Geographical clustering of cases of infection with moxifloxacin-resistant *Clostridium difficile* PCR-ribotypes 012, 017 and 046 in Sweden, 2008 and 2009

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We report the results of two nationwide surveillance studies of *Clostridium difficile* infection conducted during 2008 and 2009 in Sweden. The first study aimed to identify and quantify the proportion of *C. difficile* isolates with decreased susceptibility to moxifloxacin, particularly those of PCR-ribotype 027. From December 2007 to September 2008, 20 of 28 regional laboratories sent 585 isolates to the Swedish Institute for Infectious Disease Control for typing. A majority of the isolates (454 of 585; 78%) belonged to four PCR ribotypes (012, SE37, 017 and 046), all clustered in geographical regions. Only two type 027 isolates were found, both from the same patient. In the second study, involving all 28 regional laboratories, all consecutive *C. difficile* isolates collected during two time periods in 2009 (n=364) were typed and tested for susceptibility to clindamycin, erythromycin, moxifloxacin, metronidazole and vancomycin. The three most common PCR ribotypes were SE21, 001 and 020 (22% of all isolates). Types 012, 017, and 046 were geographically clustered and associated with decreased susceptibility to moxifloxacin, clindamycin and erythromycin. The extent of moxifloxacin prescription was highly variable among counties, indicating a need for careful monitoring of prescription rates to follow its role in *C. difficile* epidemiology.

Introduction

Clostridium difficile infection is a common nosocomial problem in elderly patients following antibiotic treatment [1]. *C. difficile* is typically isolated from faeces of 1–5% of healthy individuals, but colonisation increases upon admission to hospitals. Hospital-based studies performed in 1999 to 2001 showed that nosocomial spread of *C. difficile* was rare in Swedish hospitals in which infection control routines were implemented, although there were small outbreaks associated with multiresistant PCR-ribotype 012/SE17 [2–4]. Many outbreaks involving other *C. difficile* types have been reported in Europe and the rest of the world [5–8],

particularly those caused by moxifloxacin-resistant PCR-ribotype 027/PFGE NAP1 [9–17]. The expansion of certain *C. difficile* types during 2004 to 2008 prompted us to initiate a broader nationwide *C. difficile* surveillance strategy in Sweden. First, in an aim to find virulent type 027, a screening of *C. difficile* isolates with reduced susceptibility to moxifloxacin was performed during 2008. Second, a surveillance programme, including epidemiological typing and antibiotic susceptibility testing, was launched in 2009. Third, a web-based system for continuous voluntary laboratory reporting was initiated in 2009. This article presents the epidemiological situation of *C. difficile* in Sweden in 2008 and 2009 with respect to the occurrence of various PCR ribotypes and their antibiotic susceptibility.

Methods

Screening of *C. difficile* isolates with decreased susceptibility to moxifloxacin (2008)

An invitation to participate in the study, including instructions, methods and study protocol, was sent to all 28 regional clinical laboratories in Sweden. Faecal specimens sent by the clinicians for routine *C. difficile* detection were tested in the regional laboratories according to their standard methodology (ELISA/EIA was the most commonly used method, in 24 of the 28 laboratories, followed by cell cytotoxicity assay). Sample material that was positive for *C. difficile* was inoculated anaerobically on taurocholate-cefoxitin-cycloserine-fructose agar (TCCFA) for 48 hours and toxigenic *C. difficile* colonies were subcultured on blood agar. Susceptibility to moxifloxacin was tested using disc diffusion (5 µg) on IsoSensitest agar (Oxoid, United Kingdom) supplemented with 5% defibrinated horse blood and 20 µg/ml beta-NAD incubated at 36 °C for 48 hours. *C. difficile* strains with an inhibition zone diameter >16 mm (ATCC9689) and 6 mm (Ø07-1702) were used as controls. All *C. difficile* isolates with zone diameter <16 mm were sent to the Swedish Institute for Infectious Disease Control for typing. A protocol with

strain number, diameter of the inhibition zone, and, if possible, the total number of screened isolates were included each shipment. Upon arrival, samples were inoculated on both TCCFA and blood agar and incubated anaerobically at 36 °C for 48 hours. Isolated strains were verified for susceptibility to moxifloxacin using the disc diffusion method described above and Etest (AB Biodisk, Sweden). Of 641 sent samples, 37 were excluded because *C. difficile* could not be isolated and/or the sample was contaminated; a further 19 isolates

were omitted due to an inhibition zone diameter >16 mm, giving a final 585 *C. difficile* isolates. The study period was from 7 December 2007 to 1 September 2008. Since no patient data were included, we could not judge how many patients or how many cases of *C. difficile* infection there were during the study period.

