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Molecular epidemiology of community- and hospital-associated *Clostridioides difficile* infections in Jönköping, Sweden, October 2017 – March 2018

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Clostridioides difficile infections (CDIs) in Sweden are mostly hospital-associated (HA) with limited knowledge regarding community-associated (CA) infections. Here, we investigated the molecular epidemiology of clinical isolates of CA-CDI and HA-CDI in a Swedish county. Data and isolates (n = 156) of CDI patients (n = 122) from Jönköping county, October 2017–March 2018, were collected and classified as CA (without previous hospital care or onset ≤2 days after admission or >12 weeks after discharge from hospital) or HA (onset >3 days after hospital admission or within 4 weeks after discharge). Molecular characterization of isolates included PCR ribotyping (n = 156 isolates) and whole genome sequencing with single nucleotide polymorphisms (SNP) analysis (n = 53 isolates). We classified 47 patients (39%) as CA-CDI and 75 (61%) as HA-CDI. Between CA-CDI and HA-CDI patients, we observed no statistically significant differences regarding gender, age, 30-day mortality or recurrence. Ribotype 005 (RR 3.1; 95% CI: 1.79–5.24) and 020 (RR 2.5; 95% CI: 1.31–4.63) were significantly associated with CA-CDI. SNP analysis identified seven clusters (0–2 SNP difference) involving 17/53 isolates of both CA-CDI and HA-CDI. Molecular epidemiology differed between CA-CDI and HA-CDI and WGS analysis suggests transmission of CDI within and between hospitals and communities.

Key words: Bacteriology; clinical microbiology; *Clostridioides difficile*; community-associated infections; enhanced surveillance; hospital-associated infections; molecular microbiology; whole genome sequencing.

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Clostridioides difficile (*C. difficile*) is one of the primary causes of nosocomial diarrhoea worldwide and *C. difficile* infection (CDI) has emerged as an increasingly important infectious disease (1). Hypervirulent strains such as ribotype RT027 (RT027/NAPI and B1) emerged during the 21st century and have been responsible for several outbreaks worldwide. The epidemic type RT027 is characterized by an increased cytotoxin production *in vitro* compared with other common types, and unlike historic isolates of type RT027, the epidemic types were characterized by resistance to moxifloxacin (2) (Åkerlund *et al*., https://pubmed.ncbi.nlm.nih.gov/18287318/). An increased antibiotic resistance profile may confer a selective advantage during high antibiotic consumption as this is observed in several strains considered to be hypervirulent (3). CDI is primarily regarded as a hospital-associated (HA) infection for which the major reservoirs are hospitalized patients, patients with a history of antibiotic exposure or contaminated hospital facilities. However, community-associated (CA) infections can account for up to one third of all CDI cases, but the reservoirs of CA-CDI are not well studied (4).

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Possible reservoirs in the community include soil, water, animals, meats and vegetables (5-8) and CA-CDI most likely might also be a reservoir for transmission to healthcare facilities.

Sweden is a country with a widely diverse CDI epidemiology, and RT027 has only occurred sporadically and caused few local outbreaks. From 2012 to 2016, the incidence of CDI in Sweden has decreased by 25%, and during the same period hypervirulent and multi-resistant strains have almost disappeared, resulting in an increased type diversity (9). After 2016, the incidence decrease has plateaued at 64 cases per 100,000 inhabitants in 2017 and 2018. To further decrease the CDI incidence in an epidemiological diverse setting, we need to study the epidemiology of CA-CDI and how it may affect HA-CDI. In this cross-sectional study, the epidemiology of CA-CDI and HA-CDI was studied for a six-month period in Jönköping County, Sweden, by analysing ribotype distribution, recurrence, antibiotic susceptibility, mortality and CDI treatment. PCR ribotyping, whole genome sequencing (WGS) and epidemiological data were used to determine possible connections between CA-CDI and HA-CDI. Ribotype-specific epidemiology can provide new insights to tailor preventive measures to further decrease the CDI incidence in Sweden.

