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Clinical and laboratory features of the most common *Clostridium difficile* ribotypes isolated in Belgium

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SUMMARY

Background: Previous studies comparing *Clostridium difficile* infection (CDI) due to different ribotypes have been conflicting, and many have only compared small numbers of cases or few ribotypes.

Aim: To compare patient and episode characteristics for CDI due to different ribotypes.

Methods: The ribotyping results from 3333 toxin-producing isolates collected from 110 Belgian hospitals between October 2010 and December 2015 were matched to clinical data from the national CDI surveillance database. Data for ribotypes with at least 100 occurrences were compared. In addition, the national reference laboratory quantitatively measured the level of toxin production in five randomly chosen cultured isolates for each of the most common ribotypes.

Findings: Ribotypes with more than 100 occurrences were R014, R020, R002, R078, R027, R005 and R106 (Brazier classification). The median age for all patients was 79 years [patients with R027, 83 years ($P < 0.001$); patients with R106, 73 years ($P < 0.001$)]. In total, 10% of episodes were recurrences; values were higher for R027 (22%) and R106 (18%). CDI due to R078 was not significantly more likely to be community associated than healthcare associated (28% vs 24%; $P = 0.1$). Complications occurred in 7% of all episodes, and 12% for those with R027 and R078. However, after adjusting for age, onset outside the hospital and recurrence, R027 was no longer associated with complications [odds ratio (OR) 1.3, 95% confidence interval (CI) 0.7–2.4], unlike R078 (OR 1.7, 95% CI 1.0–2.6; $P = 0.04$). A positive stool toxin test and greater levels of toxin production in the cultured isolates were more likely for R078 and R027.

Conclusion: Out of the seven most common ribotypes in hospital patients, R078 and R027 were associated with higher rates of complications. Infections with R027 and R106 were more likely to be recurrent. The presence of toxin in stools was most likely with R078, R027 and R106, with highest levels of toxin production *in vitro* for R078 and R027. R060 produced the lowest levels of toxin *in vitro*.

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Introduction

Clostridium difficile is the leading cause of healthcare-associated diarrhoea in industrialized countries. Patients with *C. difficile* infection (CDI) are likely to spend an extra one to three weeks in hospital compared with those without CDI, at an estimated additional cost of €14,000/per patient.[1] Both ribotype 027 (R027) and ribotype 078 (R078) have been labelled 'hypervirulent' on account of their increased toxin production compared with other ribotypes. They have both been linked with more severe outcomes or complications, and R027 has been linked with recurrent disease, but evidence remains conflicting.[2] Little is known about the clinical characteristics or toxin production of other ribotypes. Studies have measured relatively rare events due to CDI (e.g. death, pseudomembranous colitis and recurrence), but most have only included small numbers of cases. Also, many of these studies have been too small to compare outcomes of infection between more than two or three different strains.

Case-based surveillance of CDI has been ongoing in Belgian hospitals since 2006. The proportion of hospitals reporting isolates with the strain R027 has decreased from 34% in 2009 to 16% in 2015. During the same period, the proportion of hospitals reporting isolates with R078 has increased from 11% to 42%.[3]

The aim of this study was to compare episodes of CDI due to the most frequent ribotypes found in Belgium in terms of patient and episode characteristics and toxin production.

Methods

Sources of data and case definitions

This study used data collected as part of routine surveillance of *C. difficile* in Belgian hospitals (110 in total), according to the national protocol.[4] Participating hospitals collect and report minimal clinical data for each CDI episode (age, sex, date of hospital admission, date of symptom onset, whether the episode was recurrent, probable place of acquisition, clinical evolution during hospital stay, and presence of toxin in stool or culture).

CDI stool samples or cultures were sent from local laboratories to the reference laboratory for typing. After exclusion of samples from which *C. difficile* could not be cultured (4%) or samples where the *C. difficile* cultured isolate was not cytopathogenic (4%), 3333 toxin-producing strains remained for analysis. The predominant strain from each isolate was then ribotyped.

