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Original article

First case of autochthonous *Clostridium difficile* PCR ribotype 027 detected in Spain☆

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ABSTRACT

Introduction: *Clostridium difficile* ribotype 027 (Cd027) has caused outbreaks in the United States, Canada, and Europe since 2001. In Spain, the importance of Cd027 is still unknown. In 2007, we began active surveillance of Cd027 to determine its incidence in our hospital.
Methods: From January 2007 to April 2012, isolates of *C. difficile* by multiplex PCR were studied to detect toxin genes. Binary toxin-positive isolates were characterized using PCR-ribotyping. Cd027 were further characterized by toxino-typing, sequencing of tcdC gene, and MLVA (multilocus-variable-number-tandem-repeat-analysis).
Results: Only 8 strains were Cd027 from 3666 isolates of *C. difficile* analyzed during the study period. These strains were isolated from 4 patients: a Spanish patient previously hospitalized in the UK, a pregnant laboratory technician, a British tourist, and a Spanish patient without epidemiological antecedents for acquiring Cd027. MLVA typing of Cd027 isolates revealed 4 different patterns. The first patient had 2 episodes of diarrhea caused by different Cd027. The strains from the first episode of patient 1 and the strain from patient 2 were grouped in the same clonal cluster (these cases were previously published as laboratory transmission), while strains from patients 3 and 4 were genetically unrelated to each other, and to the strains from patients 1 and 2.
Conclusion: We report the first finding of an autochthonous case of non-severe Cd027 infection. Our results indicate that Cd027 diarrhea is uncommon in our area, and it appears mainly as imported cases. MLVA typing enables us to distinguish different genotypes among our Cd027 isolates.

Primer caso autóctono de *Clostridium difficile* del ribotipo 027 detectado en España

Palabras clave:
Clostridium difficile
PCR ribotipo 027
Caso autóctono
España

RESUMEN

Introducción: *Clostridium difficile* del ribotipo 027 (Cd027) ha causado importantes brotes en EE. UU, Canadá y Europa desde 2001. Actualmente su importancia en España es poco conocida. En 2007, nuestro grupo inició la búsqueda de Cd027 para determinar su incidencia en nuestro hospital.
Métodos: Desde enero de 2007 hasta abril de 2012 se estudiaron todos los aislados de *C. difficile* mediante PCR multiplex de los genes de las toxinas. Las cepas toxina binaria positivas se caracterizaron por PCR-ribotipado. Las cepas de Cd027 encontradas se genotiparon por toxinotipo, secuenciación del gen tcdC y MLVA.
Resultados: Durante el periodo de estudio se analizaron 3.666 cepas de *C. difficile* de las que solo 8 fueron Cd027. Estas cepas se aislaron de 4 pacientes: una paciente española previamente hospitalizada en el Reino Unido, una técnico de nuestro laboratorio, una turista británica y un paciente español sin antecedentes de riesgo para haber adquirido Cd027. Mediante MLVA obtuvimos 4 patrones de tipado

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diferentes. La primera paciente tuvo 2 episodios de diarrea causados por cepas diferentes de Cd027. Una de estas cepas fue la misma que la de nuestra técnico de laboratorio (este caso está publicado como una transmisión de laboratorio). Las cepas de los pacientes 3 y 4 tuvieron MLVA únicos.
Conclusión: En este trabajo describimos el primer autóctono de diarrea causada por Cd027. Nuestros resultados indican que es infrecuente en nuestro medio y que aparece principalmente como casos importados. El tipado por MLVA nos ha permitido diferenciar genotipos diferentes entre los aislados de Cd027 de nuestro hospital.

Introduction

Hypervirulent epidemic strains of *Clostridium difficile* belonging to ribotype 027 (toxinotype III, toxin A + B + binary +) have been reported to cause important outbreaks of severe infection in the United States and Canada since 2001.[1,2] Since 2003, *C. difficile* ribotype 027 (Cd027) has been detected in major outbreaks or sporadic cases in several European countries.[3] In Spain, data describing the molecular epidemiology of *C. difficile* are scarce, and the importance of Cd027 is still unknown.[4,5]

In this report, we describe the first Spanish autochthonous case of Cd027 infection and present an additional 3 cases of diarrhea caused by Cd027 strains isolated at our institution.