MATERIALS AND METHODS

Study design and case definition

This was a cross-sectional study of laboratory-confirmed CDI patients resident in Jönköping County, Sweden, which has a catchment population of 356,291 as of September 2017. The diagnosis of CDI in 122 patients was performed in any of the three hospitals in Region Jönköping County between 1 October 2017 and 31 March 2018. A laboratory-confirmed CDI case was defined as a person having diarrhoea or toxic mega colon with a stool sample positive for *C. difficile* by nucleic acid amplification test using GeneXpert® (Cepheid, Sunnyvale, CA, USA).

Medical records were reviewed to categorize primary cases as either CA- or HA-CDI according to the definition of the European Centre for Disease Prevention and Control (ECDC) (10): a CA-CDI case was defined as a patient with symptom onset (1) in the community and without any hospitalization in the previous 12 weeks, or (2) within 48 h of admission to a hospital and without any hospitalization in the previous 12 weeks. A HA-CDI case was defined as a patient with (1) symptom onset after three days of hospitalization or (2) symptom onset in the community with hospital contact in the previous 4 weeks. A case was defined as 'not classified' when symptoms began between four and twelve weeks after discharge from a hospital. *C. difficile* recurrence was determined as yes or no by assessing the medical records for a new episode of CDI more than two weeks and less than eight weeks after the previous CDI episode. Patients that entered the study with a recurrent episode, were categorized as CA- or HA-CDI based on their primary episode.

Patient data and isolate collection

Patients' age, gender, treatment against CDI, recurrent infection and 30-day mortality were obtained retrospectively from patients' medical records. *C. difficile* isolates were recovered from patients' stool samples through isolation on CLD agar (including D-Cycloserine 250 mg/L and Cefoxitine 8 mg/L) and subsequently on blood agar, for 48 h under anaerobic conditions. Isolates were verified as *C. difficile* using MALDI-ToF microFlex (Bruker, Billerica, MA, USA) and stored in skimmed milk at −80°C and upon completion of collection sent to the Public Health Agency of Sweden for further analysis.

PCR ribotyping and whole genome sequencing

PCR ribotyping of *C. difficile* isolates was performed using capillary PCR ribotyping as previously described (11). All isolates (n = 53) of the common ribotypes RT002, RT005, RT023, RT020 and RT078 were analysed by WGS to investigate potential transmission of CDI between patients. WGS was performed on the Ion Torrent platform at the Public Health Agency of Sweden, followed by SNP analysis as described elsewhere (12). A minimum spanning tree was constructed on the basis of all SNP differences using MSTgold (13). Isolates with 0–2 SNPs difference were defined as a cluster based on analyses of *C. difficile* evolutionary rate (14).

Antibiotic susceptibility testing

Antibiotic susceptibility testing was performed as described previously (12). Briefly, isolates were grown on Mueller–Hinton fastidious agar, and minimum inhibitory concentration (MIC) values for moxifloxacin, erythromycin, clindamycin, metronidazole and vancomycin were determined using Etests (bioMérieux, Marcy l'Etoile, France). The epidemiologic cut-off breakpoints for resistance were as follows: metronidazole MIC >2 mg/L, vancomycin MIC >2 mg/L, erythromycin MIC >2 mg/L, clindamycin MIC >16 mg/L and moxifloxacin MIC >4 mg/L, according to the European Committee on Antimicrobial Susceptibility Testing (EUCAST).

Analysed variables

The following variables were analysed as binary variables in this study: sex, recurrent infection and 30-day mortality. 30-day mortality was defined as yes or no if death occurred within 30 days after CDI onset. Age was grouped into six categories (0–14, 15–44, 45–64, 65–74, 75–84, >84). Treatment against CDI was categorized depending on whether the patient received the treatment antibiotics metronidazol, vancomycin, fidaxomycin, a combination of those or no treatment.

The isolates were categorized into the seven most frequent ribotypes (RT070, 023, 078, 014, 020, 002, 005) and the remaining isolates were grouped into the category "Others." Resistance against the antibiotics—tetracycline,

moxifloxacin, erythromycin, clindamycin, metronidazole and vancomycin—was reported as yes or no. Each resistance was analysed as separate binary variable.

