Epidemiology of Clostridium difficile infections in the Netherlands and Europe: implications for surveillance and control

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To all hospital
cleaning personnel

Chapter 1

Introduction

Clostridium difficile infection (CDI) was discovered in 1978 as an important cause of antibiotic-associated diarrhoea and pseudomembranous colitis [1]. CDI became the most common healthcare-associated infection in Northern-America and Europe during the antibiotic era, especially after global spread of a fluoroquinolone-resistant ribotype 027 strain originating from the Canadian province Quebec in 2003 [2, 3]. The rise of CDI in Northern-America and Europe urged the use of epidemiological surveillance systems to monitor disease dynamics and rapidly detect outbreaks [3]. In Europe, national surveillance activities were supported by the European Centre for Disease Prevention and Control (ECDC) gradually moving towards standardised epidemiological surveillance with molecular typing systems for CDI [4]. However, valid estimations of the infection burden of CDI in Europe were hampered by the heterogeneity and insufficiency of diagnostic algorithms for CDI, lack of standardised typing systems and incomplete surveillance methodologies. This thesis includes two studies conducted within a project named 'the European CDI Surveillance Network' (ECDIS-Net) focussing on enhancement of CDI surveillance and laboratory capacity for CDI in Europe. In the Netherlands, a sentinel epidemiological surveillance system monitors the incidence of CDI and the occurrence of outbreaks in hospitals, but not in other healthcare facilities or in the community. This thesis describes (spatial) trends in the epidemiology of CDI in the Netherlands according to sentinel surveillance, in particular for children and the potentially zoonotic *C. difficile* ribotype 078. Data of a community-based case-control study to estimate the burden of CDI in the community, was used to apply spatial scan statistics to detect CDI clustering beyond the hospital setting. Finally, this thesis provides directions for future epidemiological surveillance systems of CDI, both in the Netherlands and Europe.

Clostridium difficile

COMMENSAL AND PATHOGEN

Clostridium difficile is a ubiquitous gram-positive, spore-forming, rod-shaped bacterium, firstly observed in the bacterial flora of meconium from healthy neonates in 1935 by Hall and O'Toole [5]. The bacterium was called "*Bacillus difficile*" for its difficulty to be cultured, growing exclusively under anaerobic conditions. The bacterium did not cause any symptoms in the neonates studied, but appeared to be highly pathogenic in animal models due to production of an exotoxin [5]. It was not just a matter of chance that *C. difficile* was firstly isolated from neonates; *C. difficile* colonisation is the highest during early life in humans (prevalence of 26%, with a range of 18-90%) [6, 7].

C. difficile did not appear to be an important cause of disease before the antibiotic era [8 – 11]. The introduction of antibiotics, especially clindamycin in the 1960s, led to an increase of antibiotic-associated colitis [11 – 13]. In 1978, Bartlett et al. recognised toxins of *C. difficile* to be the cause of antibiotic-associated pseudomembraneous colitis in adults [1]. The bacterium was renamed "*Clostridium difficile*" (κλωστήρ: Greek for rod) [14]. Recently, the bacterium has been reclassified as *Clostridioides difficile* [15]. Subsequent studies confirmed antibiotic use to be the foremost risk-factor for *C. difficile* infection (CDI) [11]. Antibiotics create a niche for *C. difficile* growth and toxin production in the gut due to loss of other microbial communities that compete for nutrients and/or interfere with more specific metabolic pathways (e.g. bile acid metabolism) [16, 17]. During or soon after antibiotic use, the odds for getting CDI increase a 7–10-fold, and gradually normalise during a period of 3 months [18, 19]. The risk is dose dependent [20] for and the highest for (third-generation) cephalosporins and clindamycin [21, 22]. Disruption of the gut microbiota is not the exclusive preserve of antibiotics; also other drugs, such as proton pump inhibitors, affect the gut microbiota and increase the risk for CDI [23].

C. difficile belongs to the Peptostreptococcaceae family [15, 24] within the genus of Clostridia with more than 150 species. Around 15 of these species, including *C. difficile*, *C. perfringens*, and *C. tetani*, are capable to produce toxins, and thereby cause infection in humans [14]. *C. difficile* is known for its potential to produce a toxin A (tcdA), a toxin B (tcdB), and a binary toxin (*C. difficile* transferase; CDT) [25]. Some *C. difficile* subspecies carry one or more toxin genes ('*toxigenic*' strains) and these toxin genes can be acquired and lost though time [26]. Toxin A and B inactivate regulatory proteins (Rho-GTPases) of the cytoskeleton, and thereby cause apoptosis of the epithelial cells in the gut [27, 28]. Binary toxin is a ADP-ribosylating toxin that induces formation of microtubule-based protrusions that might facilitate adherence and colonisation [29, 30]. Its role in *C. difficile* virulence is a topic of debate [31]. *C. difficile* contains many other factors to invade and survive hostile circumstances [32]. For example, specific adhesion molecules on the cell surface of *C. difficile* were found to facilitate the binding and release from target cells [33]. The ecological fitness of *C. difficile* –linked to its ability to cause disease

and emerge– varies per genotype [34]). The exceptional ecological fitness of a genotype named PCR ribotype 027/NAP1 facilitated its worldwide transmission since 2003 and ability to cause outbreaks [2, 34].

Like other clostridia, *C. difficile* sporulates to survive the aerobic conditions outside the hosts gut, indispensable for transmission to other hosts (Figure 1) [35]. *C. difficile* spores are metabolically dormant and have a spore coat covered by an exosporium, causing resistance to other environmental stress-factors (e.g. antiseptics) as well [35, 36]. Besides, sporulation helps the bacterium to resist antibiotics and the host immune system after digestion [35]. *C. difficile* spores are excreted by both symptomatic as asymptomatic humans and animals [37, 38]. Yet, symptomatic patients have higher levels of skin and environmental contamination than asymptomatic carriers (Risk Difference 20%) [39].

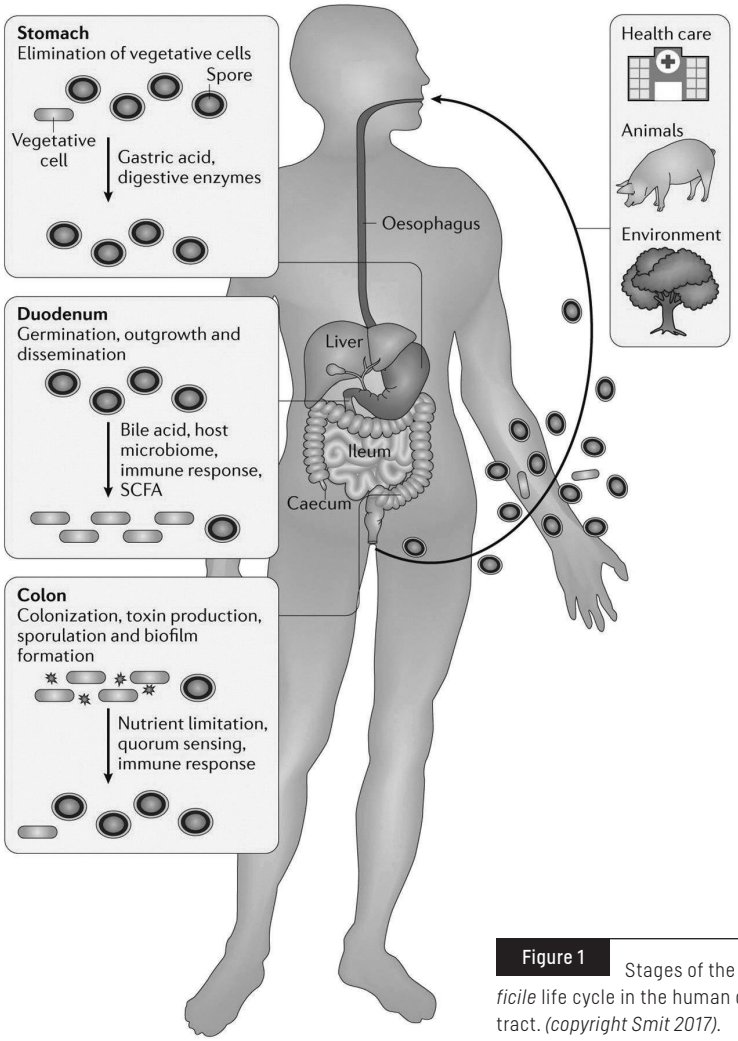

Figure 1 Stages of the *Clostridium difficile* life cycle in the human gastrointestinal tract. *(copyright Smit 2017).*