Materials and methods

Since January 2007, our laboratory has performed active surveillance of Cd027 based on multiplex polymerase chain reaction (PCR) to detect *C. difficile* toxin genes in all strains of *C. difficile* isolated in our hospital. *tdcA* conserved fragment, *tcdA* deleted fragment, *tcdB*, *cdtA*, *cdtB*, and *16SrRNA* (internal control) were amplified by PCR following a method adapted from other authors.[6,7]

As Cd027 is characterized by binary toxin production, all binary toxin-positive strains were ribotyped.[8] Strains belonging to ribotype 027 were further characterized by toxinotyping,[9] sequencing of *tcdC*,[10] and multilocus-variable-number of tandem repeat analysis (MLVA).[11] MLVA patterns were interpreted using summed tandem repeat differences (STRD).[11] Susceptibility to erythromycin, clindamycin, moxifloxacin, rifampin, imipenem, metronidazole, and vancomycin was assessed using the E-test in *Brucella* agar.

By April 1st, 2012 we had studied 3666 strains of *C. difficile* of which 444 (12%) had binary toxin genes. A total of 360 strains (10%) belonged to toxinotype V ribotype 078/126, and only 8 (0.21%) belonged to toxinotype III ribotype 027 (Fig. 1, ribotyping pattern compared to epidemic strains kindly provided by E.J. Kuijper). All Cd027 strains had a single 1-bp deletion at position 117 and an 18-bp deletion in the *tcdC* gene that is characteristic of the epidemic

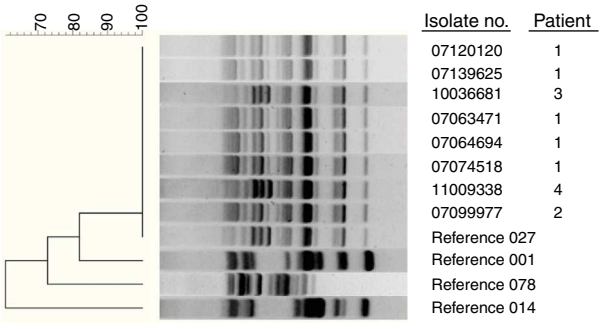

Fig. 1. Similarity dendrogram of ribotyping of the Cd027 strains.

C. difficile strain ribotype 027. These strains were isolated from 4 patients (Table 1).

Results

Case descriptions

Case 1: The first case of Cd027 infection in our institution was a Spanish woman living in the UK, where she was being treated for alveolar proteinosis and systemic lupus erythematosus. She returned to Spain to undergo lung transplantation. During her stay in our medical ICU she developed 2 different episodes of severe[12] *C. difficile* infection (considering as different 2 episodes of *C. difficile* diarrhea separated more than 8 weeks[5]). Detection of *C. difficile* toxin A and B antigens in feces was positive (Immunocard, Meridian Bioscience), cytotoxicity assay and toxigenic culture were positive too. Five different isolates of Cd027 were obtained (Table 1). The patient survived her episodes of *C. difficile* infection but died soon afterwards from other complications of her underlying diseases. Despite the fact that the first episode occurred 20 days after admission to our ICU and 3 months after the last hospitalization in the UK, the epidemiological history led us to consider the Cd027 infection as imported. Data from this case were partially reported in 2008.[13]

Case 2: A healthy pregnant laboratory technician working with the *C. difficile* isolates of case 1 developed *C. difficile* diarrhea shortly after being treated with oral fosfomycin for urinary tract infection. *C. difficile* toxin A and B antigens were detected (Immunocard, Meridian Bioscience), and the cytotoxicity assay and toxigenic culture from feces were also positive. This case was previously reported as a laboratory-acquired *C. difficile* infection.[13]

Case 3: A British tourist was admitted to our hospital emergency department in March 2010 with clinical features of acute appendicitis. She had developed acute diarrhea during the previous 5 days. Diarrhea persisted during her post-surgical stay, and a study of *C. difficile* was performed. *C. difficile* toxin A and B antigens were not detected (Immunocard, Meridian Bioscience) and the results of the direct cytotoxicity assay was negative. The patient's previous medical history was unclear, although she appears to have had chronic diarrhea for more than 20 years and underwent several operations in the UK more than 10 years ago (cholecystectomy, hysterectomy, and colonic resection by rectocele). We do not know if this patient had recent contact with health-care institutions in UK. This case was also considered to be imported.