Statistical analysis

We dichotomized the outcome as CA-CDI or HA-CDI; isolates from patients not classified were excluded. Univariate and multivariate logistic regression models were used to evaluate the patient variables sex, age, recurrent infection, 30-day mortality and treatment associated with CA-CDI. Ribotypes and antibiotic resistance were included as explanatory variables in univariate logistic generalized estimating equation (GEE) to account for the repeated measures of recurrent CDIs for each patient. Risk ratios (RR) for each variable were calculated and considered significantly associated with CA-CDI if the p-value was <0.05 based on Wald-test. Statistically significant variables were included in a multivariable model to calculate adjusted risk ratios (adj RRs) with 95% confidence intervals (CIs). All statistical analysis was performed in Stata version 15 (StataCorp., College Station, TX, USA).

Ethical statement

The study protocol was reviewed and approved by the Stockholm Regional Ethics Review Board (dnr 2017/1496-31/2).

RESULTS

Study population

During the six months study period (1 October 2017 to 31 March 2018), 165 isolates from 131 patients were collected. Nine patients were excluded from the analysis since they could not be classified as HA- or CA-CDI, resulting in 156 isolates from 122 patients. The number of isolates reflects the number of CDI episodes that the 122 patients had during the study period.

The gender distribution among the total number of patients was similar (Table 1) and the median and mean age for all cases were 73 and 65, respectively (range 0–97). A majority (62%) of the cases were classified as HA-CDI (Table 1). Of the 29 patients (24%) with recurrent infections, 18 had one recurrent episode and five and three patients had three and four recurrent episodes after their primary infection, respectively. For three patients, the number of episodes could not be determined as the primary infection occurred before the study period.

Twenty-two (18%) patients died within 30 days after CD infection. The majority of CDIs were treated with metronidazole or vancomycin, whereas a combination of the two or fidaxomycin was rarely used (Table 1).

Demographic factors associated with CA-CDI or HA-CDI

The mean age for HA-CDI and CA-CDI cases was 71 and 57 years, respectively, and CA-CDI was more common among men and in the younger age groups (0–14 and 15–44), whereas HA-CDI was more frequent among older patients (age group 75–84 and >84, Table 1). Higher proportions of patients with CA-CDI (30%) had recurrent infections compared with HA-CDI (20%) whereas 30-day mortality was lower for CA-CDI (13%) than HA-CDI (21%).

Univariate analysis identified a weak association of CA-CDI and being male, which disappeared in multivariate analysis (Table 1). Both univariate and multivariate analysis showed that CA-CDI was associated with receiving no treatment compared with receiving metronidazole (Table 1). No significant association between CA-CDI and the other characteristics (age group, type of infection, 30-day mortality) was found. Of note, only HA-CDI patients received the recommended treatment for severe CDI during the study period, that is a combination of vancomycin and metronidazole.

Ribotypes and antibiotic resistance in CA-CDI compared with HA-CDI

All CDI isolates from this study (n = 156) were PCR-ribotyped and analysed for antibiotic resistance.

In total, 41 different RTs were found, 19 different among CA-CDI, 36 different in HA-CDI during the study period and 14 RTs were common to both. The most common RTs were RT070, 023, 078, 014, 020, 002 and 005 (Fig. 1). RT005 and RT020 were more common among CA-CDI patients than those having HA-CDI (RR 3.1, 95% CI 1.79–5.24; $p < 0.001$ and RR 2.5, 95% CI 1.31–4.63; $p = 0.005$). There were no significant differences among other RTs and CA-CDI and HA-CDI.

Regarding antibiotic resistance, we observed lower levels of resistance against tetracycline and clindamycin among CA-CDI than HA-CDI (Fig. 2). However, the associations were not significant (RR 0.3, 95% CI 0.77–1.28; $p = 0.106$ and RR 0.3, 95% CI 0.04–1.79; $p = 0.173$, respectively).

Five multidrug resistant (MDR), *C. difficile* isolates were detected during the study period, all from HA-CDI. Three were classified as MDR3 (resistant against erythromycin, clindamycin and tetracycline; ribotypes RT203, RT012 and one unknown type x184) and two isolates as MDR4 (resistant against moxifloxacin, erythromycin, clindamycin and tetracycline; ribotype RT017 and x184). None of the isolates was resistant against metronidazole or vancomycin.

