Increased number of *Clostridium difficile* infections and prevalence of *Clostridium difficile* PCR ribotype 001 in southern Germany

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In recent years, *Clostridium difficile* infection (CDI) has emerged as an increasing problem, both in in- and outpatients. In a rural region of southern Germany, the annual number of *C. difficile* toxin (Tcd)-positive patients has increased from 95 to 796 in the period from 2000 to 2007. Simultaneously, the proportion of positive tests among all Tcd examinations has risen from 7.0% to 12.8%, indicating that the higher number of affected patients was not solely due to an increase in the number of assays. Elevated numbers of CDI have recently been associated with outbreaks of the ribotype 027 strain, particularly in North America. This strain has also been isolated in Europe, including in Germany. Ribotyping and PCR testing for binary toxin genes of *C. difficile* strains isolated from in- and outpatients demonstrate a predominance (59%) of *C. difficile* ribotype 001, which exhibits antibiotic resistance to erythromycin, ciprofloxacin, and moxifloxacin, but lacks binary toxin genes. In summary, in our region of Germany, the number of patients affected by CDI has increased, probably due to spread of *C. difficile* ribotype 001.

Introduction

Numbers of *Clostridium difficile* infections (CDI) are increasing in- and outside of Europe [1-5]. CDI in North America and partly also in western Europe have often been attributed to outbreaks caused by the hypervirulent strain NAP1/027 containing the binary toxin genes cdtA and cdtB [1,3,6]. Recently, this strain has also been isolated from patients in western Germany [7]. Different *C. difficile* strains are isolated in different European countries, suggesting a prevalence of particular strains in local settings [8-10].

CDI is usually regarded as a nosocomial infection that can be minimised by robust infection control practices and good antibiotic stewardship. In some hospitals in Europe it has become the most frequent nosocomial disease and consequently, analyses of *C. difficile* epidemiology were restricted to hospital outbreaks [11]. However, community-acquired cases of CDI have been observed for a few years now [12,13]. Interestingly, *C. difficile* strains associated with CDI in hospitalised patients were different from the ones isolated from community cases [13].

Our laboratory is located in a rural area in southern Germany. In this region, CDI is noticed as a growing nosocomial problem with sporadic fatal cases. However, the available information about the real extent of this apparent increase in CDI is limited. Furthermore, no studies have been done on distinct *C. difficile* strains in Germany or defined regions in Germany. We therefore collected data on the number of patients known to excrete *C. difficile* toxin (Tcd) in stool and on the number of patients analysed for Tcd. PCR was performed on *C. difficile* isolates from outpatients and from patients treated in two hospitals located in southern Germany, in order to gain knowledge about the epidemiological background of these regional strains.

Here we present data about the prevalence of a quinolone- and erythromycin-resistant *C. difficile* ribotype 001 strain in southern Germany.

Patients and methods

Laboratory and hospitals setting

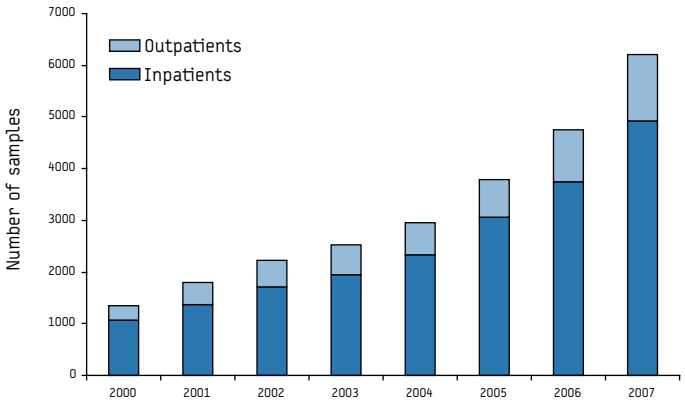
The Synlab Medical Care Service analyses laboratory samples from about 40 hospitals and more than 2,000 physicians serving outpatients. In 2006 a total of 161,000 microbiological samples were examined. *C. difficile* was isolated from Tcd-positive stool samples from patients diagnosed at two hospitals (A and B) and from outpatients. Hospital A is a primary health care hospital with 270 beds comprising two tertiary university hospital facilities (cardiology, gastroenterology). In 2006, 10,793 patients were admitted to that hospital (74,146 patient days). Hospital B is a primary health care hospital with 135 beds, and 4,886 patients (34,811 patient days) were admitted to that clinic in 2006.

