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Clostridium difficile presence in Spanish and Belgian hospitals

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ABSTRACT

Clostridium difficile is recognised worldwide as the main cause of infectious bacterial antibiotic-associated diarrhoea in hospitals and other healthcare settings. The aim of this study was to first survey *C. difficile* prevalence during the summer of 2014 at the Central University Hospital of Asturias (Spain). By typing the isolates obtained, it was then possible to compare the ribotype distribution at the Spanish hospital with results from the St Luc University Hospital in Belgium over the same period. The prevalence of positive cases reported in Spain and Belgium was 12.3% and 9.3% respectively. The main PCR-ribotypes previously described in Europe were found in both hospitals, including 078, 014, 012, 020 and 002. In the Spanish hospital, most of the *C. difficile*-positive samples were referred from oncology, acute care and general medicine services. In the Belgian hospital the majority of positive samples were referred from the paediatric service. However, a high percentage of isolates from this service were non-toxigenic. This study finds that the presence and detection of *C. difficile* in paediatric and oncology services requires further investigation.

1. Introduction

Clostridium difficile is currently one of the most largely studied pathogenic bacteria in the world and is considered as the major cause of nosocomial antibiotic-associated diarrhoea and colitis in industrialised countries [1]. Clinical manifestations of *C. difficile* infection (CDI) range from mild or moderate diarrhoea to fulminant and sometimes fatal pseudomembranous colitis [2]. Normally, the diarrhoea has been described to appear 48–72 h post infection and characterised as non-haemorrhagic and watery, accompanied with abdominal pain, fever and leucocytosis [3]. However, the worst outcomes are sepsis and death, which is observed in 17% of CDI cases [4]. The highest incidence and mortality rate is usually reported among patients of advanced age who have had a stay in a healthcare setting [5].

A recent review of CDI cost-of-illness attributes a mean cost ranging from $8911 to $30,049 per hospitalised patient in the USA [6] and around €3000 million total per annum in Europe [7]. In addition, in many hospitals the diagnosis strategy remains suboptimal and a proportion of infections may remain undiagnosed [8]. In the past decade, an increase in the incidence and severity of the infection has been reported in various healthcare settings among many countries [9]. This situation was attributed to the emergence of a new epidemic and hypervirulent *C. difficile* strain, identified as PCR-ribotype 027 (NAP1 or North American pulsed field type 1) [10]. Since 2003, in the United States and Canada, studies have shown an increase in the number and severity of CDI cases, including an increase in the case fatality, mortality and colectomy rates [11]. The situation presented by studies in North America is mirrored in Europe. In 2008, the PCR-ribotype 027 was detected in 16 European countries and caused outbreaks in Belgium, Germany, Finland, France, Ireland, The Netherlands, Switzerland and the United Kingdom [11,12]. However, in a further epidemiology study conducted in Europe, the most prevalent PCR-ribotypes were identified as 014/020 (15%), 001 (10%) and 078 (8%), while PCR-ribotype 027 was less prevalent (5%) [12]. Surveillance data for Belgium from 2008 to 2010 showed a stable incidence of CDI in Belgian hospitals, and even a decrease in 2010. In addition, PCR-ribotype 027 was the most prevalent type during the years 2007–2009 [13]. A further study reporting CDI ribotype distribution in Belgian hospitals between 2008 and 2010 described a decrease in cases caused by PCR-ribotype 027 (from 55% in 2008 to

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28% in 2010). In contrast, the proportion of other PCR-ribotypes involved in CDI increased, such as ribotype 014 (from 20% in 2008 to 33% in 2010) and ribotype 078 (from 11% in 2009 to 23% in 2010) [14]. Meanwhile, a prospective study conducted in 2009 in the region of Barcelona (Spain) identified the main PCR-ribotypes associated with CDI as 241 (26%), 126 (18%), 078 (7%) and 020 (5%), while PCR-ribotype 027 was not detected [15]. In a later study conducted in the region of Madrid (Spain) from January to June 2013, most of the isolates associated with a CDI case possessed binary toxin and were classified as PCR-ribotype 078/126 (90.7%) [16]. Consistent with these reports, Weber et al. [17] studied *C. difficile* clinical isolates recovered at the reference hospital of the Balearic Islands (Spain) between August 2007 and April 2011. The authors detected a total 43 different PCR-ribotypes with a higher prevalence of types 014 (34%), 078 (13%) and 001 (5%). As in other Spanish studies, none of the isolates were identified as PCR-ribotype 027.

The aim of this study was to survey the *C. difficile* circulation during the summer of 2014 at the Central University Hospital of Asturias (Spain), a provincial hospital located in the North of Spain. By typing of all the isolates obtained, it was then possible to compare the ribotype distribution at the Spanish hospital with results from the St Luc University Hospital in Belgium over the same period.

2. Methods

2.1. Hospital selection, data and sampling

The Central University Hospital of Asturias (HUCA) located in Oviedo (Asturias, Spain), is the referral hospital of the Health Service of the Principality of Asturias. Overall, the hospital has 17 buildings with a total of 1324 beds, 29 operating rooms, 203 consultation rooms (for outpatients) and 123 emergency rooms.

During the 4-month period from July to October 2014, all samples from outpatients and hospitalised patients suspected of being infected with *C. difficile* were tested. Stool consistency of samples was evaluated using the Bristol Stool Chart (BSC). Samples were documented for data relating to clinical history, diagnosis and treatment received, including the prescription of antimicrobial agents. Numerical identification was used for all samples to guarantee patient anonymity.