Table 1. Number of cases (n) and risk ratios (RR) of demographic characteristics and antibiotic treatment of CDIs in Jönköping, October 2017 – March 2018

Characteristics	All n = 122		No. (%) of cases							
			HA-CDI n = 75	(62)	CA-CDI n = 47	(39)	RR (95% CI)	p Value	Adj RR (95% CI)	p Value
Gender										
Male	59	(48)	31	(41)	28	(60)	1.6 (0.99–2.50)	0.054	1.5 (0.94–2.38)	0.090
Female	63	(52)	44	(59)	19	(40)	ref.	ref.	ref.	ref.
Age group (years)										
0–14	4	(3.3)	0		4	(8.5)	–	–	–	–
15–44	21	(17)	10	(13)	11	(23)	1.3 (0.67–2.57)	0.433		
45–64	20	(16)	12	(16)	8	(17)	ref.	ref.		
65–74	22	(18)	12	(16)	10	(21)	1.1 (0.56–2.30)	0.722		
75–84	28	(23)	22	(29)	6	(13)	0.5 (0.22–1.30)	0.169		
>84	27	(22)	19	(25)	8	(17)	0.7 (0.34–1.63)	0.457		
Recurrence										
Yes	29	(24)	15	(20)	14	(30)	1.4 (0.85–2.17)	0.195	–	–
No	93	(76)	60	(80)	33	(70)	ref.	ref.		
30-day mortality										
Yes	22	(18)	16	(21)	6	(13)	0.7 (0.32–1.37)	0.268	–	–
No	100	(82)	59	(79)	41	(87)	ref.	ref.		
Treatment (of primary infection)										
Metronidazole	73	(60)	48	(64)	25	(53)	ref.	ref.	ref.	ref.
Vancomycin	24	(20)	16	(21)	8	(17)	0.97 (0.51–1.86)	0.935	1.0 (0.53–1.87)	0.977
Fidaxomicin	3	(2.5)	1	(1.3)	2	(4.3)	1.9 (0.82–4.60)	0.129	1.6 (0.62–4.15)	0.331
Combination[1]	5	(4.1)	5	(6.7)	0		–	–	–	–
None	10	(8.2)	3	(4.0)	7	(15)	2.0 (1.22–3.42)	0.007	2.0 (1.21–3.21)	0.006
Unknown	7	(5.7)	2	(2.7)	5	(11)	n.a.	n.a.	n.a.	n.a.

Adj RR, adjusted risk ratio; CA, community-associated; CDI, *Clostridioides difficile* infection; CI, confidence interval; HA, hospital-associated; n.a., not applicable; ref., reference; RR, crude risk ratio.
[1]Metronidazole & Vancomycin.

Whole genome sequencing and single nucleotide polymorphism analysis

All isolates (n = 53) of the common ribotypes RT002, RT005, RT023, RT020, RT078 were analysed using WGS to detect single nucleotide polymorphisms (SNPs). Isolates were compared with each other and to historical *C. difficile* isolates from Sweden when available. Isolates with 0–2 SNPs difference were defined as a cluster (14). Isolates from cases that could not be classified as HA- or CA-CDI were also included in WGS analysis (n = 4). Recurrent infections with the same ribotype were excluded. In total, 7 clusters were identified, involving 17/53 isolates (32%) from our study population (plus two historical isolates, Fig. 3).

Clusters consisted of CA-CDI cases only (1/7), HA-CDI cases only (2/7), CA- and HA-CDI cases (3/7) and CA- and non-classified CDI cases (1/7). For RT002 isolates (n = 16), we identified four clusters (cluster C1–C4; Fig. 3A); two clusters contained one HA- and one CA-CDI isolate each (C1 and C2) and cluster 3 (C3) consisted of two HA-CDI isolates which differed by one SNP. The largest cluster (C4) consisted of three CA- and one HA-CDI isolate clustering with two historical isolates from a geographical region neighbouring Jönköping which have been sequenced because of a suspected outbreak.

One cluster with three cases (2 CA-CDI and 1 non-classified isolate; C5) was detected among RT020 isolates (Fig. 3B) and one cluster with two cases (2 CA-CDI; C6) among RT005 isolates (Fig. 3C). No cluster was observed for RT023 (Fig. 3D). One cluster of two HA-CDI isolates (C7) which differed by 2 SNPs was identified for RT078 (Fig. 3E).

