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Incidence and characterization of *Clostridium difficile* in a secondary care hospital in Spain

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ABSTRACT

Introduction: *Clostridium difficile (C. difficile)* is a major nosocomial infectious agent in hospitals. Previous studies have addressed the high proportion of infection episodes that are overlooked in health care facilities.

Objective: the main aim of this study was to characterize *C. difficile* clinical cases that occurred in a secondary care hospital during a five-month period.

Material and methods: for this purpose, a total of 137 stool samples from the same number of patients with diarrhea were analyzed for the presence of *C. difficile* by culture techniques. An enzyme immunoassay (EIA) test for the detection of *C. difficile* and its toxins was also used in 50 cases (36.5%) for diagnostic purposes.

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Results: a total of 14 (10.2%) *C. difficile* isolates were obtained, of which nine (64.3%) were toxigenic. A mean incidence of 3.2 episodes of *C. difficile* infections (CDI) per 10,000 patients-days was estimated for the study period. Around 56% of the CDI cases were determined as hospital-acquired, whereas 44% originated in the community. Among these, only two episodes (22.2%) were detected in the hospital by the EIA test, which indicated that the hospital CDI detection protocol needed to be revised. One unusual *C. difficile* isolate was negative for all toxin genes examined and also for the non-toxigenic strain assay, which highlights the need to perform genome sequencing to study its pathogenicity locus insertion site organization. A stable metronidazole-resistant *C. difficile* strain and three strains showing multidrug resistance were detected in this study, suggesting that *C. difficile* antimicrobial susceptibility surveillance programs should be established in this health-care facility.

Key words: *Clostridium difficile.* Humans. Metronidazole. PCR-ribotyping. Immunoassay.

INTRODUCTION

Clostridium difficile (C. difficile) is an anaerobic gram-positive bacillus and the principal causative agent of nosocomial diarrhea in developed countries. This organism also causes 10-25% of antibiotic associated diarrhea (1). The main predisposing factors for *C. difficile* infections (CDI) include advanced age, a stay at a health care facility and the use of broad-spectrum antibiotics (2). Although metronidazole and vancomycin are the first choice antibiotics for CDI treatment, *C. difficile* strains increasingly show a low susceptibility or resistance to these antibiotics, especially metronidazole (3). The epidemiology of *C. difficile* has changed and now is considered as one of the main nosocomial infectious agents. *Clostridium difficile* is also increasingly reported as a cause of community-acquired infections (CA-CDI) and some common ribotypes (RT) (e.g., RT078) have been isolated from animals and the environment, suggesting an inter-species transmission (4).

The Center for Disease Control and Prevention (CDC) included *C. difficile* within the immediate urgent threat to public health category due to its unique relationship with antibiotic use and resistance (5). In Europe, *C. difficile* accounts for 172,000 cases/year with an attributable mortality of 9% (6). Besides, Spain seems to be one of the many European countries that have experienced a significant growth of CDI (7). In 2014, the first autochthonous non-severe CDI produced by ribotype 027 was reported in this country (8). Since then, the incidence of this type has increased in Spain and has produced several outbreaks in different hospitals (9).

Previous studies have addressed the high proportion of CDI episodes that are undiagnosed or misdiagnosed in health care facilities (2). This common situation occurs as a consequence of non-optimal laboratory procedures for the diagnosis of *C. difficile* clinical cases or the lack of clinical suspicion, mainly in young people or non-hospitalized patients (10). Therefore, the implementation of surveillance programs that are able to detect and control endemic and epidemic *C. difficile* strains and to assess their antimicrobial susceptibilities is of utmost importance.

The main aim of this study was to detect and characterize the CDI clinical episodes occurring in a secondary hospital during a five-month period. Thus, CDI incidence was estimated and antibiotic susceptibility, toxin genotype and molecular characterization (PCR-ribotyping, toxinotyping and multi-locus sequence typing or MLST) of *C. difficile* isolates were studied. In addition, the number of CDI cases overlooked was estimated via an analysis of all unformed stools, regardless of the request of the charge practitioner and the origin of the samples.

