, van Rheenen C, Dankert J (2001) Nosocomial outbreak of *Clostridium difficile*-associated diarrhoea due to a clindamycin-resistant enterotoxin A-negative strain. Eur J Clin Microbiol Infect Dis 20: 528-534.
47. Rajabally N, Kullin B, Ebrahim K, Brock T, Weintraub A, Whitelaw A, Bamford C, Watermeyer G, Thomson S, Abratt V, Reid S (2016) A comparison of *Clostridium difficile* diagnostic methods for identification of local strains in a South African centre. J Med Microbiol 65: 320-327.
48. Helmi H, Hamdy G (2006) Prevalent PCR ribotypes and antibiotic sensitivity of clinical isolates of *Clostridium difficile*. Egyptian Journal of Medical Microbiology: DOI:10.1099/JMM.0.05207-0.
49. Quesada-Gómez C, Mulvey Mr, Vargas P, Del Mar Gamboa-Coronado M, Rodríguez C, Rodríguez-Cavillini E (2013) Isolation of a toxigenic and clinical genotype of *Clostridium difficile* in retail meats in Costa Rica. J Food Prot 76: 348-351.
50. Davies KA, Ashwin H, Longshaw CM, Burns DA, Davis GL, Wilcox MH (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. Eurosurveillance 29: doi: 10.2807/1560-7917.ES.2016.21.29.30294.
51. Freeman J, Vernon J, Morris K, Nicholson S, Todhunter S, Longshaw C, Burns DA, Davis GL, Wilcox MH (2015) Pan-European longitudinal surveillance of antibiotic resistance

among prevalent *Clostridium difficile* ribotypes. Clin Microbiol Infect 21: 248.e9-248.e16.
52. Seugendo M, Janssen I, Lang V, Hasibuan I, Bohne W, Cooper P, Daniel R, Gunka K, Kusumawati RL, Mshana SE, von Müller L, Okamo B, Ortlepp JR, Overmann J, Riedel T, Rupnik M, Zimmermann O, Groß U (2018) Prevalence and strain characterization of *Clostridioides (Clostridium) difficile* in representative regions of Germany, Ghana, Tanzania and Indonesia - a comparative multi-center cross-sectional study. Front Microbiol 9: 1843 .
53. Jalali M, Khorvash F, Warriner K, Weese JS (2012) *Clostridium difficile* infection in an Iranian hospital. BMC Res Notes 5: 159.
54. Khoshdel A, Habibian R, Parvin N, Doosti A, Famouri F, Eshraghi A, Hafizi M (2015) Molecular characterization of nosocomial *Clostridium difficile* infection in pediatric ward in Iran. Springerplus 4: 627.
55. Alzahrani N, Al Johani S (2013) Emergence of a highly resistant *Clostridium difficile* strain (NAP/BI/027) in a tertiary care center in Saudi Arabia. Ann Saudi Med 33: 198-199.
56. Janezic S, Ocepek M, Zidaric V, Rupnik M (2012) *Clostridium difficile* genotypes other than ribotype 078 that are prevalent among human, animal and environmental isolates. BMC Microbiol 12: 48.
57. Al-Nassir WN, Sethi AK, Nerandzic MM, Bobulsky GS, Jump RLP, Donskey CJ (2008) Comparison of clinical and microbiological response to treatment of *Clostridium difficile*-associated disease with metronidazole and vancomycin. Clin Infect Dis 47: 56-62.
58. Pituch H, Braak N van den, Leeuwen W van, Belkum A van, Martirosian G, Obuch-Woszczatyński P, Luczak M, Meisel-Mikołajczyk F (2001) Clonal dissemination of a toxin-A-negative/toxin-B-positive *Clostridium difficile* strain from patients with antibiotic-associated diarrhea in Poland. Clin Microbiol Infect 7: 442-446.
59. Arca-Suárez J, Galán-Sánchez F, Cano-Cano F, García-Santos G, Rodríguez-Iglesias MA (2018) Antimicrobial susceptibility and molecular typing of toxigenic clinical isolates of *Clostridium difficile* causing infections in the south of Spain. Anaerobe 54: 146–150.
60. Zhao L, Luo Y, Bian Q, Wang L, Ye J, Song X, Jiang J, Tang YW, Wang X, Jin D (2020) High-level resistance of toxigenic *Clostridioides difficile* genotype to macrolide-lincosamide-streptogramin B in community acquired patients in Eastern China. Infect Drug Resist 13: 171-181.
61. Lew T, Putsathit P, Sohn KM, Wu Y, Ouchi K, Ishii Y, Tateda K, Riley TV, Collins DA (2020) Antimicrobial susceptibilities of *Clostridium difficile* isolates from 12 Asia-Pacific countries in 2014 and 2015. Antimicrob Agents Chemother 64: e00296-20.
62. Imwattana K, Knight DR, Kullin B, Collins DA, Putsathit P, Kiratisin P, Riley TV (2020) Antimicrobial resistance in *Clostridium difficile* ribotype 017. Expert Rev Anti Infect Ther 18: 17-25.

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