PREVENTION OF CDI

The presence of toxigenic *C. difficile* in the gut does not lead to infection in hosts with an adequate colonisation resistance and may even protect against CDI (Risk Ratio 0.3) [40, 41]. CDI occurs when growth and toxin production of commensal or recently acquired *C. difficile* surpasses the host resistance. The host resistance is influenced antibiotic use, age, underlying diseases and other drugs that affect the gut microbiota or immune system; the most well-described risk factors for CDI [42]. In hospitalised patients whom usually have a reduced colonisation resistance for CDI, exposure to symptomatic CDI patients and/or environmental contamination increases the risk for CDI (Hazard Rate 1.2) [43, 44]. Environmental *C. difficile* exposure in hospitals can be significantly reduced by daily and/or terminal bleach disinfection, and multifaceted campaigns to improve hand hygiene [45]. Restrictive antibiotic stewardship programmes (aiming to persevere the patient resistance for CDI) can reduce the incidence of CDI by 48-49%, although the evidence for this finding is weak [46, 47]. The largest preventative effects has been shown for daily and terminal bleach disinfection, antibiotic stewardship, and bundled interventions (e.g. improved hand hygiene, contact precautions, environmental cleaning, and antibiotic stewardship) [45, 48]. During an outbreak, these measures are usually intensified [49]. Recent developments in the field of high resolution molecular typing methods (whole-genome sequencing) resulted in a shift of previously generally accepted hypothesis on nosocomial CDI transmission. It was found that only 20-45% of hospital CDI results from direct transmission from other symptomatic patients [50 – 52]. Alternative reservoirs –within and beyond healthcare facilities– were not elucidated and need further investigation. Reservoirs include '*one or more epidemiologically connected populations or environments in which the pathogen can be permanently maintained and from which infection is transmitted to the target population*' [53]. Reservoir populations of *C. difficile* include several animal species (e.g. pets) and asymptomatic infant and adult carriers [54]. Besides, the living environment is widely contaminated with *C. difficile* spores; 25% of the parks, 17% of the households and 17% of the hospitals contains spores of toxigenic *C. difficile* (in southern United States) [55]. In addition, *C. difficile* spores are isolated from water and food in varying percentages [36, 37]. Therefore, tracing the attribution of different *C. difficile* sources to CDI is extremely complex but would be useful to reduce exposure, especially in those with a decrease of colonisation resistance. Antibiotic stewardship in the outpatient setting is important as in hospitals; e.g. a 10% reduction of antibiotic use could prevent 17% of the community-acquired CDI cases [56].

DIAGNOSING CDI

The range of symptoms caused by toxigenic *C. difficile* is wide and overlaps other gastrointestinal infections. Severe damage of the colon by *C. difficile* toxins results in elevated yellow-white nodules or plaques consisting of neutrophils, nuclear debris and other inflammatory elements on the mucosal surface ("pseudomem-

branes") observed by endoscopy [57] potentially leading to ileus, toxic megacolon, septic shock and death. The majority of the outpatients diagnosed with CDI has persistent diarrhoea, watery stools, weight loss, abdominal pain, and watery or slimy stools [58 – 60]. About 4-8% of the outpatients are hospitalised because of CDI and death due to CDI is rare (<1%) [58, 59, 61]. In contrast, hospital patients with CDI more often have systemic signs of infection indicative of severe disease [62] such as leucocytosis (29-50%) and fever (37-56%) [63, 64]. Within 30 days, 13% of the hospital patients with CDI decease, and 10% of these deaths were found to be related to CDI [65]. Children tend to have milder disease compared to adults [66]. Laboratory diagnosis of CDI is one of the most discussed topics in CDI surveillance. The main problem is the absence of a single diagnostic test that has optimal test characteristics (high sensitivity and specificity) and is easy and quick to perform. Besides, research and communication on CDI diagnostics is complicated by the presence of two golden standards; a cell cytotoxicity assay detecting free toxin in faeces and a cytotoxigenic culture detecting toxigenic *C. difficile*. In 2009, a two-step laboratory algorithms was recommended for diagnosis of CDI by the ESCMID diagnostic guideline for CDI [67]. Moreover, the guideline endorsed CDI laboratory testing of merely unformed stool samples using the '3-day rule' (testing samples of all diarrhoeic patients ≥72 hrs admitted to a healthcare facility). A revised ESCMID diagnostic guideline was published in 2016, e.g. abandoning use of toxin EIAs (without detection of GDH) as a first step of the diagnostic algorithm for CDI and adding an optional third step for indistinct cases (CDI or carriage of toxigenic *C. difficile*) [68].

CDI TREATMENT

CDI can resolve by discontinuation of the inciting antibiotic. When specific treatment is indicated, it is recommended to treat a first CDI with an oral ten-day course of metronidazole in mild cases, or a ten-day course of vancomycin in severe cases [69 – 71]. Metronidazole produces free radicals that break DNA strands and cause cell death of several anaerobic bacteria [72], whereas vancomycin inhibits cell-wall synthesis in gram-positives by binding to the peptidoglycan precursor [73]. Vancomycin has a higher symptomatic cure rate than metronidazole (79 vs. 72%; Risk Difference 7%) but is more expensive [74] and has more profound negative effects on the microbiota [75]. *C. difficile* resistance to both antibiotics is rare [76], but needs further clinical evaluation. Nine other antibiotics are available for CDI [74], among them fidaxomycin that has a lower recurrence rate compared to vancomycin in cases other than *C. difficile* ribotype 027 [69, 77] and mainly has its place for treatment of recurrent CDI [69]. Decision-making (supported by prediction models), involves consideration of disease severity, disease recurrence, causative factors, feasibility of oral treatment, *C. difficile* type and antibiotic susceptibility [69].

The antithesis of treating an antibiotic-induced disease with antibiotics [78] stimulated the development of new CDI treatments. The most striking 'novel' treatment

of CDI includes transfer of a healthy donors stool sample into the gut of an infected patient (faecal microbiota transfer; FMT) effective in 94% of the patients with recurrent CDI [79]. In 2015, a National Donor Feces Bank (NDFB) was initiated to provide FMT for Dutch hospitals in a standardised manner [80]. Alternative preventative or treatment modalities in development that focus on the host resistance against CDI are vaccination, human monoclonal immunotherapy [81], toxin binders, administration of non-toxigenic *C. difficile* strains [82] and microbiome-based strategies (e.g. administration of *Firmicutes* spores) [83, 84].

Epidemiological surveillance systems

AIMS AND METHODOLOGIES

Epidemiological surveillance systems originate from the mid-20th century and involve "*the systematic collection, analysis, interpretation and timely dissemination of health data for the planning, implementation and evaluation of public health programmes*" [85, 86]. Surveillance systems aim to generate "*a public health response leading to the control and prevention of adverse health events, or to a better understanding of the process leading to an adverse outcome*" (e.g. disease risk factors or transmission) [86]. Epidemiological surveillance systems are categorised into disease-specific, syndromic, and event-based surveillance [87]. Sub classifications refer to the method of data collection, e.g. differentiating healthcare provider-based from laboratory-based surveillance, active from passive surveillance, and prospective from retrospective surveillance [88]. Yet, 'retrospective surveillance' seems like a contradiction-in-terms that should be avoided. Another sub classification differentiates sentinel from national surveillance [89]. Sentinel surveillance refers to collection of data from a representative fraction of the population to monitor disease trends in a larger area. A sentinel approach is useful when high-quality data is needed that cannot be obtained through passive surveillance [89] and the workload and/or costs of national surveillance is too high. The methodology of an epidemiological surveillance system is adapted to its aim; control of an infectious disease with a high disease burden requires comprehensive national surveillance, whereas elimination of a rare infectious disease requires more detailed information [89]. A surveillance system can also be applied at a regional or local level, e.g. within a single hospital, to guide the local infection control measures.