2.2. C. difficile rapid detection

Initial screening for *C. difficile* presence was performed using a rapid membrane enzyme immunoassay for the simultaneous detection of *C. difficile* glutamate dehydrogenase antigen and toxins A and B (Cdiff QuickChek Complete® TechLab, Blaclsburg, USA). In the case of doubtful results or glutamate dehydrogenase antigen testing positive and toxins A and B testing negative, GenomEra CDX System *C. difficile* (Abacus Diagnostica, Turku, Finland) was performed for rapid identification of toxin B. These tests were applied only in semisolid, mushy stools and watery/entirely liquid faeces (Bristol stool chart levels 4 to 7) while samples outside this range were discarded. This analysis constituted the routine protocol followed in the hospital laboratory for the diagnosis of CDI. If various stool samples were received from the same patient, a second analysis was only performed if the first *C. difficile* screening was made at least one month prior.

2.3. Culture, identification and characterisation

All specimens received in the laboratory for *C. difficile* testing were cultured regardless of their classification in the BSC. Culture was carried out as described previously [18]. Briefly, approximately 0.1 g of faeces was spread directly on cycloserine cefoxitin fructose agar taurocholate medium (CCFAT), freshly prepared in the laboratory. Plates were incubated anaerobically for 48–72 h at 37 °C. The anaerobic atmosphere in the jar was created using AnaeroGen™ sachet (Oxoid, Dardilly, France) and checked using an anaerobic indicator BR0055B (Oxoid). An enrichment step was also performed. One gram of faeces was inoculated into 9 ml of CCFT (cycloserine cefoxitin fructose taurocholate) broth and incubated anaerobically for 72 h at 37 °C. A 10 μl aliquot of the enriched broth was spread on CCFT plates and incubated anaerobically at 37 °C for 48–72 h. One presumptive colony per plate was subcultured onto blood agar 5% Sheep Blood (Biorad, Temse, Belgium) and checked using a *C. difficile* latex agglutination rapid test Kit DR 1107A (Oxoid). Detection of a species-specific internal fragment of the *tpi* gene, toxin A and B genes, and CDT (*cdtA*) was performed according to the multiplex PCR protocol [18]. Sterile water and PCR-ribotype 027 strain were used as negative and positive controls respectively. Further toxin profile characterisation, deletions in the regulator gene *tcdC,* and *gyrA* mutation (a gene associated with moxifloxacin resistance) were determined using the Genotype Cdiff system (Hain Lifescience, Nehren, Germany) according to the manufacturer's instructions. The supernatant from each pure culture was tested for cytotoxicity assay (TcdB) using confluent monolayer MRC-5 cells, as previously described [18].

All strains were ribotyped as described by Bidet et al. [19]. Amplicon sizes were analysed by capillary electrophoresis and profiles obtained were compared with those of reference strains from the European collection (Cardiff International number, Brazier classification) and with our own database (nomenclature beginning with UCL).

2.4. Antibiotic resistance

Susceptibility of the isolates to metronidazole, moxifloxacin and tetracycline was determined by Etest strips (Lucron ELITech Group, Zottegem, Belgium) on Brucella Blood Agar with hemin and vitamin K1 (Becton-Dickinson Benelux NV, Erembodegem, BE) according to the manufacturer's instructions. Plates were anaerobically incubated at 37 °C for 48 h. The resistance (r) breakpoints for metronidazole (Met r $\geq$ 32 μg/ml), moxifloxacin (Mox r $\geq$ 8 μg/ml) and tetracycline (Tet r $\geq$ 8 μg/ml) were those recommended by the Clinical and Laboratory Standard Institute (CLSI) [20]. *Bacteroides fragilis* ATCL was included as a quality control.

2.5. Surveillance data in Belgium

During the same study period (from July to October 2014) analysis of *C. difficile* ribotype distribution was made at the St Luc University Hospital (Brussels, Belgium) in order to compare PCR-ribotypes with those obtained in Spain. The Belgian hospital is an academic acute care hospital with a total of 1000 beds and the National Reference Center for *C. difficile* in humans in Belgium. All stools received in the laboratory were tested for the presence of *C. difficile*. Multiple stool samples from the same patient were all tested. Initial screening was made using Cdiff QuickChek Complete® (TechLab). Culture of positive samples was performed on CHROMagar *C. difficile* Colorex™ (CHROMagar, BioTrading, Keerbergen, BE) in order to isolate the strain (without enrichment, planting faeces directly on agar). Plates were incubated anaerobically for 24 h at 35 °C. All cultures were read with a binocular stereomicroscope, with the light beam through the Petridish under a certain angle. Strains were ribotyped as described above. The toxin gene profile of the strains and PCR-ribotype distribution in the Belgian hospital were then compared with those found in the

Spanish ward.

3. Results

3.1. C. difficile detection and strain characterisation in HUCA, Spain

During the four-month study period, a total of 249 samples were screened for *C. difficile* presence using both the rapid enzyme test and culture analysis. Twelve additional samples were only examined by culture because they were classified outside of the range established (between 4 and 7) on the BSC. The overall prevalence of *C. difficile* in the faecal microbiota of patients studied was 12.3% (32/261). Of these, 69% were from adults aged more than 65 years old. Only following clinical suspicion, and a positive result for toxins A and/or B by rapid-test detection (Cdiff QuickChek Complete® or GenomEra CDX System *C. difficile*), a patient was considered to suffer from infection. With this approach, a total of 22 patients were diagnosed with CDI.

Altogether, 7 of the 32 *C. difficile*-positive samples detected (22%) were referred from the oncology unit. However, the medical services which sent the most samples for the screening of *C. difficile* during the study period were the acute care unit (28/261; 10.7%) and general medicine service (37/261; 14.2%) (Table 1). From these two services, *C. difficile* was isolated from six and five patients respectively. Regarding the type of faeces, six patients (2.3%) suspected of CDI presented bloody stools but all tested negative for the bacterium. Most of the positive patients had mushy, watery or liquid stools (n = 24). However, two patients with formed stools were also colonised with toxigenic *C. difficile* strains (Table 1).