Epidemiolologic analysis of *C. difficile* in South Germany

Numbers of Tcd-positve stool samples and numbers of Tcd-positive patients were evaluated by the Hybase system (Cymed AG, Bochum, Germany) linked to the laboratory data system "promed open" (mcs, Eltville, Germany). Hybase (http://www.cymed.de/download_hy.php) is a computer programme that supports the surveillance of bacterial pathogens, e.g. calculation and documentation of the number of notifiable bacterial species.

Figure 1

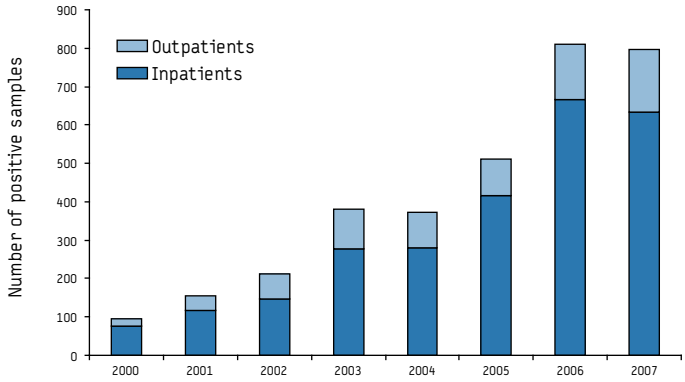
Number of patients examined by Tcd ELISA, southern Germany, 2000–2007



Tcd: *C. difficile* toxin; ELISA: enzyme-linked immuno-sorbent assay.

Figure 2

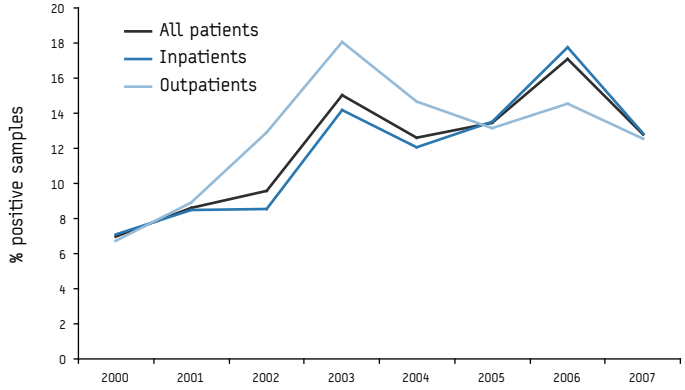
Number of Tcd-positive patients, southern Germany, 2000–2007



Tcd: *C. difficile* toxin.

Figure 3

Percentage of Tcd-positive patients, southern Germany, 2000–2007



Tcd: *C. difficile* toxin.

Data (age, sex, outpatients versus inpatients, taking into account where they were treated) from patients with Tcd-positive stool in 2006 were documented.

C. difficile toxin analysis, culture and antibiotic susceptibility testing

Stool samples from inpatients of hospitals A and B were collected between May 2006 and March 2007 and tested for *C. difficile*. Samples from outpatients were collected between March and April 2007.

Tcd was examined by using an enzyme-linked immunosorbent assay (ELISA) detecting toxin A and B (R-Biopharm AG, Darmstadt, Germany). Bacterial cultures were grown on *C. difficile*-selective agar containing cefoxitin and cycloserin (Heipha, Eppelheim, Germany; www.heipha.de/db/files/209e.pdf) under anaerobic conditions.