INTRODUCTION OF NATIONAL SURVEILLANCE OF CDI

Historically, the use of epidemiological surveillance systems for CDI has been triggered by the occurrence of outbreaks. One of the first publications on surveillance of CDI describes the implementation of active hospital-wide surveillance after the recognition of a CDI cluster in Belgium in 1988 [90]. Interestingly, the authors note that surveillance outcomes were initially biased due to unawareness and underdiagnosis of CDI. After addressing these issues, surveillance and molecular typing led to improved prevention of CDI. In the Netherlands, a first laboratory-based surveillance study was conducted in 2005 [91]. This study coincided with the occurrence of PCR ribotype 027 CDI outbreaks in the Netherlands [92]. In 2006-2009, Dutch national surveillance was limited to severe cases of CDI and outbreaks and a three-year surveillance study [93]. In 2009, continued Sentinel Surveillance for CDI was implemented in a subset of hospitals in the Netherlands (Figure 2). Aims are to:

- obtain continuous incidence rates of CDI in hospitals in the Netherlands
- identify and characterize new circulating PCR ribotypes
- correlate new circulating PCR ribotypes with changes of epidemiology, and clinical syndromes of CDI

The Dutch Sentinel Surveillance for CDI is a disease-specific, healthcare-provider based surveillance system that targets acute care hospitals only. Inclusion of patients is based on the presence of clinical symptoms of CDI and laboratory confirmation. The surveillance system is exceptional in Europe for its unselective PCR ribotyping of all submitted *C. difficile* strains. Hospital participation in Sentinel Surveillance for CDI is voluntary. Outcomes of Sentinel Surveillance for CDI are used to prioritise and monitor national control of CDI, especially transmission and outbreaks of ribotype 027 and other *C. difficile* strains with an increased ecological fitness. Hospital get direct notification of molecular typing results in case of ribotype 027 and/or outbreaks and can receive additional support for local infection control measures. Healthcare facilities not participating in Sentinel Surveillance for CDI are able to submit samples for (free of charge) PCR ribotyping in case of severe CDI or suspected outbreaks.

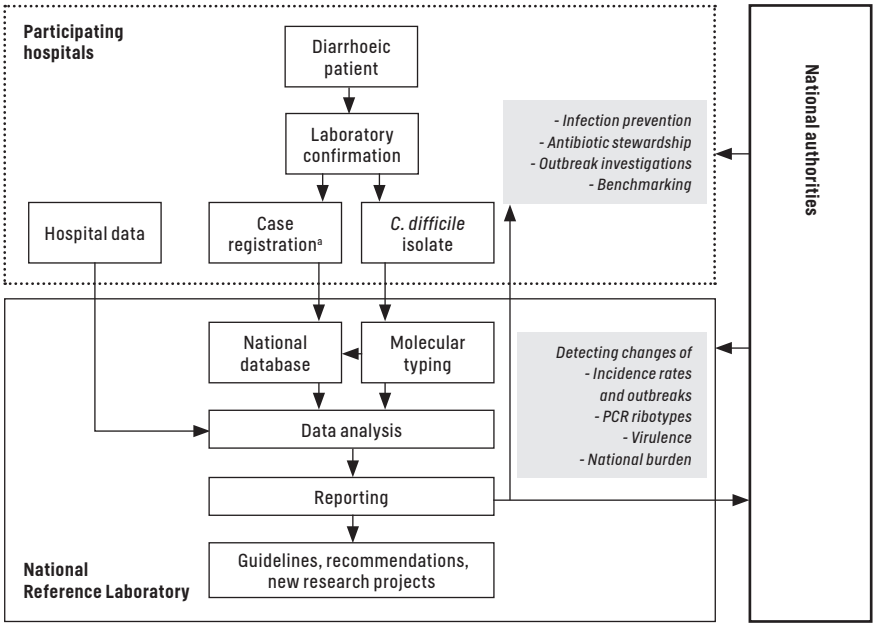

Figure 2 Flow chart of Sentinel Surveillance for CDI in the Netherlands. The National Reference Laboratory for *C. difficile* is a collaboration of the Department of Medical Microbiology of the Leiden University Medical Centre in Leiden and the National Institute for Public Health and the Environment (RIVM) in Bilthoven. [a]The Orisis registration system of the RIVM is used for case registration with clinical and epidemiological data, and linkage of molecular typing results.

SURVEILLANCE OF CDI IN EUROPE

In Europe, national surveillance activities of European countries were supported by ECDC after recognition of the emergence of ribotype 027 in Europe in 2006, gradually moving towards standardised epidemiological surveillance and molecular typing systems [4]. In the same year, ECDC and the ESCMID study group for *C. difficile* publised 'interim' surveillance definitions for CDI [3]. European countries were advised to adapt their national surveillance system to their local situation, selecting for laboratory-based surveillance or healthcare provider-based surveillance in specific, targeted populations. In 2005, a first pilot of multicountry two-month survey in Europe was conducted [94]. This study underlined the need for implementation of a standardised case-definition and harmonisation of laboratory diagnostics for benchmarking. The need for routine surveillance was reinforced in the ESCMID guideline to control transmission of CDI in 2008, also in the absence of outbreaks [95]. In that year, a first periodic European surveillance for CDI illustrated the added value of multicountry surveillance in Europe [64]. However, the continued heterogeneity of diagnostic, molecular typing and surveillance methodologies hampered implementation of European-wide surveillance.

By way of comparison, The Centers for Disease Control and Prevention (CDC) integrated CDI surveillance as separate component in the Healthcare-Associated Infections Community Interface (HAIC) of the Emerging Infections Program (EIP) in the United in 2009 [96]. Before that time, surveillance was implemented in the National Nosocomial Infection Surveillance System initiated in 1970 [97]. The CDI surveillance component of EIP is population-based and aims to estimate the burden of health-care associated CDI and in the community, describe other epidemiological aspects of CDI and characterize *C. difficile* strains [96].
In 2011, 14 of the 31 European countries (45%) had adopted CDI surveillance and methodologies were heterogeneous. Continued integration of microbiological data was limited [98]. A multistate CDI surveillance system, such as implemented in the United States[99], was considered the only viable option to monitor and control CDI in Europe. Objectives of such a European surveillance system of CDI were agreed to [100]:

- estimate the incidence of CDI in European acute care hospitals
- assess the burden of CDI in European acute care hospitals
- provide participating hospitals with a standardised tool to measure and compare their own incidence rates with those observed in other participating hospitals
- assess adverse outcomes of CDI including death
- describe the epidemiology of *C. difficile* at the local, national and European level, in terms of factors such as antibiotic susceptibility, PCR ribotype, presence of toxin A (TcdA), toxin B (TcdB) and binary toxin, morbidity and mortality of infection, and the detection of new/emerging types

The 'European CDI Surveillance Network' (ECDIS-Net) aimed to optimise and test the feasibility of a surveillance protocol for European Surveillance of CDI in 2010-2014.

USE OF MOLECULAR TYPING FOR SURVEILLANCE

Molecular typing methods aim to identify relatedness of pathogens and are used to test epidemiological hypotheses on transmission events [101]. Hence, molecular typing is vital for outbreak investigations [102]. Molecular typing also supports monitoring of 'outbreak-associated' or more virulent genotypes in an endemic setting. Several typing methodologies for *C. difficile* were developed and implemented all over the world, targeting different parts of the *C. difficile* genome [103 – 105]. PCR ribotyping is the traditional typing method in Europe. It targets the intermediary region of the 16S and 23S rRNA genes with a variable length, present in multiple copies of the ribosomal operon in the genome [106, 107]. Identical PCR ribotypes have an equivalent banding pattern after visualising the DNA fragments [106]. Currently, 219 PCR ribotypes can be discriminated by the national reference laboratory for *C. difficile* in the Netherlands [108]. Capillary gel-based electrophoresis ribotyping has an improved performance compared to conventional (agarose gel-based) PCR ribotyping for inter-laboratory standardization [109]. Moreover, the electronic portability of capillary ribotyping results is considered to dissolve the current problem of limited reference/central databases and contribute to more rapid detection of internationally emerging PCR ribotypes [109]. *C. difficile* transmission events are hallmarked by identical PCR ribotypes, but far more discriminatory typing methods are required to confirm transmission events and to study outbreaks [103, 105]. One of these methods is Multiple-Locus Variable-Number Tandem-Repeat Analysis (MLVA), targeting seven regions with short tandem repeats spread in the *C. difficile* genome [110]. The total number of differences in repeat copy number at each locus (summed tandem-repeat difference; STRD) describes the genetic relatedness of the studies isolates [111]. Whole genome sequencing –of which the application for *C. difficile* was firstly reported in 2010 [112]– has the capability to distinct strains at a single nucleotide level; single nucleotide variants (SNV) [103]. The application of whole-genome sequencing led to important insights in the evolution and (drivers of) transmission of CDI [2, 50, 112].