Using rapid detection, 22 isolates tested positive for *tcdB* gene while 6 isolates were found to be non-toxigenic. Characterisation of colonies obtained after culture of samples showed 27 toxigenic isolates (presence of toxins A and B). Of these 27 toxigenic isolates, 6 had binary toxin genes. None of the isolates presented a single base deletion at position 117 in the regulator *tcdC* gene. Two isolates showed an 18 bp deletion and eight presented a 39 bp deletion in the regulator *tcdC* gene. Twenty different PCR-ribotypes were detected. Only nine isolates had a ribotype profile associated with a Cardiff collection reference number (002 (n = 3), 078 (n = 2), 012, 070, 023 and 020). The remaining isolates were associated with an internal nomenclature (UCL), with a total of 14 different PCR-ribotypes identified. The only non-toxigenic PCR-ribotype was associated with the ribotype UCL9. In addition, this ribotype was the only that presented three types of deletions in the regulator *tcdC* gene (117 bp, 39 bp and 18 bp deletions) (Table 2). The same results (the presence of *C. difficile* in the sample with the same PCR-ribotypes) were obtained with and without enrichment of faeces. None of the patients were identified as carriers of more than one PCR-ribotype.

None of the isolates showed resistance to metronidazole. For tetracycline, eight isolates were fully resistant: PCR-ribotype 078 (two isolates), PCR-ribotype UCL16b (one isolate), PCR-ribotype UCL5a (four isolates) and PCR-ribotype 36a (one isolate). Resistance for moxifloxacin was detected in all isolates of PCR-ribotypes 078, UCL5a and UCL9. All of these isolates (PCR-ribotype 078, UCL9 and UCL5) presented a mutation in *gyrA* gen (Table 2).

3.2. C. difficile detection and strain characterisation in St Luc University Hospital, Belgium

Between July 2014 and October 2014 a total of 880 stool specimens were analysed from patients of the St Luc University Hospital suspected of having CDI. The national prevalence for *C. difficile* reported from the Belgian Reference Center was 9.3%. A total of 127 *C. difficile*-positive samples were obtained from 87 patients. Seventeen of these positive patients (19.5%) were referred from the paediatric service (including eight from the paediatric haematology unit and four from the intensive neonatology unit). The other medical services with significant numbers of *C. difficile*-positive patients were general internal medicine (n = 9; 10.3%), consultation (n = 8; 9.2%), pneumatology-gastroenterology (n = 8; 9.2%), nephrology-neurology (n = 7; 8%), surgery (n = 5; 5.7%) and

Table 1
Clinical data comparison between *C. difficile*-colonised and non-colonised patients.

	C. difficile-negative patients (%)	*C. difficile*-positive patients (%)
Total (%)	229 of 261 (87.7)	32 of 261 (12.3)
Mean age in years	60.5	63,6
Sorted by age		
>65 years	137 (60)	22 (68.8)
20-<65 years	76 (33.2)	7 (22)
>10–20 years	7 (3.1)	3 (9.4)
>3, ≤10 years	4 (1.7)	0 (0)
≤3 years	5 (2.2)	0 (0)
Sorted by gender		
Male	125 (54.6)	22 (68.8)
Female	104 (45.4)	10 (31.3)
Sorted by service		
Oncology	4 (1.7)	7 (22)
Acute care unit	22 (9.6)	6 (18.8)
General medicine	32 (14)	5 (15.6)
Nephrology	14 (6.1)	2 (6.3)
Digestive	20 (8.7)	2 (6.3)
Haematology	5 (2.2)	2 (6.3)
General emergencies	11 (4.8)	2 (6.3)
Paediatric emergencies	6 (2.6)	1 (3.1)
Surgery	1 (0.4)	1 (3.1)
Urology	2 (0.9)	1 (3.1)
Other	112 (48.9)	3 (9.4)
Sorted by type of sample		
Bloody stools	6 (2.3)	0 (0)
Mushy, watery or entire liquid stools (Bristol Stool Chart 6–7)	163 (62.5)	24 (75)
Smooth and soft stools (Bristol Stool Chart 4–5)	82 (31.4)	6 (18.8)
Formed stools (Bristol Stool Chart 1–3)	10 (3.8)	2 (6.3)

Table 2
Detailed information on *C. difficile* isolates at the HUCA hospital (Spain), including molecular characterisation and antibiotic resistance of the isolates.