Identification of *C. difficile* was performed on rapid ID 32 A system (identification system for anaerobes, Biomerieux, Nürtingen, Germany). Antibiotic susceptibility was tested using ATB ANA strips (susceptibility test for strict anaerobic bacteria, Biomerieux, Nürtingen, Germany) according to the manufacturer´s instructions or alternatively in an E-test procedure (for erythromycin, ciprofloxacine, moxifloxacine, cefotaxime; AB-Biodisk, Solna, Sweden). E-test results were confirmed at the German consiliary laboratories for *C. difficile* (Mainz) or gastrointestinal infections (Freiburg). Presence of binary toxin genes was examined at the German consiliary laboratory for *C. difficile* (Mainz) according to Stubbs et al. [14].

Ribotyping of *C. difficile* strains

Ribotyping was performed at the German consiliary laboratory for gastrointestinal infections (Freiburg). PCR ribotyping was performed according to he protocol of Bidet et al. [15] resulting in so-called "ribotype Freiburg". In previous comparative analyses, representative isolates of each ribotype Freiburg had been sent to the Anaerobe Reference Laboratory in Cardiff for re-typing according to the "Cardiff" PCR ribotyping library in order to establish the correlation between ribotype Freiburg and the commonly used ribotype nomenclature of Stubbs et al. [16]. It was therefore possible to relate local PCR results not only to "ribotype Freiburg" but also to European C. difficle ribotypes.

Results

Over the past years, reported numbers of patients affected by *C. difficile* infection (CDI) have increased markedly in Germany [4]. Figures 1-3 show a comparison of the number of stool samples tested for *C. difficile* toxin (Tcd) with the number of Tcd-positive stool samples in the period between 2000 and 2007.

The number of patients analysed for Tcd increased by 458% (from 1,358 to 6,214; Figure 1), but the actual number of Tcd-positive samples increased by 838% in the same period of time (from 95 to 796; Figure 2). The percentage of Tcd-positive patients increased from 7.0% in 2000 to 12.8% in 2007, with two peaks in 2003 (15.0%) and in 2006 (17.1%; Figure 3). As demonstrated by Figure 3, the peak in 2003 predominantly resulted from a high proportion of Tcd-positive outpatients (18.0%). In contrast, the peak in 2006 was caused by Tcd-positive inpatients (17.8%).

In summary, these data indicate that the increasing numbers of CDI in this region are real and not simply a result of increasing

analysis efforts. Furthermore, not only hospitalised patients but also non-hospitalised patients were affected by CDI.

Previous reports have identified high age as an important risk factor for contracting CDI [4,5]. A representative list concentrating on the age and sex distribution of patients who had Tcd-positive stools in 2006 is shown in Table 1.

A total of 784 patients were registered in our database, 17.3% of which were outpatients. Looking at the median age, the majority were elderly patients. Interestingly, the median age of outpatients (69 years) was lower than that of inpatients (77 years). In addition, Tcd-positive women tended to be older than Tcd-positive men.

To assess the cause for the increasing numbers of Tcd-positive patients via spread of hypervirulent *C. difficile* 027, ribotyping of *C. difficile* was performed on isolates from Tcd-positive stool samples previously collected from outpatients and from patients treated in two different hospitals in southern Germany (Hospitals A and B).

As shown in Table 2, at least seven different *C. difficile* ribotypes could be identified. While *C. difficile* ribotype 001 was isolated from 11 patients, other types were only isolated once from a single patient. *C. difficile* ribotype 001 was isolated from inpatients of both hospitals and was also common in outpatients indicating a predominance of this strain in this region.

Ribotype 001 *C. difficile* lacked the binary toxin genes but was resistant to quinolone antibiotics (ciprofloxacine, moxifloxacine) as well as to erythromycin, cefotaxime (MIC >16 µg/mL) and clindamycin. However, ribotype 001 strains were susceptible to ampicillin/sulbactam, piperacillin, piperacillin/tazobactam, imipenem, vancomycin and metronidazole.