PATIENTS AND METHODS

Study design

A five-month prospective study of *C. difficile* infection was performed between August and December 2013 at the Hospital Royo Villanova (HRV, secondary care hospital, Zaragoza, Spain). The study was performed with the permission and also under the supervision of the Ethics Committee of Clinical Research of Aragón (CEICA, CP05/2014). The HRV has 266 beds (ten in the Intensive Care Unit [ICU]) and serves an area of approximately 200,000 inhabitants. The presence of *C. difficile* was assessed in all diarrheic samples (inpatients and outpatients) submitted to the Microbiology Service for toxigenic culture, regardless of the request of the charge physician. Samples from children younger than two years were excluded from the study due to the uncertain role of *C. difficile* toxins in this age group (10). The CDI diagnostic protocol established in the hospital was followed at the time of the sampling. Furthermore, diarrheic feces studied by microbiological culture from patients under antibiotic treatment and/or hospitalized were also tested using a membrane enzyme immunoassay (EIA), following the manufacturer's instructions. This test is designed for the simultaneous detection of *C. difficile* glutamate dehydrogenase antigen (GDH) and toxins A and B (C. DIFF QUIK CHEK COMPLETE®, Techlab, Orlando, FL, USA) (2).

A CDI case was defined as a case of diarrhea or toxic megacolon and a positive laboratory assay for *C. difficile* toxins in stools or a toxin-producing *C. difficile* isolate (detected by culture or other

means) (11). In addition, information about the origin of the case (HC-CDI: healthcare-associated; CA-CDI: community-associated; and UA-CDI: unknown association) (11), gender, age, antibiotic treatment, prior diseases within three months before the start of diarrhea (12) and previous CDI episodes were collected from each patient from medical records.

The presence/absence of disbiosis was also recorded for each patient when available. In this study, the presence of disbiosis was defined as the absence of the growth of enterobacteria on routine microbiological media used for feces culture, overgrowth of Gram-positive microorganisms and/or yeast and the absence of lactose positive bacteria. The media used for this purpose were blood agar, *Salmonella Shigella* agar, Hektoen agar and Selenite broth (data not shown).

Bacterial isolation and molecular characterization

Isolation of *C. difficile* from stool samples, molecular characterization of the strains obtained (i.e., *tpi* housekeeping and toxin genes detection by PCR), the identification of non-toxigenic strains and PCR-ribotyping were performed. In addition, the minimum inhibitory concentration (MIC) to vancomycin (VA; range: 0.016-256 µg/ml), metronidazole (MZ; range: 0.016-256 µg/ml), moxifloxacin (MX; range: 0.02-32 µg/ml), erythromycin (ER; range: 0.016-256 µg/ml), clindamycin (CM; range: 0.016-256 µg/ml) and tetracycline (TC; range: 0.016-256 µg/ml) was also performed, as previously described (13). The breakpoints for clindamycin, moxifloxacin and metronidazole resistance were those established by the Clinical and Laboratory Standards Institute (CLSI) (14) for anaerobic bacteria. The breakpoint for tetracycline was ≥ 8 µg/ml (15). The remaining breakpoints were based on the literature (16). A PCR-RFLP (restriction fragment length polymorphism) based toxinotyping protocol was performed for all the toxigenic strains with the purpose of analyzing the variability of A and B toxin genes (A3 and B1 fragments, respectively) (17). Furthermore, strains with unexpected results, i.e. negative PCR results for *tcdA* and *tcdB* genes and also non-toxigenic assay, were further studied by toxinotyping (A1, A2, A3, B1, B2 and B3 fragments) in order to detect the possible presence of toxin gene fragments.

The population structure (phylogeny) of the *C. difficile* strains obtained in this study was studied by multi-locus sequence typing (MLST) (18). The allele and sequence type (ST) designation was determined from the *C. difficile* PubMLST website (http://pubmlst.org/cdifficile/). A maximum likelihood tree with 1,000 bootstrap replicates was constructed based on the Kimura 2-parameter model (19). A total of 37 strains were used for this purpose: 14 from this study (Hu isolates) (Table

1), four isolates from other studies from our research group (RC10, 5754, RF17 and E6) (20,21) and 19 from the PubMLST database, in order to provide a context for the *C. difficile* population (ST1, ST3, ST5, ST11, ST32, ST37-39, ST41, ST67, ST96, ST122, ST177-181, ST200 and ST206). The percentage of trees in which the associated taxa clustered together is shown next to the branches. Initial tree(s) for the heuristic search were obtained by applying the neighbor-joining method to a matrix of pair-wise distances estimated using the maximum composite likelihood approach. Evolutionary analyses were conducted in MEGA7 (22).