Isolate number	Rapid detection GDH	Rapid detection toxin B[c]	Rapid detection toxin B[d]	Culture detection	PCR-ribotype	CE	*tcdA tcdB*	*cdtA cdtB*	*tcdC*[a] MUT117	*tcdC*[a] *39bp*	*tcdC*[a] *18bp*	*gyrA*[b] MUT	Metronidazole	Moxifloxacin	Tetracycline
10404	+	−	+	+	UCL23b	+	+	−	−	−	−	−	−	−	−
10405	+	+	+	+	002	+	+	−	−	−	−	−	−	−	−
10406	−	−	NT	+	078	+	+	+	−	+	−	Mut1A	−	+	+
10407	+	+	−	+	UCL16b	+	+	−	−	−	−	−	−	−	+
10408	+	+	+	+	UCL16	+	+	−	−	−	−	−	−	−	−
10409	+	−	−	+	UCL9	−	−	−	+	+	+	Mut1A	−	+	−
10410	+	−	+	+	012	+	+	−	−	−	−	−	−	−	−
10411	+	+	+	+	078	+	+	−	−	+	−	Mut1A	−	+	+
10412	+	−	−	+	UCL16	+	+	−	−	−	−	−	−	−	−
10413	+	+	+	+	UCL16°	+	+	−	−	−	−	−	−	−	−
10414	+	+	+	+	UCL55a	+	+	−	−	−	−	−	−	−	−
10415	+	+	+	+	070	+	+	−	−	−	−	−	−	−	−
10419	+	+	+	+	014	+	+	−	−	−	−	−	−	−	−
10420	+	+	+	+	002	+	+	−	−	−	−	−	−	−	−
10421	+	−	+	+	UCL489	+	+	−	−	−	−	−	−	−	−
10422	+	+	+	+	UCL5a	+	+	+	−	+	−	Mut1A	−	+	+
10423	+	+	+	+	002	+	+	−	−	−	−	−	−	−	−
10454	+	+	+	+	023	+	+	+	−	+	+	−	−	−	−
10425	+	−	−	+	UCL5a	+	+	+	−	+	−	Mut1A	−	+	+
10426	+	−	+	+	UCL36a	+	+	−	−	−	−	−	−	−	+
10427	−	−	NT	+	UCL499	+	+	−	−	−	−	−	−	−	−
10428	+	+	+	+	UCL16i	+	+	−	−	−	−	−	−	−	−
10429	+	+	+	+	UCL5a	+	+	+	−	+	−	Mut1A	−	+	+
10430	+	+	+	+	UCL108	+	+	−	−	−	−	−	−	−	−
10431	−	−	NT	+	UCL5a	+	+	+	−	+	−	Mut1A	−	+	+
10432	+	−	+	+	020	+	+	−	−	−	−	−	−	−	−
10433	+	+	+	+	UCL483	+	+	−	−	−	−	−	−	−	−
10155	−	−	NT	+	UCL283	+	+	−	−	−	−	−	−	−	−
10559	+	−	+	−	−	−	−	−	−	−	−	−	−	−	−
10497	+	−	+	−	−	−	−	−	−	−	−	−	−	−	−
10584	+	−	−	−	−	−	−	−	−	−	−	−	−	−	−
10287	+	−	−	−	−	−	−	−	−	−	−	−	−	−	−

MUT: mutation; CE: cytotoxicity assay using confluent monolayer MRC-5 cells; NT: not tested.
+: Positive result.
-: Negative result.
[a] Presence of deletions in the regulator gene *tcdC* (118bp-39bp-17bp).
[b] Presence of mutation in the *gyrA* gene associated with moxifloxacin resistance.
[c] Cdiff QuickCheck Complete TechLab.
[d] GenomeEra CDX System *C. difficile*.

subacute geriatrics (n = 5; 5.7%). The oncology service referred three positive patients (3.4%). Twenty-one patients were *C. difficile*-positive in more than one sampling. The mean age of positive patients was 45 years old. However, 19 positive patients were children less than 10 years old, with a mean age of 1 year and 6 months in this group, and 9 of these patients were less than 1 year old. If the paediatric group is analysed separately, the mean age of positive adult patients was 60 years old (Table 3).

Eighty-three isolates (65%) were positive for toxigenic culture and toxins A and B. Forty-four isolates were identified as non-toxigenic (Table 3). Overall, 37 different PCR-ribotypes were detected. Eight of these had ribotype profiles associated with the Cardiff collection under reference numbers 015 (n = 1), 078 (n = 14), 106 (n = 8), 014 (n = 5), 020 (n = 9), 056 (n = 13), 012 (n = 6) and 002 (n = 4). The remaining isolates were associated with an internal nomenclature (UCL), with a total of 29 different PCR-ribotypes identified, including all the non-toxigenic isolates (PCR-ribotypes UCL 36, UCL 9, UCL 110, UCL 122, UCL 257, UCL 384, UCL 46d) (Table 3).

4. Discussion

C. difficile continues to be the most common cause of healthcare-associated infection in the developed world. A previous European *C. difficile* infection hospital-based survey has shown that the incidence of CDI and the distribution of causative PCR-ribotypes differed greatly between hospitals [21]. In Spain, the number of toxin-positive cases reported varied between 5.5% and 5.6% (2008) [22], 9% (2008) [21] and 6.0%–6.5% (2013) [22]. In this study the prevalence was higher than has been previously found in Spain. The number of *C. difficile*-positive specimens was 32 (12.3%), but in 1 of these a non-toxigenic strain was identified. In addition, two other positive cases detected only by rapid test were toxin-negative. Therefore, the final percentage of toxin-positive cases in the Spanish hospital was established as 11.1% (29/261). While in the other surveys [21,23] *C. difficile* was more commonly detected in females, in this study 68.8% of positive samples were from male patients.

All diarrhoeal non-duplicate specimens submitted to the diagnostic laboratory were tested, even if they were discarded from the routine *C. difficile* detection protocol due to their consistency (samples labelled outside levels 4 to 7 in the BSC). Two positive samples were detected in the analysis of these additional samples (n = 12); however, the overall prevalence was almost the same (12.3% (32/261); 12% (30/249)). In a recent study conducted in Australia, while the number of *C. difficile*-positive specimens increased with the analysis of all diarrhoeal specimens (including non-requested samples), the overall prevalence with the analysis of all samples was lower than that identified by routine testing [23]. In a further study conducted in Spain, the authors found that CDI remained a highly neglected disease because of the absence of clinical suspicion or the lack of sensitive diagnostic testing in some

Table 3
Detailed information on *C. difficile*-positive patients at the St Luc University Hospital (Belgium), including molecular characterisation of the *C. difficile* isolates.