The burden of CDI in hospitals

After the discovery of *C. difficile* as a pathogen in 1978, CDI was soon recognised as the foremost cause of antibiotic-associated diarrhoea in hospitals, causing 15-25% of all cases [102]. The regional incidence rate of CDI ranged from 1.1-7.9 cases per 10,000 patient-days in the United States according to data from the National Nosocomial Infections Surveillance System in 1978-2001 [97]. A gradual increase of the incidence of CDI was noted for IC units and smaller hospitals. In Canada, the incidence rate of CDI was estimated at 6 cases per 10,000 patient-days in 1997 (corresponding to a prevalence of 13% among all diarrhoeic inpatients) by surveillance part of the Canadian Nosocomial Infection Surveillance Program [113]. In 2002, the incidence rate of CDI increased dramatically in the region of Quebec in Canada to 15 cases per 10,000 patient-days [114, 115]. Almost a quart (23%) of the affected patients died within 30-days compared to 7% of matched controls [115]. Continuing signs of increased severity of CDI and the occurrence of outbreaks led to the recognition of the emergence of an earlier uncommon ribotype 027/NAP 1 strain in Canada and the United States in 2005 [116, 117].

In the Netherlands, the endemic incidence of CDI remained unreported until 2005 but sporadic outbreaks were noticed [118 – 120]. In 2005, an excessive number of CDI outbreaks occurred with a high impact on the local incidence rates and mortality [91, 121 – 125]. Also other European countries, i.e. the United Kingdom, Belgium and France, were affected by CDI outbreaks [94]. These outbreaks were related to the global spread of the ribotype 027/NAP1 strain from Canada and the United States towards Europe [2]. Transmission of ribotype 027 had been reported by 11 and 16 European countries in 2007 and 2008, respectively [126]. Introduction of ribotype 027 could be linked to international travels in some countries. The exceptional virulence of ribotype 027 was attributed to increased toxin production (associated to its *tcdC* gene mutation) and altered antibiotic resistance [126]. Retrospective investigation using whole-genome sequencing demonstrated that fluoroquinolone resistance acquired by two separate ribotype 027/NAP1 lineages contributed to its emergence and undermined the role of the *tcdC* gene mutation [2].

The Netherlands was the first European country that reported a decrease of ribotype 027 in 2006 by national surveillance data [93]. The incidence rate of CDI in hospitals in the Netherlands stabilised at 3 cases per 10,000 patient-days and ribotype 027 caused not more than 3% of all CDI (virtually all relating to healthcare). However, transmission of ribotype 078 caused concern due it high abundance, especially compared to other countries [93]. A European surveillance estimated the incidence of healthcare-associated CDI at 4 cases per 10,000 patient-days in 2008. Ribotype 027 was isolated from 5% of CDI, and less prevalent than e.g. ribotype 078 [64] The United Kingdom had a remarkable high prevalence of ribotype 027, but succeeded to reduce the national incidence rate of CDI and prevalence of ribotype 027 by antibiotic stewardship (e.g. restricting fluoroquinolone prescribing) in additional to other infection prevention control measures in 2007-2013 [127]. ECDC estimated the overall number of healthcare-acquired CDI at

123,997 (95% CI: 107,697-441,969) in 2011-2012 [128]. Yet, thereafter ribotype 027 emerged in Eastern-Europe [129].

In the United States however, 31% of healthcare-associated CDI and 19% of community-associated CDI was caused by ribotype 027/NAP1 in 2011 [99]. It was estimated that approximately 453,000 patients were affected by CDI in 2011 (95% confidence interval: 397,100 -508,500) of which 29,000 succumbed (Figure 3). CDC denoted CDI as one of the three urgent 'antibiotic resistance threats' in the United States in 2013 [130].

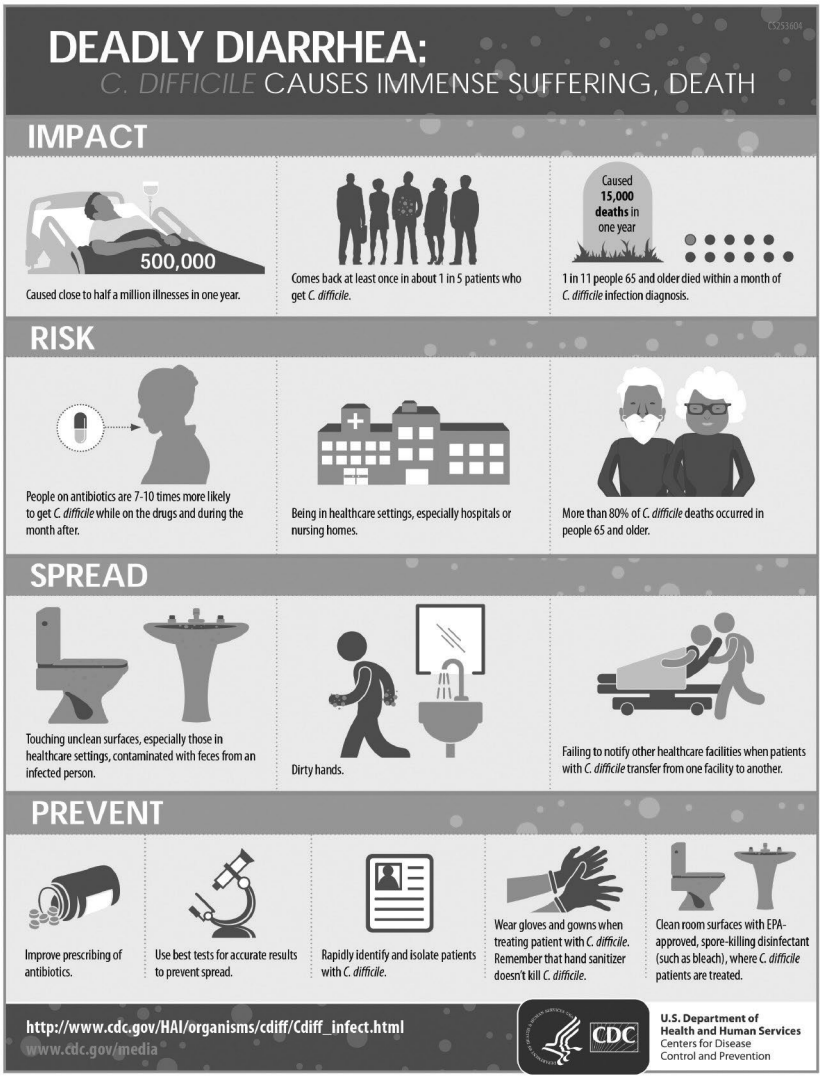

Figure 3 Infographic of the Centers for Disease Control and Prevention (CDC) on prevention of CDI.

CDI beyond hospitals

As hospitals, long-term care facilities (LTCF) host a population at high risk for developing CDI, due to frequent antibiotic consumption, advanced age, comorbidities and suboptimal infection prevention. LTCF residents can facilitate *C. difficile* transmission between hospitals and LTCF [111]. In the United States, the number of CDI cases in nursing homes in 2012 was estimated at 112,800 (95% confidence interval: 93,400-131,800), circa one quarter of all CDI cases [131]. These numbers may be biased due to the fact that *C. difficile* laboratory testing in nursing homes is not always part of daily routine. A large proportion of the patients (76%) had been hospitalised ≤12 weeks before CDI and the very old (>85 yrs) were at particular risk [131]. In Europe, the burden of healthcare-associated infections in LTCF is monitored by repeated point-prevalence surveys. CDI was not one of most common HAI or isolated microorganisms. The CDI-specific burden was not reported but approximated 37,900 (0.9% of all HAI) on the basis of available data [132]. There are no national estimates of CDI in LTCF in the Netherlands, but outbreaks have been reported [3].