Discussion

Worldwide – as well as in Germany – there is a discussion about increasing case numbers of CDI-affected patients [1-5]. In this study we demonstrate that the number of Tcd-positive patients increased markedly in southern Germany in the period between 2000 and 2006. It was assumed that this might be a result of intensified examination efforts, as from 2000 to 2007, the total number of stool samples examined for Tcd per year increased, too. However, the percentage of Tcd-positive patients also increased markedly during this period (from 7.0 to 12.8%) showing a maximum in 2006 (17.1%). This higher ratio indicates that the increased number of Tcd-positive patients is a real phenomenon and not solely due to the fact that examination efforts were stepped up.

Between 2006 and 2007, the number of CDI-affected patients remained constant, although the number of patients examined for CDI increased. This finding suggests that the intensified infection control measures may have been successful in preventing the nosocomial spread of *C. difficile*. However, the possibility to separate between nosocomial and community acquired CDI is limited by the lack of patient data.

In agreement with earlier studies [4,5], Tcd-positive stool samples were mainly obtained from elderly patients. The fact that 136 of 784 Tcd-positive patients (17.3%) in 2006 were outpatients clearly shows that CDI was not restricted to hospitalised patients. On the other hand, the median age of Tcd-positive inpatients was higher than that of Tcd-positive outpatients, an indication that CDI in younger people has a milder course and does not require hospital admission.

The *C. difficile* 027 strain was detected in Germany for the first time in 2007 [7]. However, the ribotyping results presented here reveal that this strain was not prevalent in northern Bavaria. In contrast, multi-resistant *C. difficile* 001 were frequently found.

Table 1

Number and characteristics of in- and outpatients with Tcd-positive stool samples, southern Germany, 2006

	Outpatients	Inpatients (all hospitals)	Hospital A	Hospital B
Number of patients	136	648	45	34
CDI per 1,000 admissions			4.2	6.1
CDI per 10,000 patient days			7.0	9.0
Proportion of positive Tcd analyses (%)	14.6	17.8	16.4	13.9
Age distribution				
Age of patients, median (mean)	69.0 (62.3)	77 (73.1)	75 (72.6)	80.5 (76.6)
Number of patients <6 years	6	4	0	1
Number of patients <21 years	8	14	1	0
Number of patients 21-79 years	79	325	25	15
Number of patients >79 years	35	258	18	11
Number of patients >89 years	8	47	1	7
Sex distribution				
Number of female patients	77 (56.6 %)	373 (57.6 %)	27 (60 %)	19 (55,9 %)
Age females, median (mean)	68 (62.3)	79.0 (76.1)	79 (75,48)	81 (82.0)
Number of male patients	59 (43.4 %)	275 (42.4 %)	18 (40.0 %)	15 (44.1 %)
Age males, median (mean)	69,0 (63.3)	73.0 (69.2)	71 (68.39)	75 (69.8)

Tcd: *C. difficile* toxin

For this analysis, *C. difficile* were cultured from Tcd-positive stool samples from in- and outpatients. The hospitalised patients had been treated at two hospitals located about 200 km apart. Since *C. difficile* type 001 was also isolated from outpatients, it is obvious that this strain is predominant in southern Germany.

All tested ribotype 001 *C. difficile* proved to be resistant to erythromycin and moxifloxacine in the antibiotic susceptibility testing, a feature commonly observed for ribotypes 001, 027 and 106 [6,17]. Ribotyping and binary toxin gene analysis showed that all of these *C. difficile* strains were different from the NAP1/027 strain. Recently, it has been discussed whether ribotype 027 strains could be more virulent than other ribotypes [11,18]. Only scarce clinical information - reported anecdotally - is available about the death of several patients. Nevertheless, it is clear that severe courses of CDI in our region are not limited to ribotype 027 isolates.