Patient number	Age	Genre	Medical service	*C. difficile* isolation date	PCR-ribotype	CE	*tcdA tcdB*
01	4 years	Male	Paediatric haematology	01/07/2014	UCL36	−	−
				04/07/2014	UCL36	−	−
				08/07/2014	UCL36	−	−
				17/07/2014	UCL36	−	−
06	28 years	Male	Consultation	09/07/2014	UCL36	−	−
11	62 years	Male	Medical surgical intensive care	25/08/2014	UCL36	−	−
15	1 year	Male	Paediatric haematology	10/10/2014	UCL36	−	−
56	51 years	Female	Gastroenterology	25/08/2014	UCL36	−	−
59	81 years	Female	General internal medicine	02/10/2014	UCL36	−	−
				09/10/2014	UCL36	−	−
61	88 years	Female	Not specified	15/10/2014	UCL36	−	−
63	48 years	Female	Consultation	17/10/2014	UCL36	−	−
38	6 years	Male	Paediatric haematology	12/09/2014	UCL36a	+	+
82	35 years	Female	Urgent care	01/10/2014	UCL36a	+	+
02	4 years	Male	Paediatric haematology	01/07/2014	UCL9	−	−
03	62 years	Male	Cardiovascular surgery	07/07/2014	UCL9	−	−
04	64 years	Male	Nephrology neurology	07/07/2014	UCL9	−	−
07	20 days	Male	Intensive neonatology	28/07/2014	UCL9	−	−
08	5 months	Male	Outpatient emergency	04/08/2014	UCL9	−	−
12	2 years	Male	Paediatric haematology	26/08/2014	UCL9	−	−
				01/09/2014	UCL9	−	−
				22/09/2014	UCL9	−	−
13	10 days	Male	Intensive neonatology	16/09/2014	UCL9	−	−
14	2 months	Male	Intensive neonatology	22/09/2014	UCL9	−	−
19	2 years	Male	Not specified	23/10/2014	UCL9	−	−
16	60 years	Male	Outpatient dialysis	13/10/2014	UCL9	−	−
49	9 months	Female	Consultation	01/07/2014	UCL9	−	−
50	14 years	Female	Consultation	18/07/2014	UCL9	−	−
53	15 days	Female	Intensive neonatology	28/07/2014	UCL9	−	−
54	84 years	Female	Subacute geriatrics	04/08/2014	UCL9	−	−
58	8 years	Female	Paediatric haematology	22/09/2014	UCL9	−	−
60	8 months	Female	Paediatric	13/10/2014	UCL9	−	−
05	66 years	Male	Pneumatology gastroenterology	07/07/2014	UCL110	−	−
21	35 years	Male	Pneumatology gastroenterology	01/07/2014	UCL100b	+	+
				02/07/2014	UCL100b	+	+
62	25 years	Female	Abdominal surgery	17/10/2014	UCL122	−	−
09	79 years	Male	Subacute geriatrics	05/08/2014	UCL257	−	−
			Intensive Care Unit Neuro-traumatology	16/10/2014	UCL257	−	−
55	66 years	Female	Haematology	13/08/2014	UCL384	−	−
10	47 years	Male	Orthopaedics	19/08/2014	UCL46d	−	−
				25/08/2014	UCL46d	−	−
34	94 years	Male	Urgent care	01/09/2014	UCL48	+	+
20	76 years	Male	General internal medicine	29/10/2014	UCL122	−	−
22	40 years	Male	General internal medicine	14/07/2014	UCL 23f	+	+
23	46 years	Male	Urgent care	14/07/2014	UCL86	+	+
39	33 years	Male	Consultation	16/09/2014	UCL14	+	+
32	82 years	Male	Not specified	19/08/2014	UCL5a	+	+
86	68 years	Female	General internal medicine	29/10/2014	UCL26	+	+
72	78 years	Female	General internal medicine	18/08/2014	UCL16r	+	+
44	63 years	Male	Not specified	03/10/2014	UCL16u	+	+
30	81 years	Male	General internal medicine	18/08/2014	UCL16b	+	+
77	79 years	Female	Subacute geriatrics	08/09/2014	UCL16L	+	+
31	5 years	Male	Paediatric intensive care	18/08/2014	UCL16L	+	+
70	58 years	Female	Cardiovascular and thoracic surgery	13/08/2014	UCL16L	+	+
				29/08/2014	UCL16L	+	+
				01/09/2014	UCL16L	+	+
				01/09/2014	UCL16L	+	+
				07/10/2014	015	+	+
29	1 year	Male	Not specified	11/08/2014	UCL16°	+	+
				12/08/2014	UCL16°	+	+
				13/08/2014	UCL16°	+	+
				02/09/2014	UCL16°	+	+
				03/09/2014	UCL16°	+	+
				24/09/2014	UCL16°	+	+
				27/10/2014	UCL16°	+	+
57	17 years	Female	Paediatric haematology	30/08/2014	UCL266	−	−
68	36 years	Female	Maternal Intensive Care	23/07/2014	UCL381	+	+
52	77 years	Female	Nephrology neurology	22/07/2014	UCL468	−	−
			Nephrology neurology	04/08/2014	UCL468	−	−
			Subacute geriatrics	30/08/2014	106	+	+
51	34 years	Female	Stomatology neurology	22/07/2014	UCL471	−	−
28	11 years	Male	Paediatric haematology	11/08/2014	UCL475	+	+

(*continued on next page*)

Table 3 (*continued*)