In the general population, the incidence of CDI in the community was estimated at 0.67 cases per 10,000 person years (95% CI 0.58–0.78) in the Netherlands in 2010-2012, comparable to *Salmonella* spp. This corresponded to a prevalence of 1.5% amongst community residents that visit their general practitioner with diarrhoea and submit a stool sample for laboratory testing [59, 60]. In another Dutch study, 4.2% of community residents with gastro-intestinal complaints were positive for toxigenic *C. difficile* using PCR, higher than *Salmonella* spp [133]. In Denmark, the community incidence of CDI was 2.3 cases per 10,000 person years [58]. In Minnesota, United States, the incidence of community-acquired CDI was estimated at 0.96 cases per 10,000 person years in 1991-2005 in a population-based study [134]. While recent literature underlines the increasing burden of CDI in the community, the substantial community burden of CDI has been confirmed decades ago –prior to the emergence of ribotype 027/NAP1–if tested for [135]. Yet, the minority of the diarrhoeic community patients were tested for CDI at that time [135, 136].

Community patients with CDI have a rather different risk profile compared to hospital patients [134, 137]. Antibiotic use is the foremost risk-factor for CDI as in hospitalised patients, but absent in a considerable proportion (30-60%) of the community patients [59, 60, 137, 138]. It was suggested that other disrupting factors of the microbiome cause CDI in this subpopulation of non-exposed patients (e.g. proton-pump inhibiters) [139]. As mentioned before, *C. difficile* reservoirs include asymptomatic infant and adult carriers, and animals (e.g. pets). Food, water and other environmental contamination can be considered as 'sinks' rather than reservoirs, but may be part of CDI transmission paths. In previous years, molecular studies aimed to trace CDI sources. One of these studies showed that 13% of CDI in adults were genetically related to infants strains according to whole-genome sequencing [140]. Advanced age, breastfeeding and exposure to pet dogs were

found to be risk factors for *C. difficile* in children in the same study. Animal contact has been recognised to increase the risk of CDI in children <2 yrs old by others [138]. Phylogenetic results of other whole-genome sequence studies suggest that numerous long-range transmission events occur between pet dogs and humans [141], as well as pigs and humans [142, 143]. Transmission paths have not been elucidated. In the Netherlands, piglets are frequently colonised by *C. difficile* PCR ribotype 078 [144] and contamination of the farm environment has been demonstrated [145]. Persons with daily contact with pigs had a 1:4 risk to be positive for *C. difficile* in a small study, virtually all ribotype 078 [146]. In Central North Carolina, one of the largest pig producing states in the United States, environmental exposure to livestock farms increases the risk for community-acquired CDI [147]. Moreover, CDI complies with some criteria of foodborne disease. Yet, the attribution of food to CDI transmission is considered low, as illustrated for hospitalised patients [148]. *C. difficile* cannot germinate and grow in food, and outbreaks of CDI were never found to be food-related [37, 149]. Yet, regular consumption of beef was an imported risk factor (Odds Ratio 5.5) of community-acquired CDI for adults in Denmark in contrast to other food products [138]. Overall, a complex interplay of animal and human reservoir populations and environmental sources ('sinks') need to be considered for CDI transmission beyond hospitals.

Outline of the thesis

This thesis aims to describe contemporary changes of the epidemiology of CDI in the Netherlands and Europe and the subsequent introduction of a new standardised epidemiological surveillance system for CDI in Europe. Findings will be used to guide future directions of epidemiological surveillance systems for CDI –in particular for Europe and the Netherlands. This will improve estimations of the infection burden and helps to understand *C. difficile* sources and transmission routes that are needed for appropriate infection prevention control interventions.

The heterogeneity of existing epidemiological surveillance systems for CDI in Europe hampered a valid estimation of CDI burden and illustrated the need for a standardised European-wide surveillance system for CDI. However, suboptimal laboratory diagnostic capacity was considered as the foremost barrier for implementing European-wide surveillance for CDI. Besides, application of various non-standardised molecular typing methodologies prevented their use for monitoring transmission and control. In 2010, ECDC supported a 4-year project named 'the European CDI Surveillance Network' (ECDIS-Net) to enhance CDI surveillance and laboratory capacity to test for CDI. This thesis incorporates two studies that were conducted within the framework of ECDIS-Net. **CHAPTER 2** evaluates changes in local laboratory diagnostic and national typing capacity for CDI through cross-sectional surveys amongst ECDIS-Net participants in 33 European countries in 2011 and 2014. **CHAPTER 3** explores the feasibility of implementing a standardised European surveillance protocol for CDI through a three-month pilot in 14 countries in 2013. This study also illustrates the added value of collecting detailed epidemiological and microbiological data on CDI at European level. ECDIS-Net activities resulted in initiation of European Surveillance of CDI in EU/EEA countries by ECDC in 2016.

The Netherlands has a national reference laboratory in place to support epidemiological surveillance and molecular typing of CDI since the recognition of ribotype 027 outbreaks in 2005. A national Sentinel Surveillance for CDI was implemented for ongoing monitoring of the incidence of CDI and detection of new outbreaks in 2009. According to this surveillance, the incidence of CDI stabilised at 3 cases per 10,000 patient-days. Yet, the burden of CDI in children was never examined in detail but has been reported to increase in other countries. **CHAPTER 4** investigates changes of the number of reported CDI amongst children in the Netherlands. Additionally, the clinical and microbiological characteristics of CDI in hospitalised children are compared to adults to determine if additional strategies to prevent, diagnose, and treat CDI in children are needed. Overall ribotype 027 caused not more than 3% of all CDI, but the high incidence of ribotype 078 caused concern because of its potential relation to pig-farming. **CHAPTER 5** assesses the association between hospital incidence rates or ribotype 078 and provincial pig-farming. This study also incorporates the use of spatial

scan statistics to search for clustering of community-CDI that could indicate sources of CDI beyond hospitals. **CHAPTER 6** extends the use of spatial scan statistics in a community-based case-control study of CDI, with detailed data on environmental exposure of community-acquired CDI patients. These data are used to test livestock exposure as a risk factor for community-acquired CDI.

CHAPTER 7 elaborates on how advanced insights in the epidemiology, sources and transmission of CDI challenge present surveillance systems and synthesises future directions for improved surveillance systems and control of CDI.

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Chapter 2

Survey of diagnostic and typing capacity for *Clostridium difficile* infection in Europe, 2011 and 2014

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Suboptimal laboratory diagnostics for *Clostridium difficile* infection (CDI) impedes its surveillance and control across Europe. We evaluated changes in local laboratory CDI diagnostics and changes in national diagnostic and typing capacity for CDI during the European *C.difficile* Infection Surveillance Network (ECDIS-Net) project, through cross-sectional surveys in 33 European countries in 2011 and 2014. In 2011, 126 (61%) of a convenience sample of 206 laboratories in 31 countries completed a survey on local diagnostics. In 2014, 84 (67%) of these 126 laboratories in 26 countries completed a follow-up survey. Among laboratories that participated in both surveys, use of CDI diagnostics deemed 'optimal' or 'acceptable' increased from 19% to 46% and from 10% to 15%, respectively ($p < 0.001$). The survey of national capacity was completed by national coordinators of 31 and 32 countries in 2011 and 2014, respectively. Capacity for any *C. difficile typing* method increased from 22/31 countries in 2011 to 26/32 countries in 2014; for PCR ribotyping from 20/31 countries to 23/32 countries, and specifically for capillary PCR ribotyping from 7/31 countries to 16/32 countries. While our study indicates improved diagnostic capability and national capacity for capillary PCR ribotyping across European laboratories between 2011 and 2014, increased use of 'optimal' diagnostics should be promoted.