FIGURE 1

Proportion of *Clostridium difficile* PCR ribotypes^a with decreased susceptibility to moxifloxacin, Sweden, 2008 (n=585)

^a The three-digit international nomenclature is used for typing, or when reference isolates are missing, SE-type is used.

^b Ribotypes represented by fewer than three isolates per type.

National surveillance typing (2009)

An invitation to participate in the surveillance programme, including methods and study protocol, was sent to all 28 regional Swedish laboratories. All consecutive strains isolated from *C. difficile*-positive samples obtained through routine detection during 9–15 April and 21–27 September 2009 were sent to the Swedish Institute for Infectious Disease Control for typing and susceptibility testing for moxifloxacin, erythromycin, clindamycin, metronidazole and vancomycin. Of 432 samples sent, 40 were excluded because they were contaminated or not viable. Of the remaining 392 isolates, 28 were not analysed because their dates of isolation did not comply with the inclusion criteria above, giving a final total of 364 isolates.

Susceptibility testing was performed using Etest on Mueller-Hinton agar medium (BD Diagnostics) at 35°C for 48 hours. A bacterial density of McFarland 1 was used to obtain a next-to-confluent layer of *C. difficile* bacteria. There were no significant differences in minimum inhibitory concentration (MIC) values using Mueller-Hinton or IsoSensitest agar (data

FIGURE 2

Geographical distribution of dominating *Clostridium difficile* PCR ribotypes with decreased susceptibility to moxifloxacin, Sweden, 2008 (n=454)

^a The frequency is calculated as the number of isolates of the indicated type divided by the total number of screened isolates in each county multiplied by 100.

^b Due to missing numbers of total screened isolates during the study period from some laboratories, calculation of frequency was only possible for 10 of the 21 counties.

not shown). Breakpoints were chosen according to the European Committee on Antimicrobial Susceptibility Testing (EUCAST) tentative ecological cut-off for detection of isolates with resistance mechanisms, as suggested by the European Study Group for *C. difficile* [18].

PCR ribotyping

PCR ribotyping was performed according to the method of Stubbs et al. [8], with modifications described in Svenungsson et al. [4]. Reference isolates were obtained from the Cardiff-European Centre for Disease Prevention and Control (ECDC) collection of *C. difficile* strains (obtained from Ed J. Kuijper, Leiden University Center, Leiden, the Netherlands). Bionumerics software version 6.1 (Applied Maths NV, Sint-Martens-Latem, Belgium) was used for the analysis. The three-digit international nomenclature was used for typing or, when reference isolates were missing, an SE-type was given.

Prescription of antibiotics

Sales data of antibiotics were obtained from Apotekens Service AB, which is responsible for compilation of all

drug statistics, including sales, in Sweden. The data include antibiotics prescribed to patients as well as sales to hospitals and other healthcare providers.

Statistical methods

Fisher's exact test and chi-square tests were performed to examine differences in geographical distribution of ribotypes using R v. 2.10.1. Kaleidagraph v. 4.02 (Synergy software) was used for data management and graphics. To avoid type-I error due to multiple statistical tests, a significance threshold of $p=0.005$ was applied.

Results

C. difficile isolates with decreased susceptibility against moxifloxacin (2008)

A total of 20 of the 28 Swedish regional laboratories covering 15 of the 21 of the counties supplied a total of 585 isolates with reduced susceptibility to moxifloxacin. Of the 15 counties, 10 supplied data on the number of screened isolates: 546 of 2,702 isolates (20%; range: 9–34%) had decreased susceptibility to moxifloxacin. Of the 585 isolates (from the 15 laboratories), 454 (78%) belonged to four PCR ribotypes: 012, SE37, 017 and 046 (Figure 1). Types 012 and 046 were found mainly in the central and southern part of Sweden, while types 017 and SE37 were predominant in the central-east counties of Gävleborg and Stockholm, respectively (Figure 2, shown in purple). The distributions for each of the types 012, 017, 046, 078 and SE37, compared with that of other types, were highly skewed between counties: a chi-square test among counties having more than 10 isolates available for typing gave a p value of <0.001 for each of the types 012, 017, 046, 078 and SE37 (for 9 of the 21 counties). Only two type 027 isolates were found, both from the same patient.

National surveillance typing programme (2009)

Of 364 *C. difficile* isolates supplied by all 28 regional laboratories, type SE21 was found in highest number followed by types 001, 020, 078, 012 and 023 (Figure

FIGURE 3

Proportion of *Clostridium difficile* PCR ribotypes^a, Sweden, 2009 (n=364)

^a The three-digit international nomenclature is used for typing, or when reference isolates are missing, SE-type is used.

^b Ribotypes represented by fewer than three isolates per type.