The stability of metronidazole-resistant strains for those isolates that showed a breakpoint ≥ 32 µg/ml was assessed by seven serial passages over 14 days on *Brucella* blood agar plates, without antibiotics (23). Subsequently, the MIC to metronidazole was tested again via an Etest as described above. Resistance was considered as stable when the MIC of metronidazole against *C. difficile* remained (within ± 1 dilution) after the passages. Resistance was considered as unstable when resistant strains became susceptible (< 32 µg/ml) after the passages.

Data analysis

Incidence was calculated as the number of CDI episodes diagnosed at the hospital per 10,000 patients-days (including in- and outpatients) during the study period (24). Major patient characteristics including age, gender, the origin of patients (ward facility), the use of any antibiotic or comorbidity (previous three months), suspicion of CDI and disease characteristics were determined. As the age variable (continuous variable) did not follow a normal distribution, it was categorized rather than performing a mathematical transformation. This aided in the interpretation of the results obtained and the categories were based on biological criteria as follows: 15 years old was the limit of childhood, 16 to 64 years was defined as adulthood and the third category was defined as 65 years and over as this is the age limit set for the CDI risk population. The association between patient characteristics (Table 2) and infection was assessed using the Pearson's Chi-square test or Fisher's exact test for categorical variables. The MedCalc Statistical Software version 18.2.1 (MedCalc Software bvba, Ostend, Belgium) was used to perform the statistical analysis. A 2-sided *p*-value of < 0.05 was set as statistically significant.

RESULTS

Bacterial isolation and molecular characterization

A total of 137 samples from the same number of patients with diarrhea were included and analyzed for the presence of *C. difficile* by toxigenic microbiological culture over a five-month period. An EIA test was also performed in 50 cases (36.5%) in the hospital for diagnostic purposes. A total of 14 (10.2%) *C. difficile* isolates were obtained; nine (64.3%) were toxigenic (Table 1). A mean incidence of 3.2 cases/10,000 patient-days was determined in the hospital during the study period; 55.6% (5/9) of cases were HA-CDI and 44.4% (4/9) were CA-CDI.

All toxigenic strains (n = 9) harbored the *tcdA* and *tcdB* genes. However, one of them had a partial deletion in the *tcdA* gene (amplicon size 700 bp; Hu70 strain). Only one (11.1%) isolate yielded a positive result for the CDT genes *cdtA*/*cdtB*. Non-toxigenic strains (35.7%) yielded a positive result for the *cdu1*/*cdd1* PCR, except for one case which was negative for this test (Hu25 strain). This isolate was also negative to the toxinotyping scheme (A1, A2, A3, B1, B2 and B3 fragments) and was classified into ST311 (MLST), which has not been assigned to a clade yet (http://pubmlst.org/cdifficile/) (Fig. 1). A total of 13 and three different PCR-ribotypes and toxinotypes were identified, respectively. Results are summarized in table 1. Twelve different sequence types belonging to at least three distinct clades were detected. The phylogenetic tree using MEGA7 was drawn to scale, with branch lengths measured as the number of substitutions per site. The phylogenetic analysis involved 37 nucleotide sequences: 14 belonging to this study, four from previous studies of our research group and 19 from the database (Fig. 1).

Description of patients

The population studied was mainly formed by female patients (61.3%) and most were older than 16 years of age (97.8%) (median age: 64 years old; range: 5-101) and originated from the Emergency Service (51.8%). In addition to *C. difficile*, other enteric pathogens were detected (HRV, Clinical Microbiology Service) as follows: *Salmonella enterica* (8.8%), *Aeromonas* spp. (5.1%), rotavirus (1.5%) and others (2.2%; *Hafnia alvei*, *Giardia lamblia* and *Cryptosporidium* spp.). *C. difficile* was detected in two patients who also presented other enteric microorganisms (*Salmonella enterica* and rotavirus). Considering the nine toxigenic isolates detected, five strains (55.6%) were obtained from patients located in the Emergency Department, three (33.3%) in Internal Medicine and one (11.1%) in Gastroenterology. No relationship was observed between the presence of *C. difficile* and the factors considered. All results are summarized in table 2.