Patient number	Age	Genre	Medical service	*C. difficile* isolation date	PCR-ribotype	CE	*tcdA tcdB*
45	50 years	Male	Nephrology neurology	06/10/2014	UCL477	+	+
17	78 years	Male	Subacute geriatrics	17/10/2014	UCL478	–	–
18	6 months	Male	Ambulatory emergency	17/10/2014	UCL479	–	–
27	55 years	Male	Pneumatology Gastroenterology	31/07/2014	UCL472	+	+
				07/08/2014	078	+	+
64	78 years	Female	Surgery, orthopaedics and traumatology	02/07/2014	078	+	+
			Subacute geriatrics	23/07/2014	078	+	+
71	64 years	Female	Haematology	18/08/2014	078	+	+
				28/08/2014	078	+	+
40	67 years	Male	Pneumatology Gastroenterology	18/09/2014	078	+	+
				18/09/2014	078	+	+
47	30 years	Male	Pneumatology Gastroenterology	17/10/2014	078	+	+
				17/10/2014	078	+	+
				20/10/2014	078	+	+
75	53 years	Female	Oncology	01/09/2014	078	+	+
			Medical surgical intensive care	29/09/2014	078	+	+
76	60 years	Female	Nephrology neurology	01/09/2014	078	+	+
78	57 years	Female	Nephrology neurology	14/10/2014	078	+	+
				23/10/2014	078	+	+
80	92 years	Female	General internal medicine	29/09/2014	078	+	+
24	67 years	Male	Nephrology neurology	17/07/2014	014	+	+
25	90 years	Male	Subacute geriatrics	30/07/2014	014	+	+
				12/08/2014	014	+	+
33	44 years	Male	Medical surgical intensive care	27/08/2014	014	+	+
81	54 years	Female	Gastroenterology	29/09/2014	014	+	+
41	29 years	Male	Oncology	24/09/2014	020	+	+
42	11 months	Male	Paediatric transplantation	25/09/2014	020	+	+
48	1 year	Male	Consultation	20/10/2014	020	+	+
65	2 years	Female	Paediatric transplantation	04/07/2014	020	+	+
73	1 year	Female	Consultation	26/08/2014	020	+	+
79	68 years	Female	General internal medicine	09/09/2014	020	+	+
				10/09/2014	020	+	+
				12/09/2004	020	+	+
87	7 months	Female	Paediatric transplantation	30/10/2014	020	+	+
35	82 years	Male	Pneumatology Gastroenterology	08/09/2014	056	+	+
83	84 years	Female	Not specified	06/10/2014	056	+	+
				14/10/2014	056	+	+
36	62 years	Male	Oncology	09/09/2014	106	+	+
				11/09/2014	106	+	+
				12/09/2014	106	+	+
37	71 years	Male	Urgency	10/09/2014	106	+	+
46	92 years	Male	Cardiology	07/10/2014	106	+	+
26	60 years	Male	Consultation	30/07/2014	106	+	+
84	64 years	Female	Nephrology neurology	20/10/2014	106	+	+
43	76 years	Male	Neurology	01/10/2014	012	+	+
				10/10/2014	012	+	+
				27/10/2014	012	+	+
67	85 years	Female	Pneumatology Gastroenterology	16/07/2014	012	+	+
85	9 months	Female	Consultation	24/10/2014	012	+	+
66	90 years	Female	Pneumatology Gastroenterology	09/07/2014	002	+	+
			Cardiology	05/08/2014	002	+	+
69	21 years	Female	General internal medicine	11/08/2014	002	+	+
74	56 years	Female	Gastroenterology	01/09/2014	002	+	+

CE: cytotoxicity assay.
+: Positive result.
-: Negative result.

institutions. They also observed that underdiagnosis most frequently affected younger patients and patients with community-acquired CDI [22]. In the present study, no positive patients were detected in the paediatric group (less than 10 years old). However, during the study period only 9 samples were received from this service. This data may reflect that in this Spanish hospital a specific request for the diagnosis of CDI from the clinician is less common in the paediatric service than in others. The mean age of all patients studied (62.5 years old) corroborates this observation. Recent reports warn that the incidence of CDI has increasingly risen among paediatric patients [24]. Collins et al. [23] reported in one survey conducted in Western Australia that undiagnosed CDI cases only occurred among paediatric patients, and 32.3% of all CDI cases were aged <20 years. A further study also conducted in Spain showed that the isolation of *C. difficile* was common in children hospitalised for diarrhoea, especially in patients younger than 2 years old with chronic disease. Furthermore, in the same study the authors reported that the clinical picture observed in children with CDI was characterised by mild symptoms and low clinical severity [25], which may contribute to underdiagnosis in this population. In a previous survey that assessed risk factors and outcomes in children with *C. difficile*-associated diarrhoea, only 12.5% of positive samples were identified as bloody stools while 79% of positive samples were watery stools [26]. In a further study conducted in Calcutta to investigate the major clinical features of *C. difficile*-induced diarrhoea, only 17.6% of *C. difficile* cases reported bloody stools compared to 84.2% reporting watery diarrhoea [27]. These reports suggest that bloody stools are not the most common samples

associated with CDI. Concurrent with these findings, in this study it was observed that all bloody stools tested negative for the bacterium.