As per the surveillance protocol, one isolate from five consecutive patients per hospital per six-month surveillance period is typed in the national reference laboratory. In addition, because of a separate parallel study, 37 hospitals were asked to provide the reference laboratory with all of their cultured *C. difficile* isolates for ribotyping during 2015. This study matched the typing results from 3333 isolates collected between October 2010 and December 2015 with clinical data available in the national CDI surveillance database. In the few instances where more than one episode was typed per patient, only the first was kept for analysis. This study also describes the association between presence or absence of toxin in the stool (listed by tick box in the hospital clinical data set) and the level of toxin production in the cultured isolate (measured by the reference laboratory) for a limited number of samples per ribotype.

Definitions

Hospital-associated (HA) CDI: onset of CDI symptoms two days or more after admission to declaring hospital and less than four weeks after discharge, OR regular contact with a hospital (e.g. for haemodialysis) at any time in the four weeks before CDI onset.

Long-term-care-facility-associated (LTCFA) CDI: not meeting the criteria for HA CDI AND onset of CDI two days or more after admission to a LTCF and less than four weeks after discharge.

Community-associated (CA) CDI: onset in the community and no admission or regular contact with hospital or LTCF in the previous 12 weeks.

CDI of 'undetermined' origin (UO): onset of symptoms more than four but less than 12 weeks after admission to a health or care institution.

Complications: an episode resulting in pseudomembranous colitis, surgery or admission to intensive care unit; death (in hospital) less than 30 days after onset of infection, either as a direct or indirect result of CDI.

Hospital-onset (HO) CDI: an episode with onset arising on the day after admission or later — a proxy that the reason for admission was NOT CDI.

Recurrence: a new episode with onset less than eight weeks after onset of the first episode in the same patient, provided symptoms disappeared after this first episode.

Reference laboratory methods — ribotyping and toxin production

Ribotyping was carried out as described by Bidet *et al.*[5] Profiles were analysed by comparison with those of reference strains from the European collection (Brazier classification).[6] In a separate study, toxin production from cultured isolates was tested for five strains of different origins in each of the ribotypes using the chemiluminescent immunoassay automate Liaison (Diasorin, Stillwater, MN, USA), by testing serial five-fold dilutions of a 48-h growth.

Data analysis

This study describes ribotypes with at least 100 occurrences, comparing the frequency of certain characteristics for an episode due to a given ribotype with the frequency of these characteristics for all other episodes. Chi-squared test was used to assess the probability of the differences between these proportions arising by chance.

Using Stata Version 14 (StataCorp., College Station, TX, USA), a logistic model was used to identify independent risk factors for complications. All variables associated significantly ($P \leq 0.10$) with the outcome on univariate analysis were introduced into the multi-variate model.

Results

Among the 3333 ribotyped isolates, a total of 299 different ribotypes were identified. There were seven ribotypes with

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more than 100 occurrences, with the most frequently isolated ribotype being R014 ($N = 382$,12%). Out of 3297 first episodes, 1884 (57%) occurred in women. The proportion of women was similar for all the seven ribotypes under study. The distribution of ribotypes and characteristics of the episode are shown in Table I.

Sixty-seven percent of episodes were HA, 24% were CA, 4% were LTCFA and 5% were UO. Table I shows that the only significant difference between ribotypes was the proportion of episodes of R027 that were LTCFA (16/139, 12%; $P < 0.001$), which was greater than the 5% average. These 16 LTCFA cases were reported by 12 different hospitals, with two hospitals reporting more than one episode over a relatively short period. The proportion of R078 episodes that were CA was 28% (65/232), but the difference from the 24% average was not significant ($P = 0.1$). In total, 65% of all episodes had onset in the declaring hospital (HO); this was the case for 54% of R027 ($P = 0.004$), meaning that patients with R027 were more likely to be admitted to the hospital because of CDI than patients with other ribotypes

These data measure the proportion of episodes that were recurrent among those that were typed. Among 2698 episodes with non-missing recurrence information (82% of the database), 10% were recurrent. Table II shows that the ribotypes with the highest proportion of episodes that were recurrent were R027 and R106.

A positive test result for toxin in the stool (free toxin or polymerase chain reaction) was reported for 80% of all episodes. A higher proportion of episodes with R027 and R078 produced toxin in the stool, and a lower than average proportion of episodes with R020 reported toxin in the stool. Most ribotypes similarly produced high or low levels of toxin, respectively, from cultured isolates, with the exception of R106 which showed low-level toxin production from cultures but a high proportion of toxin-positive stools (Table III).