Case 4: An 82-year-old Spanish man whose only recent contact with a health care institution had been with our center for acute cholecystitis (30 days previously) and complicated urinary tract infection (10 days previously), received ertapenem followed by amoxicillin/clavulanic acid to treat both infections. The patient was admitted to the emergency department of our hospital in December 2011 with a 3-day history of fever, watery diarrhea, and abdominal pain. Glutamate dehydrogenase was positive in stools, but toxins A and B were negative (Techlab C. diff Quik Chek Complete, Blacksburg Va). GeneExpert C. diff (Cepheid, CA, USA) was then performed given positive results for toxigenic *C. difficile* presumptive 027 strain. The result of the direct cytotoxicity assay in cell culture was also positive. Stool cultures for enteropathogens

Table 1
Characteristics of patients with Cd027 infection and susceptibility of isolates.

Patient	Sample number	Date of isolation#	Patient sex/age (years)	Department	Symptoms	Underlying disease	Severe* CDI	Recurrence*	Treatment of CDI	Risk factors for Cd027	Antimicrobial susceptibility of isolates MIC (μg/ml)					
											E	Cd	Mz	V	Imp	Mox	R
1	07063471	25/4/2007	F/33	Intensive Care Unit	Acute diarrhea	Alveolar proteinosis Lupus erythematosus	Yes	Yes	Mz	Hospitalization in UK	>256	>256	0.5	1.5	>32	>32	>32
	07064694	27/4/2007							Mz		>256	>256	0.38	2	>32	>32	>32
	07074518	17/5/2007							V		>256	>256	025	2	>32	>32	>32
	07120120	13/8/2007							Mz + V		>256	>256	0.5	2	>32	>32	>32
	07139625	23/9/2007							Mz + V		>256	>256	0.75	2	>32	>32	>32
2	07099977	02/07/2007	F/32	Microbiology Laboratory	Acute diarrhea (20 stools/day)	Pregnancy	No	No	V	Occupational contact with Cd027	>256	>256	0.75	1.5	>32	>32	>32
3	10036681	02/03/2010	F/60	General Surgery	Diarrhea 5 days	Cholecystectomy Colectomy Appendicitis	No	Unknown	Mz	Hospitalization in UK	1.5	4	0.125	0.75	6	0.75	<0.002
4	11009338	30/12/2010	M/82	Internal Medicine	Acute diarrhea Fever Abdominal pain	Cholangitis Cholangiocarcinoma	No	No	Mz	None	1.5	4	0.19	1	8	1	<0.002

To define episode# and severity* of CDI we considered Refs. 5,12 respectively.
CDI, *Clostridium difficile* infection; E, erythromycin; Cd, clindamycin; Mz, metronidazole; V, vancomycin; Imp, imipenem; Mox, moxifloxacin; R, rifampin.

were negative, as was detection of rotavirus and adenovirus antigen. *C. difficile* was isolated after 48 h of incubation in agar CLO (bioMérieux). Toxigenicity was confirmed by the cytotoxicity assay and multiplex PCR. Ribotyping confirmed Cd027. The patient was treated with metronidazole, and the episode resolved with no further recurrence. His only travel abroad had been to attend a football match in Vienna in 2009.

All patients but the first one had non-severe diarrhea, and none developed important complications related to Cd027 infection.[12] Only the first patient had recurrence of *C. difficile* infection and only the first two cases were related in time (Table 1).

Characterization of isolates

All our Cd027 isolates were susceptible to metronidazole. The isolates for the first 2 patients were highly resistant to erythromycin, clindamycin, moxifloxacin, rifampin, and imipenem, although the Cd027 isolates of the last 2 patients were not so (Table 1). The vancomycin MIC varied from 0.75 μg/ml to 2 μg/ml.

MLVA typing of Cd027 isolates revealed 4 different patterns (Table 2). The strains from the first episode of patient 1 and the strain from patient 2 were grouped in the same clonal cluster (STRD $\leq$ 2). Strains from patients 3 and 4 were genetically unrelated to each other and to strains from patients 1 and 2 (STRD $\geq$ 10). Surprisingly, MLVA typing demonstrated that the strains from the 2 episodes of patient 1 were different and did not belong to the same clone (STRD $\geq$ 10), probably reflecting sequential isolation of different Cd027 strains from an initial polyclonal infection acquired in the hospital of origin in the UK.

