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Original Article

First molecular characterisation and PCR ribotyping of *Clostridium difficile* strains isolated in two Algerian Hospitals

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Abstract

Introduction: *Clostridium difficile* is the major etiological agent of nosocomial antibiotics associated diarrhoea. *C. difficile* infection is associated with considerable morbidity and mortality among hospitalized patients worldwide. Despite its known importance, there is no study on this important pathogen in Algeria.

Methodology: In this prospective study, undertaken between 2013 and 2015, faecal specimens were collected from 159 hospitalized patients with antibiotic-associated diarrhoea in two tertiary health care hospitals in Chlef, Algeria. Faecal samples were cultured on CLO plates Agar with cefoxitin, cycloserine antibiotics and sodium taurocholate. *C. difficile* suspected colonies were analysed by multiplex PCR for the detection of the toxin genes. *C. difficile* isolates were analysed by PCR ribotyping and multi-locus tandem repeat analysis. Antimicrobial susceptibility was determined by the E-test method, according to the Clinical and Laboratory Standards Institute protocol.

Results: *C. difficile* was cultured from 11 of 159 stool specimen (6.9%). Seven strains were toxigenic, mainly represented by the 020 and 014 PCR ribotypes and four non toxigenic belong to PCR ribotype 084. All 11 isolates were susceptible to both vancomycin and metronidazole and resistant to ciprofloxacin.

Conclusions: This study, which reported for the first time *C. difficile* ribotypes circulating in Algerian health care facilities, could paves the way for further more comprehensive studies on this important pathogen, and could be useful to the local health authorities to implement a surveillance program of *C. difficile* in Algeria.

Key words: *Clostridium difficile;* multiplex PCR; antibiotics; PCR ribotyping.

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Introduction

Clostridium difficile is a Gram-positive, anaerobic, rod-shaped, spore-forming bacterium, described for the first time, in 1935, by Hall and O'Tool in the stools of neonates; whereas its association with disease was not identified until 1978 [1-4].

The ubiquitous *C. difficile* bacterium is present in nature, and colonizes human and animal intestines [5]. It was frequently implicated in gastrointestinal infections in humans, ranging in severity from asymptomatic carriage to severe diarrhoea, pseudomembranous colitis, colonic perforation and death. The main risk factors of the *C. difficile* infection (CDI) are: acquisition include advanced age, antibiotic exposure, length of hospital stay and severe underlying disease [6].

C. difficile infection results after the alteration in the normal microbiota of the colon by the administration of a broad spectrum antibiotics and ingestion of spores. The ingested *C. difficile* spores germinate, colonize the gastrointestinal tract and elaborate at least two toxins, which causes an acute inflammatory response and severe damage to the intestinal epithelium [6,7].

Currently, more than 400 toxigenic and non-toxigenic PCR ribotypes of *C. difficile* have been identified. Toxigenic strains produce one (B) or two types of toxins (A and B) wich are considered as the major virulence factors of *C. difficile* responsible of the disease. Toxin A is an enterotoxin encoded by the *tcdA* gene responsible of diarrhoea, whereas the cytotoxic toxin B is encoded by the *tcdB* gene and causes inflammation of the colon, by producing cytotoxic

effects [8]. Nontoxigenic strains are non-pathogenic. The genes encoding the toxin A and toxin B are respectively located within a five-gene locus known as the PaLoc operon [9]. Certain *C. difficile* strains produce an additional binary toxin (CDT), as actin-specific ADP-ribosyltransferase, expressed from the *cdtA* and *cdtB* operon, located outside the paLoc [10].

To date, no information is available concerning *C. difficile* infection in Algeria. The aim of the present study was to report for the first time the ribotypes of *C. difficile* isolated from two hospitals in Chlef, a city in the North West of Algeria.

Methodology

Faecal specimens

Faecal specimens were collected from 159 hospitalized patients (105 males and 54 females), aged between 3 to 79 years, during the period between 2013 to 2015, in two tertiary health care hospitals: Frères Khatib and Frères Khelif of 180 and 150 beds respectively in Chlef, North West of Algeria. All patients who developed diarrhoea after hospital admission and antibiotic treatment at different wards (Internal medecine, pediatric, surgery, urology, infectious diseases) during the study period were enrolled. No specific diagnostics for the CDI was carried out for the patients. A single diarrheal stool specimen was collected from each patient in a sterile container and immediately subjected to ethanol treatment. All patient included in this study were discharged alive.