FIGURE 4

PCR ribotype patterns of the *Clostridium difficile* SE21/014/020 group^a, Sweden, 2009 (n=8)

ECDC: European Centre for Disease Prevention and Control; NT: national typing.

^a *C. difficile* strains from the 2009 study, designated NT, and the Cardiff-ECDC collection are shown. The dendrogram was produced using cluster correlation, Pearson correlation and the unweighted pair group method for arithmetic averages. The scale shows similarity as a percentage.

3). The PCR ribotype pattern of type SE21 was related to those of types SE21a/o14 and SE21b/o20 (Figure 4): because of their similarities, this group of ribotypes has sometimes been called the 'o14/o20' group (see Discussion). However, in our study we judged these as separate types. Type o12, o17 and o46 were abundant in the same geographical regions as in 2008 and the geographical distributions of these types were significantly asymmetric (Figure 5) ($p < 0.001$ each, Fisher's exact test; all 21 counties). Other types were not clustered or were only weakly so, for example, o01 ($p = 0.95$), o14 ($p = 0.022$), o20 ($p = 0.012$), o23 ($p = 0.71$), o78 ($p = 0.31$), SE21 ($p = 0.14$) and SE37 ($p = 0.23$). One type o27 isolate was detected but it showed a MIC of <4

mg/L against moxifloxacin, which is not characteristic for the epidemic and hypervirulent type o27.

In total, 57 of the 364 (16%) isolates showed decreased susceptibility to moxifloxacin ($\text{MIC} > 32$ mg/L); all other isolates displayed MIC values between 0.25 mg/L and 2 mg/L). The geographical distribution of isolates with a $\text{MIC} > 32$ mg/L was skewed ($p < 0.001$, Fisher's exact test, $n = 14$, with counties reporting fewer than 10 isolates excluded) and 46 of 57 isolates belonged to type o12, o17, o46 and SE37 (Table). In total, 60 of 364 (16%) isolates showed decreased susceptibility to clindamycin and, again, a majority (46 of 60) isolates belonged to type o12, o17, o46 and SE37 (Table). Of the 364 isolates, 62 (17%) showed decreased susceptibility to

FIGURE 5

Geographical distribution of *Clostridium difficile* PCR ribotypes associated with decreased moxifloxacin susceptibility in all 21 counties, Sweden, 2009 ($n = 57$)

^a The frequency is calculated as the number of isolates of the indicated type divided by the total number of screened isolates for each county multiplied by 100.

TABLE

Proportion of *Clostridium difficile* PCR ribotypes with decreased susceptibility to moxifloxacin, clindamycin and erythromycin, Sweden, 2009 ($n = 364$)

PCR ribotype	Number of isolates tested	Number of isolates with decreased antibiotic susceptibility ^a		
		Moxifloxacin ($\text{MIC} > 4$ mg/L)	Clindamycin ($\text{MIC} > 16$ mg/L)	Erythromycin ($\text{MIC} > 2$ mg/L)
o12	20	19	18	15
o17	11	10	11	10
o46	12	10	10	10
SE37	8	7	7	7
Other	313	11	14	20

MIC: minimum inhibitory concentration.

^a Breakpoints were chosen according to the European Committee on Antimicrobial Susceptibility Testing (EUCAST) tentative ecological cut-off for detection of isolates with resistance mechanisms, as suggested by the European Study Group for *C. difficile*.

erythromycin (Table). No isolate was resistant to metronidazole or vancomycin.

Prescription of moxifloxacin in Sweden in 2008 and 2009

The mean prescription rate of moxifloxacin per county was 0.020 defined daily dose (DDD) per 1,000 inhabitants per day for 2008 and 2009. The prescription rate differed markedly between counties (range: 0–0.088 DDD per 1,000 inhabitants per day) and was highest in the south-east of Sweden (Figure 6). Some of the counties in the south-east also had a high proportion of PCR ribotypes 012 and 046 (Figure 5). However, the prescription rate of moxifloxacin was comparatively low in the counties where types 017 and SE37 dominated.

Discussion and conclusion

PCR ribotyping has been used for studying *C. difficile* epidemiology for several years, and details of the first large *C. difficile* PCR ribotype library were published by Stubbs et al. in 1999 [8]. When the European Centre for Disease Prevention and Control (ECDC) and the Centre for Infectious Disease Control Netherlands initiated a pan-European *C. difficile* infection survey (ECDIS) study in 2008 [19], a *C. difficile* reference strain collection with defined PCR ribotypes was formed (the Cardiff-ECDC collection). One purpose of the collection was to support national laboratories in setting up local typing systems and to aid surveillance studies and international comparisons. We found that a majority of the *C. difficile* strains in Sweden was represented in this

reference strain collection. Exceptions were type SE37, for example, which was confirmed in the ECDIS study as one of six novel types (PCR ribotype 231) [19]. Another unknown type was SE21, which closely resembled types SE21a and SE21b as well as reference strains of PCR-ribotypes 014 and 020 [4]. Type SE21 was verified as a member of the 014/020 group: in total this group comprised 19% of all *C. difficile* isolates in our study in Sweden in 2009. The proportion of the 014/020 group among all *C. difficile* isolates was 16% in Europe in 2008 [19].