Clinical outcome information was available for 76% (2499/3296) of episodes; 7% of these were associated with complications, and this value was greater (12%) for R027 and R078 (Table II). The risk of complications associated with R027 on univariate analysis decreased on multi-variate analysis, unlike

Table I

Characteristics of patient and episode for the most frequent *Clostridium difficile* ribotypes isolated in Belgium 2010–2015

Ribotype (Brazier)	Age (median)		Hospital-associated[a]			Community-associated[b]			LTCF-associated[c]			Total, non-missing	Total
	Years	P^d	*N*	%	P^c	*N*	%	P^c	N	%	P^c	*N*	*N*
R014	79	0.69	257	67%	0.91	92	24%	0.79	16	4%	0.88	365	382
R020	77	0.16	191	68%	0.60	58	21%	0.26	15	6%	0.37	264	279
R002	81	0.09	164	65%	0.55	57	23%	0.75	12	5%	0.72	233	251
R078	79	0.73	146	63%	0.17	65	28%	0.10	10	5%	0.98	221	232
R027	83	<0.001	85	61%	0.13	30	22%	0.43	16	12%	<0.001	131	139
R005	81	0.2	83	71%	0.36	24	21%	0.63	1	1%	0.06	108	117
R106	73	<0.001	78	70%	0.46	24	22%	0.58	5	5%	0.93	107	111
Other	78	0.12	1206	68%	0.51	426	24%	0.64	68	4%	0.10	1700	1786
Total	79		2210	67%		776	24%		143	5%		3129	3297

CDI, *Clostridium difficile* infection.

Long-term-care-facility (LTCF)-associated CDI: not meeting the criteria for hospital-associated CDI AND onset of CDI two days or more after admission to a LTCF and less than four weeks after discharge.

Statistical testing: result for one group is compared with results for all other groups combined. [c]Chi-squared test. [d]Wilcoxon rank sum test (tests the hypothesis that two independent samples are from populations with the same distribution). Not significant if $P > 0.10$.

[a] Hospital-associated CDI: onset of CDI symptoms two days or more after admission to the declaring hospital and less than four weeks after discharge.

[b] Community-associated CDI: onset in the community and no admission or regular contact with hospital or LTCF in the previous 12 weeks.

Table II

Recurrent episodes and complications associated with the most frequent *Clostridium difficile* ribotypes in Belgium, 2010–2015

Ribotype (Brazier)	Recurrent episode				Complications			
	N with non-missing data	*N* recurrent	%	*P*	*N* with non-missing data	*N* severe	%	*P*
R014	322	29	9%	n.s.	299	26	9%	n.s.
R020	219	22	10%	n.s.	225	15	7%	n.s.
R002	207	24	12%	n.s.	190	16	8%	n.s.
R078	180	12	7%	0.14	169	20	12%	0.02
R027	115	25	22%	<0.001	110	13	12%	0.07
R005	92	5	5%	0.15	89	6	7%	n.s.
R106	87	16	18%	<0.01	75	5	7%	n.s.
Other	1476	133	9%	0.10	1,353	86	6%	0.02
Total	2698	265	10%		2499	187	7%	

n.s., not significant.

[a] Chi-squared test comparing proportion observed in one group with proportion observed in all other groups combined. n.s. if $P > 0.10$.

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Table III
Positivity of toxin test (any diagnostic method at local laboratory, e.g. polymerase chain reaction or enzyme immunoassay) on stool sample[a] and level of toxin production from cultured isolates[b] – most frequent *Clostridium difficile* ribotypes isolated in Belgium, 2010–2015