C. difficile culture

Faecal samples were cultured, after ethanol treatment, on CLO plates Agar (bioMérieux, Marcy l'Etoile, France) with cefoxitin, cycloserine antibiotics and sodium taurocholate at 37 °C for 48 h under anaerobic conditions (Don Whitley work station). *C. difficile* suspected colonies were identified based on characteristic morphology (grey-brown colonies with an irregular edge) and odour. *C. difficile*-suspected colonies were purified on blood agar, and identified with Gram-stain and API20A galleries (bioMérieux, Marcy l'Etoile, France) and confirmed by multiplex PCR**.**

DNA extraction and molecular identification

DNA extraction

Genomic DNA from bacterial cultures on blood agar medium was extracted using the QIAamp DNA Blood Mini Kit according to the manufacturer's instructions (QIAamp Mini Kit, Qiagen, Hilden, Germany).

Multiplex PCR assay

The multiplex PCR targeting the toxin A (*tcdA*), toxin B (*tcdB*), the binary toxin (*cdtA*/*cdtB)*, the glutamate dehydrogenase (*gluD*) genes, and the 16S rRNA as an internal positive control, was conducted as described elsewhere [11,12], with some modifications. The sequences of the primers used in Multiplex-PCR are listed in Table 1.

The amplification was performed in a MultiNa amplificator MCE-202 (Shimadzu Europa GmbH, Duisburg, Germany). Briefly, amplification reactions were performed in 25µL final volume containing 50 pmol of each primer, 12.50 µL of Hot Start Master Mix, and 2.5 µL DNA. The amplification was performed at 94°C for 15 minutes, followed by 35 cycles of 94°C at 45 seconds, 50°C at 45 seconds, 72°C for 1 minute

Table 1. Primers used in this study.

Name	Target	Sequence		Amplicon size (bp)
CD_cdtA-F3345	TcdA	GCATGATAAGGCAACTTCAGTGGTA	[11]	629
CD_cdtA-R3969		AGTTCCTCCTGCTCCATCAAATG		
CD_cdtB-F5670	TcdB	CCAAARTGGAGTGTTACAAACAGGTG	[11]	410
CD_cdtB-R6079A		GCATTTCTCCATTCTCAGCAAAGTA		
CD_cdtB-R6079B		GCATTTCTCCGTTTTCAGCAAAGTA		
CD_cdtA-F739A	cdtA	GGGAAGCACTATATTAAAGCAGAAGC	[11]	221
CD_cdtA-F739B		GGGAAACATTTATATTAAAGCAGAAGC		
CD_cdtA-R598		CTGGGTTAGGATTATTTACTGGACCA		
CD_cdtB-F617	cdtB	TTGACCCAAAGTTGATGTCTGATTG)	[11]	262
CD_cdtB-R878		CGGATCTCTTGCTTCAGTCTTTATTAG		
CD_PS13	16S-rDNA	GGAGGCAGCAGTGGGGAATA	[11]	1062
CD_PS14		TGACGGGCGGTGTGTACAAG		
908 CLD-gluDs	gluD	GTCTTGGATGGTTGATGAGTAC	[12]	158
909 CLD-gluDas		TTCCTAATTTAGCAGCAGCTTC		

followed by a final extension at 72°C for further 30 minutes. The final products were separated for 30 min at 120V by electrophoresis on agarose gel (1.5%) containing ethidium bromide. For normalization, a molecular size standard (100 bp; Eurogentec, Seraing, Belgium) was added in the first and the last lanes.

PCR Ribotyping

Capillary gel electrophoresis-based ribotyping of the 16-23S intergenic spacer was performed as previously described [13] with some modifications. The number of PCR cycle number was decreased to 22 cycles instead 35.