The proportion of isolates of each ribotype was plotted against the SNP distance to the closest isolate (Fig. 4), a clear pattern of ribotypes and respective SNP differences was observed. Isolates of RT002 showed the least variability (median = 1 SNP), followed by RT078 (median = 5 SNPs). SNP differences of >30 were only observed for ribotype RT005 (median = 54 SNPs). The majority of RT020 (median = 11 SNPs) and RT023 (median = 13.5 SNPs) isolates lay within 3–30 SNP difference.

DISCUSSION

Clostridioides difficile infection is one of the most important hospital- or healthcare-associated infections, however, CA-CDI is increasing (15). In

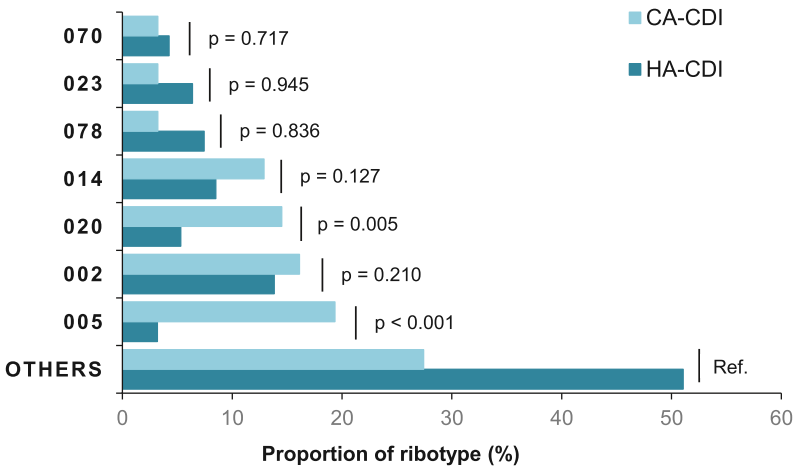

Fig. 1. Relative distribution of ribotypes among isolates from HA- and CA-infections, October 2017 – March 2018, Jönköping; CA, community-associated; HA, hospital-associated; CDI, *Clostridioides difficile* infection (p-value, Wald test).

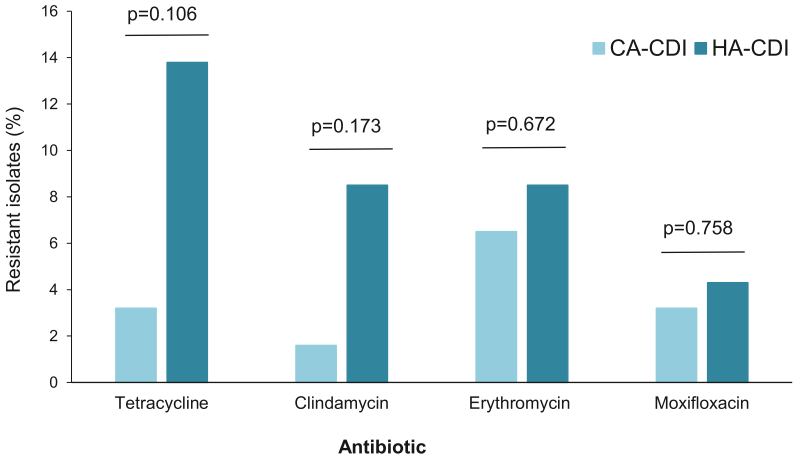

Fig. 2. Relative distribution of antibiotic resistance among isolates from HA- and CA-infections, October 2017 – March 2018, Jönköping; CA, community-associated; HA, hospital-associated; CDI, *Clostridioides difficile* infection.

Sweden, the overall incidence of CDI has decreased by more than 20% between 2012 and 2016 (9) but the cases are not classified as CA- and HA-CDI within the Swedish CDI surveillance. In this study, we identified a relative high proportion of CA-CDI (40%), that is in the higher end of the published range of 20–40% (16,17). The high proportion of CA cases may reflect functional infection control within hospitals and a poorer control in the community, which is in line with a previous study that attributed the reduced incidence of CDI in Sweden to improved infection control within healthcare settings (9).