Introduction

Since 2003, Europe has been affected by outbreaks of *Clostridium difficile* infection (CDI) associated with the emergence of PCR ribotype 027/NAP1 [1]. A decade later, *C. difficile* was the microorganism responsible for 48% of healthcare-associated gastrointestinal infections in acute care hospitals across Europe [2]. Despite being frequent, CDI remains underestimated in most European countries [3]. Underdiagnosis mainly results from a lack of awareness among medical doctors of when to suspect that patients may have CDI and use of suboptimal diagnostic algorithms at local microbiological laboratories [35]. Reference tests, i.e. toxigenic culture and cell culture cytotoxicity assay (CCA), are not suitable for routine application due to their complexity and long turnaround time [6,7]. Rapid enzyme immunoassays (EIAs) to detect *C. difficile* toxins in faeces lack sensitivity [6,8]. Highly sensitive tests such as EIA detecting glutamate dehydrogenase (GDH) – a *C. difficile*-specific enzyme [9] – or nucleic acid amplification tests (NAATs) have insufficient specificity [6,10]. To overcome underdiagnosis and suboptimal performance of stand-alone tests, the European Society of Clinical Microbiology and Infectious Diseases (ESCMID) has recommended since 2009 testing loose stools using two-step algorithms that have a highly sensitive test as the first screening step and a highly specific test as the second confirmatory test [6,11]. The 'Bristol stool scores' [12] are commonly used to categorise stool consistencies and can be used to select samples for CDI testing. ESCMID recommended performing CDI testing not only upon request of a medical doctor, but also based on other indications such as the 'three-day rule', i.e. diarrhoea after three days of hospitalisation or when diarrhoea develops after antibiotic use [6,13].

The type of diagnostic algorithm applied influences not only clinical care [14], but also CDI surveillance's sensitivity and specificity [3,14,15]. However, a consensus on when and how to test for CDI has not been established among reference and local laboratories.

Additionally, typing of *C. difficile* to understand its local or wider transmission remains non-standardised in Europe [16,17]. Numerous typing methods have become available for routine use in the last 30 years. For *C. difficile*, these include methods that use restriction enzymes (e.g. restriction endonuclease analysis, pulsed-field gel electrophoresis (PFGE)), PCR amplification of housekeeping genes (e.g. multilocus sequence typing (MLST)), of repetitive elements (repetitive-element PCR, multilocus variable-number tandem repeat analysis (MLVA)), of the pathogenicity locus (e.g. toxinotyping) or of 16S-23S rRNA intergenic spacer regions (e.g. PCR ribotyping) [16,18]. Whole genome sequencing, with its ultimate discriminatory power, can already be used for in-depth analysis of evolutionary patterns [19]. Nevertheless, PCR ribotyping still remains the standard typing method in Europe as it involves relatively simple technology and its low costs permits widespread application [16,18].

In 2010, the European Centre for Disease Prevention and Control (ECDC) launched the European *C. difficile* Infection Surveillance Network (ECDIS-Net) project, an initiative to enhance and harmonise laboratory diagnostic and typing capacity for CDI, and to support surveillance of CDI in Europe. The project consortium consisted of a team of experts involved in the first European multicountry surveillance study performed in 2008 [20]. Between 2010 and 2014, the ECDIS-Net project developed standard operating procedures for *C. difficile* culturing and PCR ribotyping, implemented a reference nomenclature database and compiled a set of reference strains to standardise PCR ribotyping. National reference laboratories were invited to participate in a workshop for culturing and typing of *C. difficile* and participated in an external quality assessment exercise.

The study presented here measured changes in capacity for diagnostic testing for CDI and typing of *C. difficile* isolates in Europe between 2011 and 2014, using surveys of European local laboratories and national coordinators participating in the ECDIS-Net project. Additionally, we aimed to obtain insight into barriers to optimal CDI laboratory diagnostics, to inform further activities of ECDC and of the ESCMID Study Group for *C. difficile* (ESGCD) in this field.

Methods

STUDY DESIGN

The Dutch National Reference Laboratory for *C. difficile* (Leiden University Medical Centre, Leiden, and the National Institute for Public Health and the Environment (RIVM), Bilthoven, the Netherlands) coordinated data collection in 2011 and in 2014 by cross-sectional surveys among two target groups: (i) local microbiology laboratories, in order to evaluate changes in routine laboratory diagnostics; and (ii) national coordinators, i.e. representatives of national or regional reference laboratories nominated by competent bodies for surveillance on the request of ECDC, to evaluate national changes in diagnostic and typing capacity for *C. difficile*. In 2011 and 2014, 32 and 33 countries participating in the ECDIS-Net project were invited to take part in the survey, respectively (in 2011, Serbia did not participate in ECDIS-Net). All surveys are available online [21].

SELECTION

There was no European register of microbiology laboratories to use for random sampling. Therefore, ECDIS-Net national coordinators were requested to invite a representative sample of the local clinical microbiology laboratories (about 10%) in each country to participate in the survey. In Austria and Norway, the laboratories were selected by random sampling; all other countries used non-random convenience sampling [22]. Selected laboratories were emailed an initial survey in October 2011: some laboratories replied in 2012. All respondents to the initial survey received a follow-up survey in June 2014.

DATA COLLECTION

Data were collected through a centralised web-based system (Questback, New York, United States). In 2011, the initial survey contained questions on several aspects of local routine diagnostics, including indications for undertaking CDI diagnostics and methodologies. Laboratories were requested to report the type of screening test primarily used for CDI diagnostics and confirmatory test (if applicable). For both, they could report more than one test. In 2014, the follow-up survey listed 10 diagnostic algorithms each designated as either 'optimal', 'acceptable' or 'incomplete' (Table 1). Laboratories were requested to estimate the percentage of samples that had been tested according to each algorithm listed, or to describe their usual diagnostic algorithm and estimate the corresponding percentage. The categorisation of CDI diagnostic algorithms was made by some of the ECDIS-Net experts who were also involved in revising the ESCMID diagnostics guidelines for CDI [6]. Algorithms designated as optimal had high sensitivity and specificity (not specifically defined), detection of free toxins in faeces and a rapid turnaround time [23]. Acceptable algorithms met the same criteria but without detecting free toxins in faeces. Any other algorithm was designated as incomplete. The 2014 follow-up survey additionally contained questions on barriers to

apply optimal or acceptable diagnostic algorithms and changes in the indications for sending samples for CDI diagnosis by medical doctors.

Table 1. Criteria for categorisation of *Clostridium difficile* infection diagnostic algorithms, survey of European countries participating in the European *Clostridium difficile* Infection Surveillance Network (ECDIS-Net) project, 2011 (n = 31)[a] and 2014 (n = 26)[a]

Categorisation of CDI diagnostics		CDI diagnostic algorithm	
		Screening test	Screening test
Optimal[b]	1[c]	NAAT	EIA toxin detection
	2–3[c]	GDH EIA and toxin detection	NAAT or toxigenic culture
Acceptable[b]	4–5[c]	GDH EIA detection	NAAT or toxigenic culture
	6[c]	NAAT	None
Incomplete[b]	7–10[c]	All other algorithms	

CDI: *Clostridium difficile* infection; EIA: enzyme immunoassay; GDH: glutamate dehydrogenase; NAAT: nucleic acid amplification test.

[a] Laboratories in 31 countries responded to the 2011 survey: Austria, Belgium, Bulgaria, Croatia, Cyprus, Czech Republic, Denmark, Estonia, Finland, France, Germany, Greece, Hungary, Iceland, Ireland, Italy, Latvia, Liechtenstein, Lithuania, Luxembourg, the Netherlands, Norway, Poland, Portugal, Romania, Slovenia, Spain, Sweden, Switzerland, Turkey and the United Kingdom (not including Wales). Serbia did not participate in the European *Clostridium difficile* Infection Surveillance Network (ECDIS-Net) project in 2011. No laboratories in Slovakia and Wales were invited to participate by ECDIS-Net national coordinators in 2011. Laboratories in 26 countries responded in 2014 (no data from laboratories in Croatia, Iceland, Latvia, Slovenia and Switzerland).

[b] Categorisation of CDI diagnostic algorithms in the second survey, in 2014 [21].

[c] Corresponding CDI diagnostic algorithms in the second survey, in 2014 [21].