None of the toxigenic *C. difficile* positive patients suffered a CDI episode during the previous eight weeks. Five of nine (55.6%) patients suffering from CDI were under antibiotic treatment at the

time of sampling or during the previous three months; the penicillin-β-lactamase inhibitor combination was the most frequently used class (Table 3). Only two (22.2%) patients did not suffer from any other underlying disease within the three months before the onset of diarrhea. The remaining patients (n = 7) suffered cardiovascular comorbidities (57.1%), gastrointestinal disease (i.e., ulcerative colitis) (42.8%), nephro-urologic pathologies (28.6%), obesity (14.3%) and/or respiratory disorders (14.3%). None of the patients had diarrhea for more than three weeks and feces were not mixed with blood or other pathological products. Four of nine patients had fever (> 38 °C) and abdominal pain. Two patients presented with vomiting and leucocytosis (≥ 15,000/µl) was detected in only one patient. Serum creatinine was elevated > 50% from the baseline before the onset of symptoms in one episode and two CDI cases were also associated with colitis (a clinically diagnosed based on the presence of diarrhea).

Nine CDI episodes were detected by toxigenic culture in this study and only four (44.4%) were diagnosed by the EIA test in the hospital via a request from the charge physician. Only one (25%) of these cases was positive for the GDH antigen and *C. difficile* toxins (A and B). Of the remaining episodes (5/9) detected by toxigenic culture, only one (20%) was diagnosed by EIA under the initiative of the Microbiology Service of the hospital, which also yielded a positive result for antigen and toxins. With regard to the cases in which the EIA test was not performed at the hospital, patients were < 65 years old, outpatients and/or were not treated with antimicrobials at the time they attended the hospital. Four of nine patients infected with toxigenic *C. difficile* detected in this study were hospitalized in the same room but none of the strains isolated belonged to the same ribotype or ST.

Antimicrobial susceptibility testing

Clostridium difficile resistance to CM, ER, MX and TC was variable, 35.7% (5/14), 28.6% (4/14), 21.4% (3/14), and 21.4% (3/14), respectively. All isolates were fully susceptible to VA (≤ 1.5 µg/ml) and MZ (≤ 0.75 µg/ml), except for one strain which was resistant to MZ (MIC 32 µg/ml after 24 hours). Slow growing MZ-hetero-resistant subpopulations were not detected. After performing seven serial passages to the MZ-resistant strain, the MIC remained at 32 µg/ml after 48 hours and 14 days, showing a stable phenotype. Furthermore, three (21.4%) isolates showed multidrug resistance (MDR) (Table 1), including the MZ-resistant strain.

DISCUSSION

A mean CDI incidence of 3.2 cases/10,000 patient-days was found in this study, which is in close agreement with the previously published data in Europe (2.45 [1], 4.1 [25] and 3.2 [26]). Among the CDI episodes detected by toxigenic culture (n = 9), only five (55.5%) were considered for study by EIA at the hospital. From these, only two (40%) yielded a positive result for the GDH antigen and toxins (2/9 in total, 22.2%). These results are in agreement with previous studies that observed a high proportion of undiagnosed or misdiagnosed CDI episodes in health care facilities. Diagnostic failures may originate due to the implementation of non-optimal laboratory procedures for CDI diagnostic (three out of five in this study) and/or the lack of clinical suspicion due to factors such as a younger age than 65 years (3/9 patients in this study), no previous/current antibiotic treatment (3/9 patients) and/or non-hospitalized patients (4/9 patients) (2,10). Thus, as previously proposed, all unformed feces should be tested for *C. difficile,* regardless of their origin (10), and CDI diagnostics should not be based exclusively on antigen detection methods (27). Other diagnostic tools that are more sensitive than EIAs (e.g., qPCR) could be used for diagnostic purposes (28).

Multilocus sequence typing is considered to be an appropriate method to study *C. difficile* phylogeny and PCR-ribotyping is useful for emergent and epidemic genotypes surveillance (29,30). In this study, PCR-ribotyping and MLST results showed a high molecular diversity among the strains obtained and only RT020 was found in more than one patient (Table 1). Both RT020 strains showed the same ST by MLST but they were not closely related in time (nine days) or space (different rooms and medical services). These results indicated that there was no clonal population established among diarrheic patients during the study period. Moreover, it could reflect the different *C. difficile* types that were in the study area during the sampling period. The isolation of four genetically different *C. difficile* strains (from the same number of patients) in the same hospital room at different times, might reflect previous observations that the source of the infection is diverse and does not necessarily originate from another symptomatic patient (31). These findings suggest the existence of other contamination sources such as asymptomatic people in the hospital or other environmental reservoirs (31).