Ribotyping further revealed that more than 50% of *C. difficile* isolates exhibited identical features, a possible indication of clonal spread within the local population. In the case of increased CDI case numbers due to admission of affected patients bearing predominantly ribotype 001, proven clonality of *C. difficile* isolates by ribotyping might erroneously suggest nosocomial spread. Under the given circumstances of many *C. difficile* isolates being clonally related, this typing method therefore provides only limited information for outbreak analyses in a defined hospital. Consequently, use of more discriminatory typing methods, e.g. multi-locus variable-number tandem repeat analysis (MLVA), may be better suited for future epidemiological studies, at least if ribotype 001 or other frequently occuring ribotypes are involved [19].

In summary, the present study shows an increase of Tcd-positive patient numbers in southern Germany. Multi-resistant *C. difficile* ribotype 001 is prevalent in southern Germany, and this strain is thought to be responsible for severe, if not fatal, cases of CDI. In due course, more discriminatory methods may be able to improve our understanding of the epidemiology of this successful strain.

Acknowledgements

The authors thank Andrea Kick, Angela Galbakioti, Corinna Kreisl, Kerstin Müller, Birgit Scherer, and Rebecca Arnold for excellent technical assistance.

Table 2

Characterisation of *C. difficile* isolates obtained from Tcd-positive stool samples, collected between May 2006 and March 2007 in southern Germany

Source, Age, Sex	Typing		Binary toxin genes		MIC (µg/ml)		
	Ribotype Cardiff	Ribotype Freiburg	*cdtA*	*cdtB*	Ery	Moxi	Cipro
A, 87, m	001	45	-	-	>256	>32	>32
B, 73, f	001	45	-	-	>256	>32	>32
A, 83, m	001	45	-	-	>256	>32	>32
B, 78, f	001	45	-	-	>256	>32	>32
B, 81, f	001	45	-	-	>256	>32	>32
A, 75, m	n.d.	n.d.	+	+	n.d.	n.d.	n.d.
A, 66, m	078	40	+	+	0.75	2	>32
A, 73, m	049	22	-	-	1.0	1	>32
A, 67, f	014	1	-	-	0.5	1,5	>32
B, 14, f	015	8	-	-	0.75	1	>32
B, 75, f	001	45	-	-	>256	>32	>32
B, 88, f	n.d.	n.d.	-	-	>256	>32	>32
A, 83, f	n.d.	n.d.	-	-	0.75	1,5	>32
A, 89, f	001	45	-	-	>256	>32	>32
Out, 63, m	042	21	-	-	0.5	1,5	>32
Out, 89, f	001	45	-	-	>256	>32	>32
Out, 64, m	001	45	-	-	>256	>32	>32
Out, 56, f	081	16	-	-	0.5	1	>32
Out, 31, f	n.d.	n.d.	-	-	>256	1	>32
Out, 77, f	001	45	-	-	>256	>32	>32
Out, 82, m	001	45	n.d.	n.d.	>256	>32	>32
U	001	45	n.d.	n.d.	>256	>32	>32

Patients had been treated either at hospital A or B or had been outpatients (Out). Minimal inhibitory concentrations (MIC) of erythromycin (Ery), ciprofloxacine (Cipro) and moxifloxacine (Moxi) were determined by E-test. Only two isolates exhibited binary toxin genes (*cdtA, cdtB*). One strain obtained from a university hospital in south-western Germany (U) was also included.
n.d. = not determined; Tcd: *C. difficile* toxin.
Ribotype Cardiff represents the European ribotype.

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This article was published on 4 December 2008.

Citation style for this article: Borgmann S, Kist M, Jakobiak T, Reil M, Scholz E, von Eichel-Streiber C, Gruber H, Brazier JS, Schulte B. Increased number of Clostridium difficile infections and prevalence of Clostridium difficile PCR ribotype 001 in southern Germany. Euro Surveill. 2008;13(49):pii=19057. Available online: http://www.eurosurveillance.org/ViewArticle.aspx?ArticleId=19057