DATA ANALYSIS

To allow comparison, data on diagnostics from the 2011 initial survey were distributed into the three categories of diagnostic algorithms defined in 2014. For each local laboratory, CDI diagnostics, i.e. CDI testing practices, were considered optimal if more than 80% of the samples followed an optimal diagnostic algorithm, and acceptable if more than 80% of the samples followed either an optimal or acceptable algorithm. CDI diagnostics of all other algorithms were considered incomplete. When a laboratory reported a three-step algorithm by applying a third diagnostic test when the screening and confirmatory tests were contradictory, this algorithm was allocated to the best-matching two-step algorithm. Changes in local laboratory diagnostic capacity were evaluated by the McNemar's test [24], and changes in the use of optimal, acceptable and incomplete algorithms in 2011 and 2014 were evaluated by a Bowker test for symmetry [24]. A sensitivity analysis was performed using two assumptions on missing data in 2014, i.e. CDI diagnostics one category inferior (Table 1) than in 2011 and CDI diagnostics one category superior than in 2011. Data were analysed using IBM SPSS statistics 20 (SPSS Inc., Chicago, United States).

SURVEY OF ECDIS-NET NATIONAL COORDINATORS

DATA COLLECTION AND ANALYSIS

All ECDIS-Net national coordinators received an initial survey in May 2011 and a follow-up survey in June 2014. Both surveys contained questions on national typing capacity (defined as any laboratory in the country performing typing) and on molecular typing methods, asking which were available in their country from a list of common methods [18].

Results

LOCAL LABORATORY CAPACITY

PARTICIPANTS

Questionnaires on local diagnostic and typing capacity for CDI were completed by 126 (61%) of 206 laboratories in 2011–12 and by 84 (67%) of these same 126 laboratories in 2014 (Table 2). A total 124 (98%) of the 126 responding laboratories in 2011–12 provided microbiological services to hospitals, of which 103 (83%) served at least one university, secondary or tertiary care hospital. In addition, 66 (53%) provided microbiological services to long-term care facilities, of which 45 provided services to nursing homes. Furthermore, 65/124 (52%; data were missing for two laboratories) provided medical services to other healthcare services (e.g. general practitioners). In 2011 and 2014, 120/126 (95%) and 83/84 laboratories (99%, among responders to both questionnaires; $p=0.50$), respectively, reported that they performed CDI laboratory diagnostics.

INDICATIONS FOR *CLOSTRIDIUM DIFFICILE* INFECTION DIAGNOSTICS

The indications for CDI diagnostics reported in 2011 are listed in Figure 1. In 2014, a change of indications for sending samples for CDI diagnosis by medical doctors was observed; 16 (19%) of 83 laboratories reported that one or two changes had occurred since 2011. Several laboratories introduced the use of Bristol stool scores to assess stool consistency for sample selection (n=5). Also, patient populations that were previously not monitored for CDI (e.g. outpatients, high-risk populations) were later explicitly included in protocols (n=3) and awareness and recognition of CDI among clinicians had improved (n=5). Other improvements of sample selection were also reported (n=5), i.e. application of guidelines for sample selection (n=3) and/or the three-day rule, i.e. diarrhoea after three days of hospitalisation (n=1), and unspecified attempts to improve sample selection (n=1).

Table 2. Response of participating European countries to local laboratory (n = 31 and n = 26, respectively) and national/subnational surveys (n = 31 and n = 32, respectively) on *Clostridium difficile* infection diagnostic and typing capacity, 2011 and 2014

Country	Number of laboratories that responded to local questionnaire/number invited		Replied to national questionnaire[a]	
	2011	2014	2011	2014
Austria	4/8	2/4	Yes	Yes
Belgium	4/9	4/4	Yes	Yes
Bulgaria	7/7	2/7	Yes	Yes
Croatia	2/4	0/2	Yes	Yes
Cyprus	3/3	3/3	Yes	Yes
Czech Republic	9/11	7/9	Yes	Yes
Denmark	3/3	1/3	Yes	Yes
Estonia	2/2	1/2	Yes	Yes
Finland	3/3	2/3	Yes	Yes
France	5/37	2/5	Yes	Yes
Germany	5/7	5/5	Yes	Yes
Greece	3/3	2/3	Yes	Yes
Hungary	8/8	8/8	Yes	Yes
Iceland	1/1	0/1	No	No
Ireland	3/5	2/3	Yes	Yes
Italy	13/14	8/13	Yes	Yes
Latvia	2/3	0/2	Yes	Yes
Lichtenstein	1/1	1/1	Yes	Yes
Lithuania	3/3	2/3	Yes	Yes
Luxembourg	2/6	1/2	Yes	Yes
Netherlands	4/6	3/4	Yes	Yes
Norway	9/13	4/9	Yes	Yes
Poland	6/6	4/6	Yes	Yes
Portugal	4/5	4/4	Yes	Yes
Romania	4/6	3/4	Yes	Yes
Serbia[b]	NA	NA	NA	Yes
Slovakia[c]	NA	NA	Yes	Yes
Slovenia	1/3	0/1	Yes	Yes
Spain	3/5	2/3	Yes	Yes
Sweden	2/3	2/2	Yes	Yes
Switzerland	1/1	0/1	Yes	Yes
Turkey	2/7	2/2	Yes	Yes
UK-England	2/6	2/2	Yes	Yes
UK-Northern Ireland	1/3	1/1	Yes	Yes
UK-Scotland	4/4	4/4	Yes	Yes
UK-Wales[c]	NA	NA	No	Yes
Total	**126/206**	**84/126**	**31**	**32**

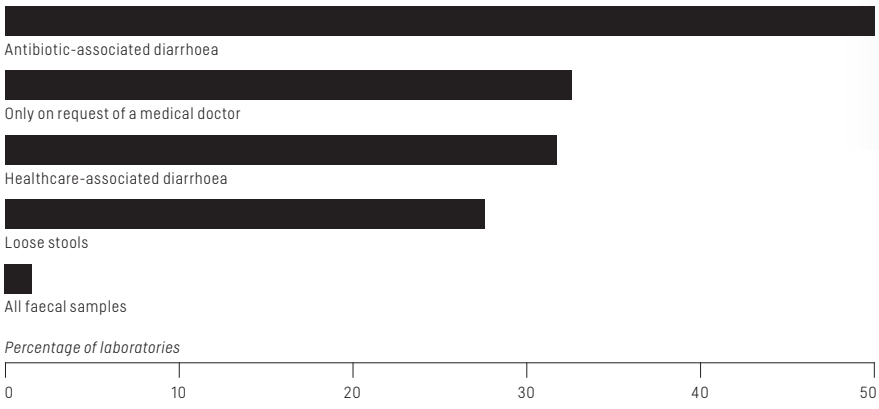
NA: not applicable; UK: United Kingdom.

[a] For the UK, data were analysed separately for England, Northern Ireland, Scotland and Wales, but the UK was counted as one country.

[b] Serbia did not participate in the European *Clostridium difficile* Infection Surveillance Network (ECDIS-Net) project in 2011.

[c] No laboratories in Slovakia and Wales were invited to participate by ECDIS-Net national coordinators.

Figure 1 Criteria for selection of faecal samples tested for *Clostridium difficile* among responding local laboratories that participated in the European *Clostridium difficile* Infection Surveillance Network (ECDIS-Net) project in 2011 (n = 120)[a]



[a] Laboratories in 31 countries responded to the 2011 survey: Austria, Belgium, Bulgaria, Croatia, Cyprus, Czech Republic, Denmark, Estonia, Finland, France, Germany, Greece, Hungary, Iceland, Ireland, Italy, Latvia, Lichtenstein, Lithuania, Luxembourg, the Netherlands, Norway, Poland, Portugal, Romania, Slovenia, Spain, Sweden, Switzerland, Turkey and the United Kingdom (not including Wales). Serbia did not participate in the European *Clostridium difficile* Infection Surveillance Network (ECDIS-Net) project in 2011. No laboratories in Slovakia and Wales were invited to participate by ECDIS-Net national coordinators in 2011.