The 16S primer was labelled at the 5'end with tetrachlorofluorescein (TET). Briefly, two microliters of DNA was used in reactions containing 12.5 µL Hot Start Taq Master Mix, 0.25 µL (10 pmol/µL) of each primer (321BacS_16S: 5'-GTGCGGCTGGATCACCTCCT-3'; 322BacAS_23S as : 5'-CCCTGCACCCTT-AATAA-CTTGACC-3') and 10 µL molecular grade water. Samples were amplified in a conventional PCR thermocycler by running at 95°C for 15 minutes as initial enzyme activation step followed by 22 cycles at 95°C for 1 minute, 57°C for 1 minute and 72°C for 1 minute, plus further 30 minutes at 72°C final elongation. PCR fragments were analysed in an ABI XL3500 genetic analyser (Applied Biosystems, Foster City, California, USA) with a 16 capillary 36 cm array 36 cm array loaded with a POP4 separation matrix (Applied Biosystems, Foster City, California, USA) Sample injection was at 5 kV for 5 seconds with a separating voltage of 15 kV for 30 minutes. A 1200LIZ standard ladder was used as an internal marker. The size of the major fluorescent peak was performed by GeneMapper Software version 5.0 (Applied Biosystems Foster City, California, USA) The ribotypes were determined by using the PCR ribotyping library (the database of the National Reference Laboratory for *Clostridium difficile* of the Netherlands in Leiden) with BioNumerics Software Version 7.1 (Applied Maths, Sint-Martens-Latem, Belgium).

Multi Locus Variable Tandem Repeat Analysis (MLVA)

The MLVA was carried out as previously described [14]. Loci A, B, C, E, F, G and H were amplified using fluorescence-labelled primers. The repeats were amplified using a duplex PCR for A&H, B&F, C&E and a single PCR for G. DNA samples (2.5 µL) were amplified in a final volume of 25 µL containing 12.5 µL Hot Start Taq Master Mix and 0.5 µL of each primer (50 µM), completed to the final volume with molecular grade water. An initial enzyme activation step of 12 min at 94°C was followed by 35 cycles of 30 s at 94°C for denaturation, 30 s at 51°C for annealing and 30 s at 72°C for elongation, plus a final elongation step for 10 minutes at 72°C. The forward primers were labelled at the 5' end with FAM, HEX or TET. PCR fragments were analysed using multi-coloured capillary gel electrophoresis on an ABI XL3500 genetic analyser (Applied Biosystems, Foster City, California, USA) with a ROX-500 ladder as an internal marker for each sample. The size of each peak was determined and analysed by the BioNumerics software version 7.1. The genetic relationship among the isolates was determined as described elsewhere [15].

Antibiotic susceptibility testing

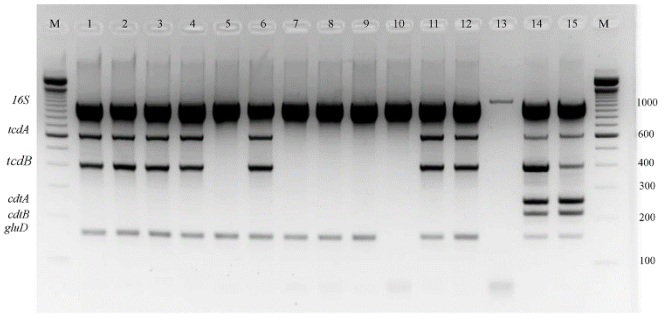
Antibiotic susceptibility test was performed by the E test method. *C. difficile* isolates were grown in an anaerobic environment on pre-reduced sheep blood agar (bioMérieux, Marcy l'Etoile, France) A bacterial suspension of 1 McFarland was prepared in PBS, then swabbed to growth as a lawn on *Brucella* blood agar. E test strips of metronidazole, clindamycin, moxifloxacin, ciprofloxacin, erytromycin, amoxicilline and vancomycin (bioMérieux, Marcy l'Etoile, France) were applied aseptically to the plates and incubated anaerobically for 24 h and 48 h at 37°C.

The resulting MIC values obtained were interpreted according to the Clinical and Laboratory Standards Institute guidelines (CLSI) breakpoints for susceptibility testing of anaerobic bacteria [16].

Results

A total of 159 stool samples were collected, from January 2013 to July 2015, from patients admitted to

Figure 1. Toxin gene profiles of 12 selected *C. difficile* strains.



Lanes 1, 2, 3, 4, 6,11 and 12: Toxigenic *C. difficile* strains *(tcdA+, tcdB+, gluD+, cdtAB-)*. Lane 5, 7, 8 and 9: Nontoxigenic *C. difficile* strains *(tcdA-, tcdB-, gluD+, cdtAB-)*. Lane 10: Negative sample. Lane13: Negative control. Lane: 14,15: Positive controls 027, 078 respectively. M: 100-bp DNA marker.

two tertiary care hospitals, Frères Khatib and Frère Khelif, in Chlef region in the North West of Algeria. All patients have developed diarrhoea after admission to hospital and antibiotic administration. Eleven (6.9%) *C. difficile* strains were isolated on the basis of PCR amplification of the *gluD*. Seven of these strains were positive by PCR amplification of the *tcdA* and *tcdB* genes, and four were negative (Figure 1 and Table 2). No binary-toxin positive strains were recovered.