In the study of Alcala et al. [22], the second cause proposed for undiagnosed or misdiagnosed CDI was the lack of sensitive diagnostic tests in some institutions. In the present study, four specimens were identified as negative for *C. difficile* and its toxins using rapid tests, but were found to be positive for toxigenic *C. difficile* strains following the culture of samples. In three additional samples toxins were not detected by rapid test but culture, isolation and characterisation of the isolates revealed the presence of at least one of the two toxins A and B. Enzyme immunoassay detection of GDH as initial screening for *C. difficile* presence has been suggested as a potential strategy. However results appear to differ based on the GDH kit used and therefore this approach remains an interim recommendation [2]. While the GenomEra *C. difficile* assay has been described to be an excellent option for toxigenic *C. difficile* detection in faecal specimens [28], in the present study toxigenic *C. difficile* strains were isolated by culture from three samples that were found negative for the toxin using this method. However, the results obtained confirm that GenomEra *C. difficile* assay is more sensitive than EIA testing for *C. difficile* toxin B, as described previously [2]. In contrast, four samples were only positive for *C. difficile* by rapid tests. Two of these positive samples were toxin negative by EIA and Genome *C. difficile* assay. Ethanol shock was not used in the course of this study, nor was alcohol selection of microorganisms conducted, and cultured colonies were observed in high numbers. Therefore, the high contamination of samples by other bacteria species may explain the failure to isolate these four strains by classical culture. For the two non-toxigenic samples identified by rapid screening, false-positive GDH test results are also plausible [29]. The use of the enrichment step in this study was shown to be not useful in the clinical samples tested, as all of the samples that tested positive after 3 enrichment days were already positive by direct culture and the same PCR-ribotypes were isolated.

The surveillance data in Belgium reported a lower prevalence than in Spain (9.3%). It should be noted that the Spanish and Belgian results must be compared with caution. In the case of the Belgian laboratory, all diarrhoeal faecal specimens were analysed, including duplicate samples from the same patient. In the Spanish hospital only non-duplicate specimens were analysed. Nevertheless, despite this important difference in the routine protocol among laboratories, the prevalence of *C. difficile* is likely to be genuinely lower in Belgium than in Spain. While incidence varies considerably between hospitals and regions, an increase in the proportion of community-associated cases and a decrease in the proportion of hospital-acquired cases of CDI between the years 2008–2014 [30] have been reported in Belgium. This data may reflect the efforts of Belgian hospitals to improve the management and prevention of CDI.

Another important difference found is the mean age of positive patients. In Spain, the mean age found in *C. difficile*-positive cases was 63.6 years old, which correlates with other surveys conducted in the country [22]. In contrast, in Belgium the mean age of adult positive patients was 45 years old. However, if the paediatric group is evaluated separately, the mean age in Belgium is 60 years old. A significant number of positive samples in the Belgian survey were from children less than ten years old (n = 19). However, only eight of the positive patients harboured toxigenic *C. difficile* strains. It has been described that during early infancy the gut microbiota complexity is poor and asymptomatic colonisation by *C. difficile* is common [31,32]. However, all the paediatric patient samples analysed in this study were diarrhoeal. These findings corroborate with previous suggestions [23] that the surveillance and the significance of *C. difficile* in paediatric groups requires further investigation. Toxigenic *C. difficile* carriage in infants could be a cause of disease, not only in paediatric populations but also in adults through close contact with.

In both Spain and Belgium, *C. difficile*-positive patients referred from oncology services all carried toxigenic strains. In a previous study, a great diversity of *C. difficile* strains associated with CDI was detected among paediatric oncology patients [33]. A further study found a probable association between certain types of tumours, the use of antibiotics and CDI incidence. The authors also emphasised the urgent need for early recognition and diagnosis of CDI in adult cancer patients [34]. PCR-ribotypes 078, 014, 012, 020, 002, UCL36a, UCL5a, UCL16b and UCL9 were isolated in both hospitals. In previous surveys in hospitals in Spain, PCR-ribotypes 078/126, 014 and 001 were the most prevalent [22]. As in previous years, PCR-ribotype 014 remains the most common in Belgium, increasing in proportion to other ribotypes and in the number of hospital sites affected since 2014. The other PCR-ribotypes more commonly detected in Belgian hospitals in 2014 were 020 and 078 [30]. In the Spanish hospital studied, there were no commonly-encountered PCR-ribotypes, suggesting there is neither regional infection nor contamination in the hospital. On the contrary, a great variety of toxigenic PCR-ribotypes was identified. Consistent with the European survey which reported that PCR-ribotype 027 was less prevalent than others [21], this ribotype was not detected either in Spain or Belgium during the present study period.

In conclusion, the data obtained shows that even with three times the number of samples analysed per month, the prevalence of *C. difficile* is lower in the Belgian hospital than the Spanish one. This data may reflect the efforts of the Belgian hospital to improve the management and prevalence of CDI, and, as previously reported, misdiagnosis or underdiagnosis of CDI in Spain due to a lack of clinical suspicion. The most common PCR-ribotypes reported in Europe were found in both hospitals. The great variety of PCR-ribotypes detected suggests there is neither regional infection nor contamination within the hospital. This study finds that the presence of *C. difficile* in paediatric and oncology services requires further investigation.

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Declaration of interest

None.

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References

[1] J.S. Brazier, Clostridium difficile: from obscurity to superbug, Br. J. Biomed. Sci. 65 (1) (2008) 39–44.
[2] H. Stuart, et al., Clinical practice guidelines for Clostridium difficile infection in adults: 2010 update by the society for healthcare epidemiology of America (SHEA) and the Infectious Diseases Society of America (IDSA), Infect. Control Hosp. Epidemiol. 31 (5) (2010) 431–455.
[3] M.M. Squire, T.V. Riley, One Health: the Human-animal-environment Interfaces in Emerging Infectious Diseases, vol. 365, Springer Berlin Heidelberg, Berlin, Heidelberg, 2013.