Unlike previous studies (18,19), we did not find an association between females being more prevalent in CA-CDI. The authors of the US study considered that this might be due to different antibiotic exposures, with females being more likely to seek medical attention and be exposed to antibiotics which is one of the most important risk factors for CDI (18). However, Sweden has low antibiotic prescription rates compared to the US, and as outpatient sales of antimicrobial have decreased more compared with inpatient sales, we hypothesize that a difference in antibiotic exposure does not account for the observed variation in gender distribution between CA- and HA-CDI (20).

The proportion of patients not receiving treatment was higher among patients in the CA-CDI group compared with the HA-CDI group. According to National guidelines, treatment against CDI should not always be given, often it is enough to discontinue the antibiotic that disrupted the normal flora of the intestine to resolve a *C. difficile*

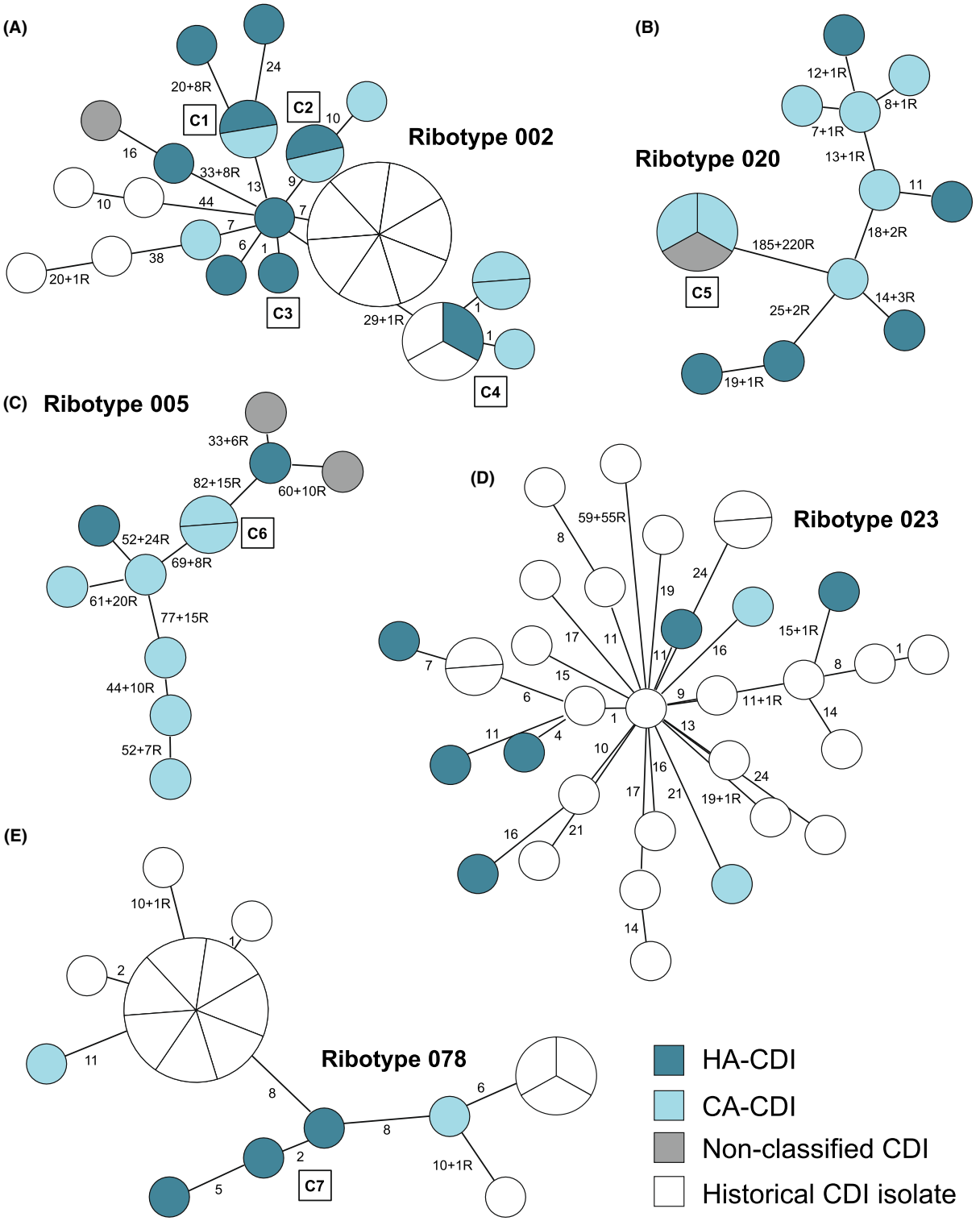

Fig. 3. Minimum spanning tree of *Clostridioides difficile* isolates. (A) RT002, (B) RT020, (C) RT005, (D) RT023 and (E) RT078. SNP differences are shown next to branches, recombination events are marked 'R'. Length of branches is not relative to relationship distance.

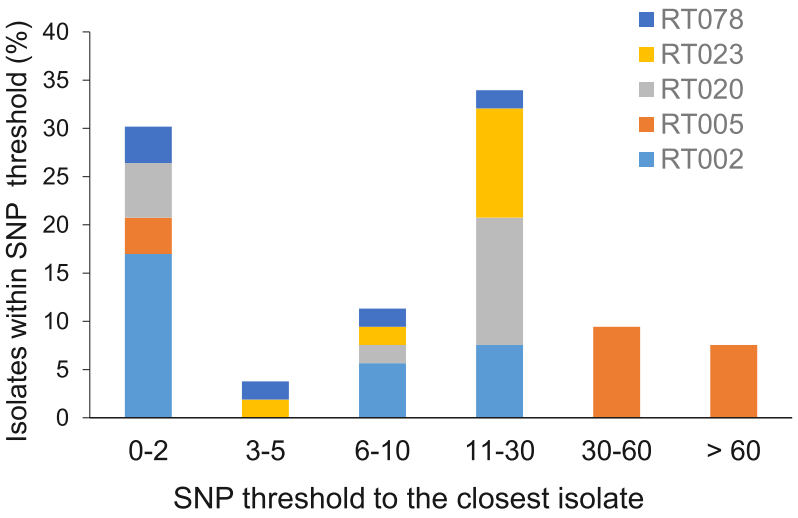

Fig. 4. Relative distribution of isolates by ribotype and SNP threshold, October 2017 – March 2018, Jönköping. SNP, single nucleotide polymorphism.

infection (21). However, not receiving treatment against CDI could further contribute to the dissemination of CDI in the community.