Six of these strains were isolated from three different wards in the Frères Khatib Hospital: men surgery (n = 3), women surgery (n = 2) and infectious diseases (n = 1); whereas 5 strains were isolated from a single ward (the interne medicine ward) in the Frère Khelif Hospital.

Overall, two different toxin profiles were detected: toxigenic tcdA+/tcdB+/*cdtAB*- (7 strains) and non-toxigenic *tcdA*$^-$/ *tcdB*$^-$/*cdtA*$^-$*B*$^-$ (4 strains).

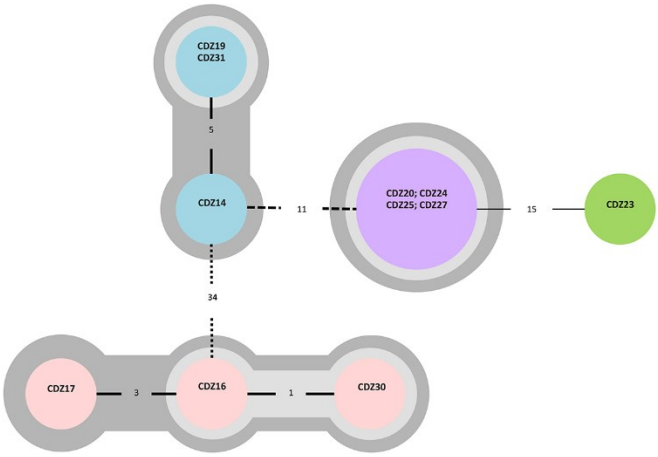
C. difficile isolates were analysed by PCR ribotyping; four distinct PCR ribotypes were identified: RT020 (n = 3), RT012 (n = 3), RT084 (n = 4), RT014 (n = 1) (Table 2). Interestingly, these ribotypes were distributed almost equally between the two hospitals with a frequency of 36% (n = 4). The RT084 was the most predominant ribotype.

The MLVA analysis allowed the distinction between the different ribotypes. All isolates belonging to the same PCR ribotype were genetically related (STRD < 10) whereas all type 084 isolates were belonging to one clonal complex (Figure 2).

Antibiotic susceptibility

The antibiotic susceptibility was interpreted according to the Clinical and Laboratory Standards Institute (CLSI) recommendations [16]. Table 3 shows the antibiotic resistance profiles of the strains. All *C. difficile* strains were sensitive to metronidazole, vancomycin, amoxicillin, moxifloxacin, and resistant to ciprofloxacin. All strains but two were sensitive to clindamycin and erythromycin.

Figure 2. Minimum-spanning-tree analysis of the *C. difficile* strains, represented by four ribotypes typed by Multilocus Variable Number tandem repeat Analysis (MLVA).



Discussion

C. difficile is one of the most important causes of health care–associated infections in the developed countries. Despite, the close proximity of Algeria and Europe, as well as the large population of Algerian migrants in Europe (mainly in France), there is no information available as to the *C. difficile* strains circulating in the Algerian healthcare system. Therefore, we conducted this study in order to isolate

Table 2. Results of the multiplex PCR and ribotyping.

Strain	Hospital	Ward	Gender (Age)	Underlying disease	Ribotype	TcdA	TcdB	CDT
CDZ14	Frères Khatib	Women surgery	F (60)	Bowel obstruction	020	+	+	-
CDZ16	Frères Khatib	Women surgery	F (40)	Appendicular mass and Crohn disease	012	+	+	-
CDZ17	Frères Khelif	Internal medicine	M (56)	Generalized peritonitis	012	+	+	-
CDZ19	Frères Khelif	Internal medicine	M (48)	Abdominal rupture	020	+	+	-
CDZ20	Frères Khelif	Internal medicine	M (48)	Anal abscess	084	-	-	-
CDZ23	Frères Khatib	Men surgery	M (28)	Peritonitis	014	+	+	-
CDZ24	Frères Khatib	Men surgery	M (17)	Peritonitis and coeliac disease	084	-	-	-
CDZ25	Frères Khatib	Men surgery	M (14)	Peritonitis	084	-	-	-
CDZ27	Frères Khelif	Internal medicine	M (50)	Appendicular mass	084	-	-	-
CDZ30	Frères Khelif	Infectious diseases	M (45)	Tuberculosis	012	+	+	-
CDZ31	Frères Khatib	Internal medicine	M (14)	Peritonitis	020	+	+	-

F: Female, M: Male.