The phylogenetic study by MLST revealed that most of the *C. difficile* isolates obtained in this study belonged to clade 1 (Table 1 and Fig. 1). However, there was one isolate whose clade is still unclear; Hu25 (RT029, ST311) (Table 1 and Fig. 1). We included previously published STs in order to provide a context for the *C. difficile* population obtained and the Hu25 isolate was shown to have a possible common ancestor with ST200. This strain represents a new toxinotype (XXXII) and

produces only toxin B (32) (http://pubmlst.org/cdifficile/). However, Hu25 was negative for all the toxin genes tested (*tcdA*, *tcdB*, *cdtA* and *cdtB*). The clade in which ST200 is located is still unclear but it has been proposed as a member of clade 6, based on the comparison of its core genome by whole genome sequencing (WGS) (33). On the other hand, ST122 has been proposed to belong to clades 6 and 1 based on MLST and WGS studies, respectively (34,35). The phylogenetic analysis based on the MLST scheme described by Griffiths et al. (2010) revealed that ST122 was closely related to clades 1 and 2 and distant from ST200 and ST311 (Fig. 1). *C. difficile* Hu25 was also negative for non-toxigenic strains by PCR. The existence of strains which harbor an atypical pathogenicity locus (PaLoc) genetic organization has been previously reported and does not present the traditional non-coding region which replaces it when it is absent (36). Thus, it seems necessary to perform further WGS analysis in order to elucidate all these aspects for the Hu25 strain.

Common ribotypes have been isolated from humans and other animal species in previous studies (4) such as those found in this study (Table 1). An example is RT078, which has been widely associated with non-human animal species (37), supporting the hypothesis of an interspecies transmission of *C. difficile*. Non-toxigenic RT010 has been frequently associated with dogs and metronidazole resistance (38-42). A non-toxigenic RT010 strain was detected in this study, which showed stable metronidazole resistance and MDR (MIC 32 µg/ml) (Table 1), thus highlighting the importance of non-toxigenic strains as antimicrobial resistance determinant reservoirs. *C. difficile* strains are increasingly reported to be resistant to metronidazole (3) and thus a wise use of this drug is of the utmost importance.

The *ermB* genes are frequently associated with clindamycin and erythromycin resistance and mobile genetic elements (43). Besides, the wide use of fluoroquinolones in human medicine is likely implicated in the widespread emergence of drug resistance to this class of antimicrobials (44). In this study, three isolates were MDR, including CM and ER resistance, and two were also resistant to MX. All these results emphasizes the importance of the implementation of *C. difficile* antimicrobial susceptibility surveillance programs in health-care facilities. However, these results and those previously discussed should be interpreted cautiously, as this study has some limitations, such as the restricted geographical distribution of the patients included and the relatively low number of samples and *C. difficile* isolates considered.

In conclusion, the CDI incidence found in this study showed that there was no ongoing *C. difficile* outbreak in the HRV nor a clonal population established among diarrheic patients during the study

period. There was a high proportion of CDI episodes that were undiagnosed or misdiagnosed due to non-optimal laboratory procedures for the CDI diagnosis and/or a lack of clinical suspicion by practitioners. Thus, all unformed feces should be tested for *C. difficile,* regardless of their origin. Furthermore, CDI diagnoses should not be based exclusively on antigen detection methods. The sources of *C. difficile* infections remain elusive in many cases and further epidemiological studies are warranted to obtain more information about this aspect and to implement more efficacious preventive measures. Several ribotypes detected in this study have been previously isolated from humans and other animal species, supporting the hypothesis of an interspecies transmission of *C. difficile*. The antimicrobial susceptibility results obtained in this study emphasized the need to perform routine antimicrobial susceptibility testing for all strains of *C. difficile* in health-care facilities. Besides, genome sequencing analyses are needed to study the PaLoc genetic organization of the unusual *C. difficile* isolate obtained.

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Table 1. Molecular characterization of *Clostridium difficile* isolates

ID	Toxin genes	NTS	Toxinotype	Ribotype	MLST	
					ST	Clade
Hu25	-	-	-	029	311	Unknown
Hu36	*tcdA*, *tcdB*, *cdtA*, *cdtB*		V	078	11	5
Hu70‡	*tcdA**, *tcdB*		VIII	017	37	4
Hu72‡	*tcdA*, *tcdB*		0	106	42	1
Hu77	-	+	-	035	107	1
Hu88	-	+	-	073	109	4
Hu109	*tcdA*, *tcdB*		0	154	110	1
Hu112	*tcdA*, *tcdB*		0	020	2	1
Hu129	*tcdA*, *tcdB*		0	020	2	1
Hu133†,‡	-	+	-	010	15	1
Hu138	*tcdA*, *tcdB*		0	110	19	1
Hu139	-	+	-	039	26	1
Hu162	*tcdA*, *tcdB*		0	014	26	1
Hu181	*tcdA*, *tcdB*		0	New	6	1

ID: sample identification; NTS: non-toxigenic strains PCR; MLST: multilocus sequence typing; ST: sequence type. *Deleted *tcdA* gene, 700bp. †Metronidazole resistant strain. ‡Multidrug resistant isolates.