[4] N.L. Crogan, B.C. Evans, Clostridium difficile: an emerging epidemic in nursing homes, Geriatr. Nurs. (New York, NY) 28 (3) (2007) 161–164.
[5] M. Arvand, et al., High prevalence of Clostridium difficile colonization among nursing home residents in Hesse, Germany, PloS One 7 (1) (2012) e30183.
[6] N. Nanwa, et al., The economic impact of Clostridium difficile infection: a systematic review, Am. J. Gastroenterol. 110 (4) (2015) 511–519.
[7] A.M. Jones, E.J. Kuijper, M.H. Wilcox, Clostridium difficile: a European perspective, J. Infect. 66 (2013) 115–128.
[8] P. Tattevin, et al., Clostridium difficile infections: do we know the real dimensions of the problem? Int. J. Antimicrob. Agents 42 (Suppl. S36–40) (2013).
[9] C.T. Evans, N. Safdar, Current trends in the epidemiology and outcomes of Clostridium difficile infection, Clin. Infect. Dis. Off. Publ. Infect. Dis. Soc. Am. 60 (Suppl. 2) (2015) S66–S71.
[10] L.C. McDonald, et al., An epidemic, toxin gene-variant strain of Clostridium difficile, N. Engl. J. Med. 353 (23) (2005) 2433–2441.
[11] J. Freeman, et al., The changing epidemiology of Clostridium difficile infections, Clin. Microbiol. Rev. 23 (3) (2010) 529–549.
[12] E.J. Kuijper, et al., Update of Clostridium difficile infection due to PCR ribotype 027 in Europe, 2008. Euro Surveillance, Eur. Commun. Dis. Bull. 13 (31) (2008).
[13] G. Van Esch, et al., Surveillance of Clostridium difficile infections in a long-term care psychogeriatric facility: outbreak analysis and policy improvement, Arch. Public Health 73 (1) (2015) 18.
[14] N. Viseur, et al., Nosocomial and non-nosocomial Clostridium difficile infections in hospitalised patients in Belgium: compulsory surveillance data from 2008 to 2010, Euro Surveill. Eur. Commun. Dis. Bull. 16 (43) (2011).
[15] D. Rodríguez-Pardo, et al., Epidemiology of Clostridium difficile infection and risk factors for unfavorable clinical outcomes: results of a hospital-based study in Barcelona, Spain, J. Clin. Microbiol. 51 (5) (2013) 1465–1473.
[16] E. Reigadas, et al., Role of binary toxin in the outcome of Clostridium difficile infection in a non-027 ribotype setting, Epidemiol. Infect. (2015) 1–6.
[17] I. Weber, et al., Molecular epidemiology and resistance profiles of Clostridium difficile in a tertiary care hospital in Spain, Int. J. Med. Microbiol. 303 (3) (2013) 128–133.
[18] C. Rodriguez, et al., Clostridium difficile in young farm animals and slaughter animals in Belgium, Anaerobe 18 (6) (2012) 621–625.
[19] P. Bidet, et al., Development of a new PCR-ribotyping method for Clostridium difficile based on ribosomal RNA gene sequencing, FEMS Microbiol. Lett. 175 (2) (1999) 261–266.
[20] Standards – CLSI: CLSI. Available at: http://clsi.org/standards/. (accessed 04.03.15).
[21] M.P. Bauer, et al., Clostridium difficile infection in Europe: a hospital-based survey, Lancet London Engl. 377 (9759) (2011) 63–73.
[22] L. Alcalá, et al., Impact of clinical awareness and diagnostic tests on the underdiagnosis of Clostridium difficile infection, Eur. J. Clin. Microbiol. Infect. Dis. Off. Publ. Eur. Soc. Clin. Microbiol. 34 (8) (2015) 1515–1525.
[23] D.A. Collins, T.V. Riley, Routine detection of Clostridium difficile in Western Australia, Anaerobe 37 (2015) 34–37.
[24] J.S. Sammons, P. Toltzis, Pitfalls in diagnosis of pediatric Clostridium difficile infection, Infect. Dis. Clin. N. Am. 29 (3) (2015) 465–476.
[25] B. Santiago, et al., Clostridium difficile isolation in children hospitalized with diarrhea. Anales de Pediatría (Barcelona, Spain: 2003) 82 (6) (2015) 417–425.
[26] V. Morinville, J. McDonald, Clostridium difficile-associated diarrhea in 200 Canadian children, Can. J. Gastroenterol. 19 (8) (2005) 497–501.
[27] M.K. Bhattacharya, et al., Clinical manifestation of Clostridium difficile enteritis in Calcutta, J. Assoc. Phys. India 39 (9) (1991) 683–684.
[28] J.J. Hirvonen, S. Mentula, S.S. Kaukoranta, Evaluation of a new automated homogeneous PCR assay, GenomEra C. difficile, for rapid detection of Toxigenic Clostridium difficile in fecal specimens, J. Clin. Microbiol. 51 (9) (2013) 2908–2912.
[29] S.D. Goldenberg, P.R. Cliff, G.L. French, Glutamate dehydrogenase for laboratory diagnosis of Clostridium difficile infection, J. Clin. Microbiol. 48 (8) (2010) 3050–3051.
[30] F. Neely and M.L. Lambert. Epidemiology of Clostridium difficile infection in Belgium Report 2015. Available at: http://www.nsih.be/download/CDIF/CDIF-AR-2015-EN.pdf. (accessed 27.11.15).
[31] C. Rousseau, et al., Clostridium difficile colonization in early infancy is accompanied by changes in intestinal microbiota composition, J. Clin. Microbiol. 49 (3) (2011) 858–865.
[32] C. Rodriguez, et al., Clostridium difficile infection and intestinal microbiota interactions, Microb. Pathog. 89 (2015) 201–209.
[33] R. Dantes, et al., Investigation of a cluster of Clostridium difficile infections in a pediatric oncology setting, Am. J. Infect. Control 44 (2) (2016) 138–145.
[34] A. Rodríguez Garzotto, et al., Risk factors associated with Clostridium difficile infection in adult oncology patients. Supportive Care in Cancer, Support Care Cancer 23 (6) (2015) 1569–1577.