Ribotype	Positive toxin test on stool[a]				Level of toxin production from cultured isolates[d] (five samples of different origin tested per strain)		
	N non-missing	Pos	%	*P*[c]	Mean	Min	Max
R014	371	288	78%	n.s.	1.74	1.60	1.86
R020	263	196	75%	0.04	0.66	0.60	0.75
R002	241	181	75%	0.08	0.68	0.61	0.75
R078	226	195	86%	<0.01	>95	>95	>95
R027	135	119	88%	0.01	>95	>95	>95
R005	110	93	85%	n.s.	0.57	0.50	0.65
R106	100	88	88%	0.03	0.38	0.30	0.45
Other	1722	1359	79%	n.s.			
Total	3168	2519	80%				

n.s., not significant.
[a] As listed by tick box in the hospital clinical dataset.
[b] As measured by the reference laboratory.
[c] Chi-squared test comparing proportion observed in one group with proportion observed in all other groups combined. n.s if $P > 0.10$.
[d] Index values refer to an arbitrary scale of relative light units (RLUs). Thus, the index value for a patient's specimen is calculated by comparing the strain's light signal (RLU) with a working curve from the light signals (RLU) obtained from calibrators. An index value of 0.9 is interpreted as negative, while a value of 1.1 is considered positive. Results between 0.9 and 1.1 are considered equivocal, and the test is repeated according to the manufacturer's instructions. Final index values do not refer to absolute values of toxin but relative values towards the comparator.

the risk associated with R078. Patients with an episode due to R027 were older than the others, more likely to have been admitted to hospital because of their CDI, and the episode was more frequently recurrent, making these variables appear as strong confounders of the association between R027 and complications.

On univariate analysis, no association was found between sex and risk of complications. HA infection was not associated with complications [univariate logistic regression odds ratio (OR) 1.2 (95% CI 0.9–1.7)], but HO infection was associated with complications [univariate logistic regression OR 1.6 (95% CI 1.2–2.1)]. HO and all other variables significant on univariate analysis were, therefore, used to build a multi-variate model to assess the adjusted OR for the association with the 'hypervirulent' ribotypes (Table IV).

The multi-variate logistic regression model showed that recurrence, older age, a positive test for toxin in the stool and admission to hospital because of CDI were associated with complications (as defined in this study) during the hospital stay. After taking these variables into account, R078 (but not R027) was associated with higher odds of complications [OR 1.7 (95% CI 1.0–2.9); $P = 0.04$].

Table IV
Risk factors for complications of *Clostridium difficile* infections, Belgium, 2010–2015

	Univariate logistic regression				Multi-variate logistic regression			
	OR	LCI	UCI	*P*	OR	LCI	UCI	*P*
Ribotype								
All other ribotypes	ref							
R027	1.7	1.0	3.2	0.072	1.3	0.7	2.4	0.467
R078	1.7	1.1	2.9	0.026	1.7	1.0	2.9	0.045
Toxin in stool								
Negative	ref							
Positive	1.7	1.1	2.6	0.022	1.6	1.0	2.6	0.038
Age (years)								
0–64	ref							
65–79	1.9	1.1	3.0	0.012	1.6	0.9	2.6	0.090
≥80	2.6	1.6	4.0	<0.001	2.6	1.6	4.1	<0.001
Non-hospital onset								
No								
Yes	1.6	1.2	2.1	0.003	1.5	1.1	2.1	0.012
Recurrent episode								
No								
Yes	2.2	1.5	3.3	<0.001	2.0	1.3	3.0	0.002

OR, odds ratio; LCI/UCI, lower/upper 95% confidence interval.

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Discussion

Main findings and comparison to previous research

This study found that hospital inpatients with CDI caused by R027 were more likely to have LTCFA CDI than patients with other ribotypes. However, the numbers were small and may indicate sporadic outbreaks in LTCF. The available data did not permit the authors to explore this further. Infection with R078 has been associated with a younger population and is more frequently found in CA disease in the Netherlands.[7] It has been linked with farm animals.[7] However, in this study, the hospital inpatients with CDI caused by R078 were no more likely to have onset in the community than in the declaring hospital, and were no younger than those infected with other ribotypes.

Episodes were more commonly recurrent for those infected with R027 and R106, and were less commonly recurrent for R014. Studies have previously linked R027 with recurrence,[8,9] but others have suggested that R027 alone is not predictive of recurrence, and recurrence more likely results from a combination of binary toxin with mutation of the toxin regulator *tcdC*.[10]

Multiple virulence factors for *C. difficile* have been identified:[11] toxin A/B, binary toxin, toxinotype, deletions in the gene *tcdC* (that is thought to negatively regulate production of toxins A and B), production of inflammatory cytokines, increased sporulation and enhanced adhesive properties of the bacterium. The predominance of each appears to vary according to strain type, experimental conditions and, perhaps as yet unidentified, host factors.