Table 2. Characteristics of patients included in the study

n = 137	n (%)	Non toxigenic strains (%)*	*p*-value
Age groups of patients			0.51
2-15 years	3 (2.2)	-	
16-64 years	67 (48.9)	3 (4.5)	
≥ 65 years	67 (48.9)	6 (9)	
Sex			0.48
Females	84 (61.3)	7 (8.3)	
Males	53 (38.7)	2 (3.8)	
Origin of patients (ward location)			0.87
Emergency Service	71 (51.8)	5 (7)	
Internal Medicine	40 (29.2)	3 (7.5)	
Gastroenterology	11 (8)	1 (9.1)	
Intensive Care Unit (ICU)	5 (3.7)	-	
Other[†]	10 (7.3)	-	
Disbiosis[‡]			0.38
Yes	7 (5.1)	1 (14.3)	
No	130 (94.9)	8 (6.1)	
Presence of other enteric pathogens			0.09
Yes	29 (21.8)	4 (13.7)	
No	108 (78.9)	5 (4.8)	

*Percentage of toxigenic strains in relation to the number of samples analysed in each group. [†] Hematology, Endocrinology, Cardiology, Preventive Medicine, Pneumology, Neurology and General Surgery. [‡]Absence of regular enteric microbiota (absence of lactose-positive enterobacteria in routine microbiological media (blood agar, *Salmonella Shigella* (SS) agar, Hektoen agar, and Selenite broth) and overgrowth of Gram positive bacteria and/or yeasts).

Table 3. Characteristics of patients with *Clostridium difficile* infection (n = 9 toxigenic isolates)

Epidemiological characteristics	n/n (%)
Outpatients (Emergency Service)	5/9 (55.6)
Epidemiological association	
HA-CDI	5/9 (55.6)
CA-CDI	4/9 (44.4)
Use of any antibiotic during previous 3 months	5/9 (55.6)
Penicillin-β-lactamase inhibitor combination (amoxicillin clavulanic acid, piperacillin/tazobactam)	5/9 (55.6)
Fluoroquinolone (levofloxacin)	1/9 (11.1)
Lincosamide (clindamycin)	1/9 (11.1)
Nitroimidazole (metronidazole)	1/9 (11.1)
Aminoglycoside (gentamycin)	1/9 (11.1)
CDI test request by practitioner (EIA)	4/9 (44.4)
Underlying diseases (previous 3 months)	
Yes	7/9 (77.8)
No	2/9 (22.2)

HA-CDI: hospital acquired *Clostridium difficile* infection; CA-CDI: community acquired *Clostridium difficile* infection; CDI: *Clostridium difficile infection*; EIA: enzyme immunoassay.

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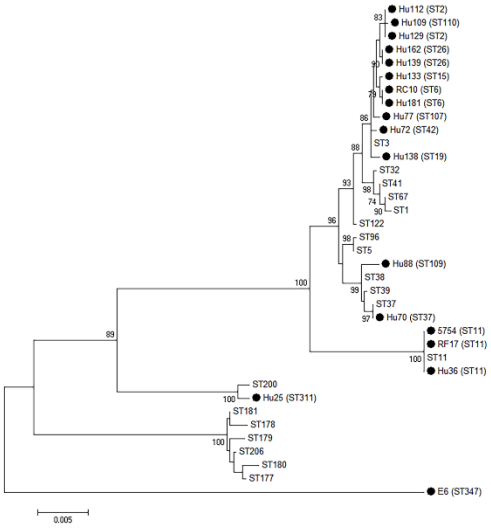

Fig. 1. Molecular phylogenetic analysis (maximum likelihood method) from concatenated MLST alleles. *Clostridium difficile* isolates corresponding to our collection are shown with a circle. ST: sequence type; Hu: human isolate; RC: rat intestinal content isolate; 5754: sow vagina isolate; RF: environmental rat feces isolate; E: exotic animal isolate.