Much interest in recent years has been focused on the worldwide spread of the hypervirulent *C. difficile* type 027 [20,21]. Outbreaks in northern Europe in 2007 to 2009 [10–11,13,22] prompted us to screen for type 027 isolates with decreased susceptibility to moxifloxacin. Of the 585 isolates analysed during 2008, only two type 027 isolates were detected (from the same patient). Furthermore, since no type 027 isolate with reduced susceptibility to moxifloxacin was identified in 2009, such isolates are probably rare in Sweden (compare with [23]). From previous geographically localised studies in Sweden in 1999 to 2001, the six most common types were 014/SE21, 012, 001, 020, 002 and 005 [2–4]. All these were still among the most common types in 2009. There were thus only small changes in the distribution of major types over a period of 10 years, a result quite different from the shift in strain types that has occurred in England [7]. One exception might be type 078, which was the fourth most common type in 2009. Type 078 may be an emerging type in Europe and is commonly found in domestic animals, suggesting transmission through the food chain [24–26].

The studies presented here have several weaknesses. All laboratories did not report a full set of data, particularly from the first screening study. This was mostly due to the fact that several laboratories were in the process of setting up culturing methods and were thus unable to supply isolates from the first day of the study period. Also, the two studies were not of identical design: the first aimed to collect consecutive moxifloxacin-resistant isolates during a longer time period and the second collected all isolates during two shorter time periods. In addition, the short study periods in 2009 implies a poor detection of seasonal variability. We also did not ask for detailed information about the number of patients or the number of cases during the study periods – thus the true prevalence could not be calculated. Despite these limitations, the regional clustering of certain types observed in 2008 was confirmed in 2009. Some of the remaining questions will be answered by the voluntary reporting system, but since all laboratories are not yet participating, it is too early to draw any conclusions from this system.

The proportion of *C. difficile* isolates with reduced susceptibility to moxifloxacin was 16% in Sweden 2009 and thus less than half of the 37.5% reported in Europe in 2005 [5]. Decreased susceptibility to moxifloxacin

FIGURE 6

Extent of prescription of moxifloxacin by county, Sweden, 2008 and 2009

has been found in several *C. difficile* types isolated in Europe and worldwide, and appears to be common in dominant types including 001, 018, 027 and 106 in, for example, England [7], Scotland [27], Germany [28] and Italy [5]. We found decreased susceptibility mainly in types 012, 017, 046 and 231/SE37. Type 012 is related to serogroup C [29] and has been reported as a common type in Sweden, Greece and Hungary [2,19] while toxin A-negative, toxin B-positive type 017 has been found to be associated with outbreaks in, for example, Poland, Canada and the Netherlands [6,30]. Regarding type 231/SE37, the proportion of this type was 0.8 % at the Karolinska University Hospital, Huddinge, Sweden, in 1999 [4], but there is no information on whether this type has caused any outbreaks in other countries.

Despite reports of increased prevalence of moxifloxacin resistance in *C. difficile* [5,31-33], including outbreaks of *C. difficile* 027 occurring shortly after replacing levofloxacin with moxifloxacin/gatifloxacin [34], it is currently unclear whether moxifloxacin use implies higher risk for *C. difficile* infection than other commonly used antibiotics [35-38]. Thus, we can neither confirm nor exclude the possibility that moxifloxacin has contributed to the selection of types 046 and 012 in certain Swedish counties. In this context, it should be pointed out that types 012, 017, 046 and 231/SE37 had decreased susceptibility also to clindamycin and erythromycin. Moreover, type 012 was shown to be resistant to many other antibiotics that were not tested in this report [32]. Thus, their success as pathogens may just reflect general selection due to the overall antibiotic use in healthcare settings [7]. Nevertheless, the cause for the variable prescription rates of moxifloxacin in Sweden needs to be investigated further.

In conclusion, in our two nationwide *C. difficile* surveillance studies in Sweden in 2008 and 2009, there was a geographical clustering of PCR-ribotypes 012, 017, 046 and a novel type (231/SE37), suggesting transmission and local outbreaks. Our results support the need for a central European or international *C. difficile* database with capability to continuously report and characterise emerging types, as well as a national surveillance system to monitor and control *C. difficile* incidence, type distribution, resistance pattern and related antibiotic use.

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