Two ribotypes, RT005 and RT020, were found to be significantly associated with CA-CDI. RT020 together with RT002 and RT056, has previously been associated to CA-CDI in a UK study, but the same study found RT005 to be equally distributed between CA- and HA-CDI (19). However, the authors found that RT005 was more commonly associated with CA-CDI patients having no hospital contact within 12 months prior to onset of disease. This finding, together with the large genomic differences between isolates of RT005 found in this study, suggests that RT005 might be to a larger extent associated with sporadic cases compared with, for example RT002 which appears to be more outbreak prone. No other ribotypes were found to be significantly associated with either CA- or HA-CDI. By comparing our data with data from the Swedish CDI surveillance from 2017/2018 (22), we found that RT002, RT005 and RT070 were more often detected in Jönköping compared to the rest of Sweden ($p = 0.065$, $p = 0.022$ and $p = 0.036$, respectively), whereas for RT014, RT020, RT023 and RT078, the distribution was similar (all $p > 0.2$) (data not shown).

By WGS, we found that 32% of all sequenced isolates belonged to a cluster. Only two of seven clusters occurred within a hospital supporting previous conclusions about adequate infection control in Swedish hospitals (9). Two clusters were found among CA-CDI and three clusters linked cases from the community and hospital. The cluster identification through WGS suggests potential transmission events between cases. However, no epidemiological link for any of the cases could be established. One explanation could be exposure to a common reservoir or the presence of asymptomatic carriers which act as intermediates in the transmission chains. Four of the seven identified clusters involved isolates with RT002. One of the clusters linked to a previous outbreak of RT002 in 2014 from a neighbouring region (23), supporting RT002 as an outbreak prone type.