Discussion

To our knowledge, we report the first autochthonous case of *C. difficile* infection caused by Cd027 in Spain. The patient had no previous contact with health care institutions in Spain or abroad; therefore, we were unable to track the origin of the strain and we ignore if this patient had contact with people (households, relatives, etc.) from environments where Cd027 is frequent.[14] The review of the other 3 episodes shows that Cd027 mainly appears as imported cases. The first 2 cases of Cd027 in our hospital were detected in 2007. The first was considered to be imported from the UK; the second involved horizontal transmission to a laboratory technician handling the first patient's stool samples.[13]

Between 2000 and 2006, rates of *C. difficile* infection increased markedly in the USA and Canada,[15,16] mainly because of the epidemic Cd027 strain.[1] Although some outbreaks of Cd027 have been described in Europe,[17,18] a surveillance study performed in 34 European nations in 2008 showed a mean incidence of nosocomial *C. difficile* infection of 4.1 cases per 10,000 patient-days (range 0.0–36.3); only 5% were caused by Cd027.[5] Similarly, a pan-European Cd027 survey revealed a relatively low prevalence in Europe that varied from country to country.[19] Cd027 outbreaks in Europe were contained thanks to awareness of the American experience and the introduction of strict control measures in several European countries.

It is difficult to know the real importance of Cd027 in Spain. Our results indicate that Cd027 infection could be distinctly uncommon in Spain. Although, it is necessary to consider that not all clinical microbiology laboratories in Spain currently culture *C. difficile*, and diagnosis of *C. difficile* infection is based mainly on the results of enzyme immunoassay as demonstrated in a recent paper by our group.[20] Besides, Spain does not have a national surveillance program to investigate C. *difficile* infection or a reference laboratory where hospitals could send *C. difficile* isolates for further characterization. These limitations would determine that Cd027

Table 2
Molecular characteristics of the Cd027 isolates studied.

Patient	Sample number	Date of isolation	Toxin profile	Toxinotype	*tcdC* molecular characteristics	MLVA[a] (No. of tandem repeats for each locus)							
					Δ18bp	Single Δ117	A6	B7	C6	E7	F3	G8	H9
1	07063471	25/4/2007	A+B+BIN+	III	+	+	19	14	37	9	5	13	2
	07064694	27/4/2007	A+B+BIN+	III	+	+	20	14	37	9	5	13	2
	07074518	17/5/2007	A+B+BIN+	III	+	+	19	14	37	9	5	14	2
	07120120	13/8/2007	A+B+BIN+	III	+	+	19	14	24	9	5	13	2
	07139625	23/9/2007	A+B+BIN+	III	+	+	19	14	24	9	5	13	2
2	07099977	02/07/2007	A+B+BIN+	III	+	+	19	13	38	9	5	13	2
3	10036681	02/03/2010	A+B+BIN+	III	+	+	33	16	23	9	5	13	2
4	11009338	30/12/2010	A+B+BIN+	III	+	+	20	22	26	8	5	12	2

[a] MLVA patterns interpreted considering STRD (summed tandem repeat differences) as previously described.[11]

could go undetected mainly considering that only a few hospitals perform molecular characterization of *C. difficile*.

These limitations are overcome in a recently reported prospective nationwide diagnostic study, in which confirmatory cultures and molecular characterization of *C. difficile* isolates were performed on all diarrheic stools arriving at 118 Spanish microbiology laboratories. An incidence of 3.8 cases of *C. difficile* infection per 10,000 patient-days was reported without isolation of Cd027.[4] In other studies recently conducted in Spanish hospitals, Cd027 was also not detected.[21,22] After intensive searching at our center, we detected a very low incidence of Cd027 strains. The reasons for this finding are both unclear and unexpected, since, annually, Spain receives millions of tourists from all over the world.

The incorporation in the routine diagnosis of *C. difficile* infection of easy-to-use systems such as GeneExpert C. diff® (Cepheid, CA, USA), which detects toxigenic *C. difficile* and presumptive Cd027, could improve detection of Cd027 strains in Spain, although laboratories should culture *C. difficile* in order to have a stock of isolates for epidemiological studies.

In conclusion, although Cd027 does not seem to be a major problem in Spain, it is important to recommend that clinical microbiology laboratories perform *C. difficile* culture, at least from toxin-positive stools or in severe infections, to enable epidemiological studies to know the real situation of Cd027 and other epidemic strains.

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Conflict of interest

The authors declare no conflict of interest.

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