Table 3. MICs of antimicrobial agents for all strains (n = 11) of *C. difficile* by the E test.

Antimicrobials	MIC interpretative criteria (µg/mL) (CLSI)			Susceptible	Intermediate	Resistant
	S	I	R			
Clindamycin	≤ 2	4	≥ 8	9 (81.8%)	0	2 (18.1%)
Erythromycin			≥ 256	9 (81.8%)	0	2 (18.1%)
Ciprofloxacin[1]	≤ 2	4	≥ 8	0	0	11 (100%)
Moxifloxacin	2		> 32	11 (100%)	0	0
Metronidazole[1]	≤ 8	16	≥ 32	11 (100%)	0	0
Amoxicillin	≤ 4/2	8/4	≥ 16/8	11 (100%)	0	0
Vancomycin[1]	≤ 2		≥ 2	11 (100%)	0	0

[a] CLSI and EUCAST did not define breakpoints for ciprofloxacin. Here instead are the breakpoints given for moxifloxacin defined by CLSI which should closely approximate the breakpoints of ciprofloxacin. [b]Breakpoints for metronidazole by EUCAST: S ≤ 2; R2 mg/L. [c] No breakpoints defined by CLSI; breakpoints presented by EUCAST.

and characterize the *C. difficile* strains from patients admitted to two Algerian tertiary care hospitals in the region of Chlef.

Our data indicate that all the RT084 strains showed identical MLVA profiles, and were isolated from patients sharing a ward (two each from 'internal medicine', Frères Khelif Hospital and 'men surgery', Frères Khatib Hospital). This may suggest patient-to-patient transmission and/or that the strain was present in the hospital ward environments.

It is also worth noting that two of our non-toxigenic isolates of ribotype 084 were isolated from children, which is similar to the results of another African study [17]. The ribotype 084 was rarely reported in the developed countries.

With a frequency of 36% (n = 4), the ribotypes 014 and 020 are equally dominant with 084. The ribotypes 014 and 020, which are known to be very much related [18], are recognised as being virulent, and are currently the main cause of CDI in the European community [19], [20]. They have also been reported to colonize a broader range of animal species in several countries including Germany, Netherlands, United States of America, and Slovenia [21,22].

The ribotype 012, which is found worldwide [20,23,24], was the third most predominant in our study with 27% (n = 3).

The similar distribution of the ribotypes in the two hospitals, suggests a possible dominance of these ribotypes in the region of Chlef.

It seems important to mention that no hypervirulent strains of ribotype 027 were found in this study.

Altogether, these data suggest that Algeria has a pattern of *C. difficile* ribotypes intermediate between those of Africa and Europe, which is consistent with its geographical location.

Interestingly, all of our *C. difficile* isolates were resistant to ciprofloxacin, which is consistent with other studies that reported a widespread, ribotype-independent, resistance of European *C. difficile* isolates to ciprofloxacin [25,26]. This resistance to ciprofloxacin could be due to the well-recognized problem of over-prescription of antibiotics in Algeria. The clinical impact of ciprofloxacin resistance of Algerian *C. difficile* isolates should be further evaluated.

Conclusion

The results of the current study confirm for the first time the existence of *C. difficile* in Algerian health care facilities, with a prevalence of toxigenic strains of 4.4% (7/159), wich is comparable to that of the Middle East (prevalence rage 4.6-13.7%) [27-32].

The high prevalence of the PCR ribotype 084 reported during the 2013-2015-study period, suggest that this ribotype is the most prevalent amongst patients with antibiotic associated diarrhoea attending two hospitals in Chlef.

Although this study has some limitations, in terms of small sample size and lack of detailed medical records of patients, nevertheless it paves the way for further more comprehensive studies, and could be useful to the local health authorities to implement a surveillance program of *C. difficile* in Algeria, similar to that implemented in other countries.

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