Another striking finding identified through WGS was the large deviations of SNPs between PCR ribotypes which suggests different reservoirs and modes of transmission (24,25). For example, RT020, RT023 and particularly RT005 showed larger average distances between isolates compared with RT002 and RT078 which are more commonly associated with outbreaks.

During the first years of the Swedish CDI surveillance (2009–2012), geographical clusters and larger outbreaks of multidrug-resistant RT012, RT017, RT046 and RT231 were observed but virtually disappeared in the following years (9,12). Resistance against multiple antibiotics including moxifloxacin is a feature also shared by RT027 (26-29). The disappearance of outbreak-related types has been noted elsewhere, for example in UK where RT027 diminished after introduction of a national surveillance programme for *C. difficile* (30). Proliferation of multidrug-resistant *C. difficile* is logical to happen in settings with high antibiotic pressure. Multidrug-resistance provides a growth advantage during ongoing antibiotic treatment not only in

individual patients but in the setting as a whole, and increases the risk of a higher load of spores to the environment (31). We found that the overall resistance was higher in HA-CDI compared with CA-CDI, although the difference was not significant. Additionally, only five isolates with multidrug resistance were found within the hospital, indicating an adequate infection control. Furthermore, few of the previous multi-resistant and outbreak-related types were found. This is markedly different from earlier time-points when multidrug-resistant RT046 was predominant in the county and prompted rigorous infection control measures to clear the outbreak (32). In line with the conclusions made in the UK study, in the absence of large outbreaks, isolates of HA-CDI reflect the reservoir of ribotypes circulating in the community (19). Interestingly, we found that resistance against clindamycin was significantly lower in isolates from Jönköping compared with isolates of the Swedish CDI surveillance from 2017/2018 (22) ($p = 0.042$). The proportion of isolates resistant to tetracycline, erythromycin and moxifloxacin was similar in both study populations (all $p > 0.1$) (data not shown).

Our study has several limitations. First, the study size was limited and therefore results should be interpreted carefully. The analysis of the molecular epidemiology should be repeated with higher numbers of CDI patients and isolates, for example in a larger national study. Second, we were not able to sequence all isolates, and there is a potential risk that we missed clusters. As *C. difficile* spores survive for long in the environment, it is important to consider that a genomic link between cases does not by default indicate new transmission events. Third, we did not have records for short-time visits to hospitals, leading to a possibility that some CA-classified cases could have been exposed to *C. difficile* spores within the hospital during said short-time visits, especially if they are recurrent for a longer time period (*e.g.* during dialysis sessions that are shorter than a full day but recurrent over a longer time period). Although *C. difficile* spores can be present in the hospital environment and asymptomatic colonization of *C. difficile* among staff and patients may occur, the risk of acquiring CDI is clearly related to the length of hospital stay (33,34). A recent report found an increased risk for CDI in family members of discharged hospitalized patients without diagnosed CDI (35). The relative risk increased from 1.30 for 1–3 days of hospital stay to 2.45 for >30 days of stay. Also, the transmission was most likely occurring within the household after discharge and not during short-time visits of family members to the hospital. While it remains crucial to identify reservoirs in the community or healthcare facilities to implement health hygiene measures that can prevent further CDI cases including asymptomatic transmission and transmission in outpatient care (35,36), we believe that short-time visits that lasts a few hours pose a low risk for CDI, and that errors in our classification of CA- and HA-cases were low. Despite the above-mentioned limitations, our study gives an indication about the molecular epidemiology of HA- and CA-CDIs. In Sweden, the overall incidence of *C. difficile* has, as in several other countries, decreased after introducing national surveillance programs and improved infection control in hospitals. However, the role of CA-CDIs should not be neglected. The high proportion of CA-CDI in our study emphasizes the need for further investigations to explore the role of common environmental sources, asymptomatic carriers and even foodborne and zoonotic transmission of *C. difficile*. Molecular methods such as ribotyping and WGS, in combination with epidemiological data, are crucial to identify transmission events. Early detection of clusters followed by adequate hygiene measures might help to limit transmission and spread in the community and subsequently enable further reduction of CDI incidence in Sweden.

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CONFLICT OF INTEREST

None.

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