This study has shown that the presence of toxin (A and B) in the stool (in vivo) is more likely for R078, R027 and R106, and is least likely for R020 among the seven most common ribotypes. Previous studies have measured levels of toxin production, and found that R027 produced significantly more toxin A and B *in vitro* (from *C. difficile* cultures) than R014 and other strains,[12,13] toxin production was highly variable amongst the same ribotypes,[14] and R106 produced high toxin levels *in vitro* (but less than R027) compared with R001 and R012.[12] Neither of the two studies that tried to link in-vitro toxin production with clinical outcome[13,14] could find a correlation, but both of these studies were very small. Faecal toxin levels may be more relevant to clinical outcome and have been associated with increased severity of diarrhoea, although no correlation could be identified between levels of faecal toxin and infecting ribotype in a small study.[14]

Although results of smaller studies have been conflicting,[15] the present results concur with those of larger studies with over 1000 cases[7,16,17] with the finding that infection with R078 is associated with severe outcomes. According to the present data, much of the effect of R027 on adverse outcome during hospital stay is related to increased age, recurrence and admission to hospital because of CDI. These last two factors are indicators of higher severity of the disease, and could be seen as factors in the causal pathway between infection with R027 and complications in hospital, rather than confounders. This is also supported by a large UK-based study[17] which found the highest mortality in people infected with R078, although higher mortality in this study persisted for R027 even after adjustment for age and baseline biomarkers. One large UK-based study did not detect increased mortality in those infected with R078.[15] However, it is notable that the number of cases infected with R078 was less than 100, and the number with adverse outcomes was small, as only 30-day mortality was included as an outcome measure. This study supports other research that increasing age is predictive of poor outcome regardless of ribotype.[15,16,18]

Strengths and limitations

A major strength of this study is that it is one of the largest to date. This is an important factor when considering relatively rare events such as complications of CDI, and comparing outcomes for small groupings such as ribotype. Thus, the authors were able to discriminate between a larger number of ribotypes than many previous studies. The conclusions of studies will depend on which comparator groups are used. For example, studies comparing R027 with all other ribotypes may draw very different conclusions than studies comparing R027 and R078 with all other ribotypes. Other studies compare 'clades', and use a different grouping system again.

A limitation of this study was the high number of missing data for the main outcome (complications) and recurrences. However, given that these data are recorded before ribotype is known and subsequently linked to patient data, any consequent bias would be non-differential (similar between ribotypes and thus not affecting comparisons). In addition, data from all over the country for several years were used, thus making the study more generalizable.

Although no data on comorbidities were available, there is no reason to believe that different ribtoypes are associated with different comorbidities. This study was not attempting to measure attributable mortality related to CDI. However, apart from some objective criteria such as pseudomembranous colitis, 'severity of disease' relied subjectively upon clinician assessment of whether CDI contributed to death, directly or indirectly.

Another limitation is that these results could hide the effects of co-infection with more than one ribotype per patient, as attempts were only made to isolate one ribotype from any isolate, albeit the predominant strain in the culture.

These data are restricted to cases admitted to hospital either because of their CDI infection or where onset occurred after admission for a different reason (defined as HO). As a result, the cases included as non-HO are likely to be more severe than those managed in the community and possibly those coincident to another reason for hospital admission. To account for this, HO was used as a confounding factor in the multi-variable model for the association between ribotype and complications.

In conclusion, this study showed that R078 and R027 in hospitalized patients are associated with more complications than R014, R020, R002, R005, R106 and all other ribotypes combined. Increased age is a strong confounder on the relationship between R027 and complications. Recurrence and admission because of CDI are associated with both R027 and complications, but may be considered factors in the causal pathway rather than confounders. This study found that R078 in hospitalized patients is not more likely to be CA or associated with a younger population. Episodes were more commonly recurrent

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for infection with R027 and R106, and were least commonly recurrent for infection with R014. R078, R027 and R106 isolates were more commonly associated with a positive test for toxin in stools, and R020 was least commonly associated with a positive test. Levels of toxin production in the cultured isolates were also greatest for R078 and R027, but R060 only produced low levels of toxin. Further work on toxin production and factors other than ribotype would be beneficial.

Conflict of interest
None declared.

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