C. DIFFICILE INFECTION DIAGNOSTICS

In 2011, 17 (14%) of 120 laboratories had optimal CDI diagnostics, 12 (10%) acceptable diagnostics and 91 (76%) incomplete diagnostics (Table 3). Incomplete algorithms included use of EIA toxin detection for screening with or without a confirmatory test, or a combination of EIA GDH and toxin detection without other tests for confirmation. Among laboratories responding to both the 2011 and 2014 surveys and that performed CDI diagnostics at both time-points (n = 81), the percentage of laboratories with optimal CDI diagnostics increased from 19% to 46% and that with acceptable CDI diagnostics from 10% to 15% while the percentage of laboratories with incomplete CDI diagnostics decreased from 72% to 40% ($p < 0.001$; Table 3). Two laboratories without any diagnostics in 2011 had optimal and incomplete CDI diagnostics, respectively, in 2014.

Table 3. Laboratories participating in the European *Clostridium difficile* Infection Surveillance Network (ECDIS-Net) project according to their diagnostics category, 2011 (n = 120)[a] and 2014 (n = 81)[a]

Categorisation of CDI diagnostics[b]	All laboratories that provided data	Only laboratories that provided data in both 2011	
	2011 n (%)	2011 n (%)[d]	2014[c] n (%)[d]
Optimal	17 (14)	15 (19)	37 (46)
Acceptable	12 (10)	8 (10)	12 (15)
Incomplete	91 (76)	58 (72)	32 (40)
Total	**120 (100)**	**81 (100)**	**81 (100)**

CDI: *Clostridium difficile* infection.

[a] Laboratories in 31 countries responded to the 2011 survey: Austria, Belgium, Bulgaria, Croatia, Cyprus, Czech Republic, Denmark, Estonia, Finland, France, Germany, Greece, Hungary, Iceland, Ireland, Italy, Latvia, Lichtenstein, Lithuania, Luxembourg, the Netherlands, Norway, Poland, Portugal, Romania, Slovenia, Spain, Sweden, Switzerland, Turkey and the United Kingdom (not including Wales). Serbia did not participate in the European *Clostridium difficile* Infection Surveillance Network (ECDIS-Net) project in 2011. No laboratories in Slovakia and Wales were invited to participate by ECDIS-Net national coordinators in 2011. Laboratories in 26 countries responded in 2014 (no data from laboratories in Croatia, Iceland, Latvia, Slovenia and Switzerland).

[b] CDI diagnostics were considered 'optimal' if > 80% of the samples followed an 'optimal' testing algorithm, and 'acceptable' if > 80% of the samples followed either an 'optimal' or 'acceptable' testing algorithm. CDI diagnostics of all other laboratories were considered 'incomplete'. The diagnostic algorithms are described in Table 1.

[c] Two laboratories that did not perform CDI laboratory diagnostics in 2011 were not included. These laboratories indicated in the 2014 questionnaire that they used optimal and incomplete CDI diagnostics, respectively.

[d] The percentages in this column do not add up to 100 due to rounding.

SENSITIVITY ANALYSIS

Laboratories with optimal CDI diagnostics in 2011 were more likely to respond to the 2014 survey (15/17) compared with those with acceptable (8/12) or incomplete diagnostics (58/91). Under the negative assumption that all non-responding laboratories in 2014 applied CDI diagnostics one category inferior in 2014 compared with that of 2011, the percentage of laboratories with optimal diagnostics would have increased from 14% to 31%, that with acceptable diagnostics would have increased from 10% to 12%, and that with incomplete diagnostics would have decreased from 76% to 58% between 2011 and 2014 ($p < 0.001$). Conversely, if all non-responding laboratories had CDI diagnostics one category superior in 2014 compared with 2011, the percentage of laboratories with optimal diagnostics would have increased from 14% to 36%, that with acceptable diagnostics would have increased from 10 to 38%, and that with incomplete diagnostics would have decreased from 76 to 27% between 2011 and 2014 ($p < 0.001$).

BARRIERS TO OPTIMAL/ACCEPTABLE DIAGNOSTICS FOR *C. DIFFICILE* INFECTION

Barriers to applying optimal or acceptable algorithms were examined in 2014. Of the 33 laboratories with incomplete CDI diagnostics, 17 indicated that materials or tests were too costly, six indicated receiving insufficient reimbursement for tests from insurers and five had insufficient availability of trained staff. Of the 50 laboratories that had optimal or acceptable CDI diagnostics, 10 also indicated that materials or tests were too costly, seven indicated receiving insufficient reimbursement from insurers and five had insufficient availability of trained staff. Ten laboratories that responded in 2014 indicated that they disagreed with the project's designations of the CDI diagnostic algorithms as optimal, acceptable or incomplete.

NATIONAL/SUBNATIONAL CAPACITY

PARTICIPATING COUNTRIES

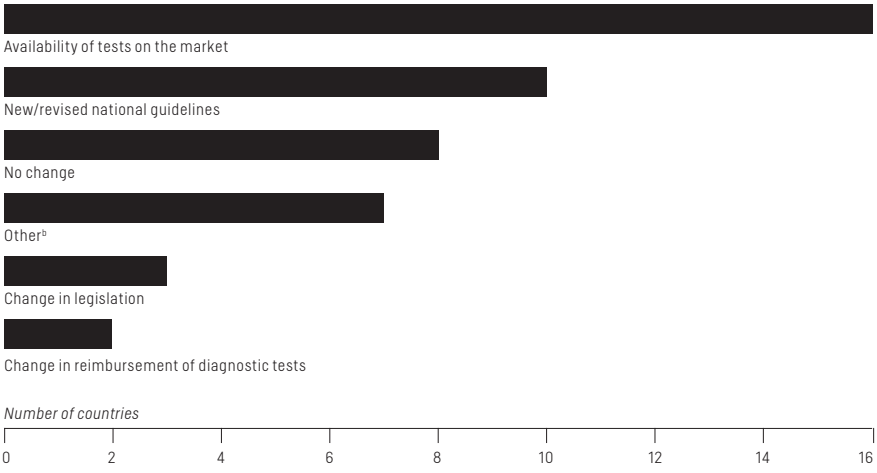
The national coordinators of 31 and 32 countries responded to the national survey in 2011 and 2014, respectively (Table 2). Data were collected separately for the four countries within the United Kingdom (UK), i.e. England, Northern Ireland, Scotland and Wales, but the UK was counted as one country.

CHANGES IN NATIONAL DIAGNOSTIC CAPACITY

In 2014, eight of the 32 responding countries (France, Germany, Greece, Latvia, Luxembourg, Sweden, Switzerland, Turkey) reported no change in national/subnational laboratory diagnostics for CDI. Conversely, 24 countries reported one or more changes in national/subnational laboratory diagnostics for CDI since 2011 (Figure 2). Specifically, 16 countries had experienced a change in availability of commercial diagnostic tests (Bulgaria, Czech Republic, Estonia, Hungary, Ireland, Italy, Lichtenstein, Lithuania, the Netherlands, Norway, Poland, Portugal, Romania,

Serbia, Slovenia, UK), 10 countries had new or revised guidelines for CDI diagnostics (Austria, Czech Republic, Denmark, Estonia, Hungary, Ireland [25], Italy, Lithuania, Romania, UK) and three countries had changes in relevant legislation (Hungary, Poland, Romania). Three countries (Belgium, Croatia, Czech Republic) had implemented changes in reimbursement policies for diagnostic tests. Greece had limited access to and reimbursement of materials in both 2011 and 2014. In 2012, the UK implemented 'harmonised' diagnostics using GDH screening (or NAAT) and EIA toxin detection (or CCA) in all its laboratories [26].

Figure 2 Reported changes affecting national/subnational laboratory diagnostic capacity for *Clostridium difficile* infection between 2011 and 2014 in participating European countries (n = 32)[a]



[a] Austria, Belgium, Bulgaria, Croatia, Cyprus, Czech Republic, Denmark, Estonia, Finland, France, Germany, Greece, Hungary, Ireland, Italy, Latvia, Lichtenstein, Lithuania, Luxembourg, the Netherlands, Norway, Poland, Portugal, Romania, Serbia, Slovakia, Slovenia, Spain, Sweden, Switzerland, Turkey and the United Kingdom (data were analysed separately for England, Northern Ireland, Scotland and Wales, but counted as one country). No data were available for Iceland.

[b] Seven countries reported other changes in national laboratory diagnostics: Slovenia was developing new national guidelines for CDI at the time of the second survey; Romania started a national surveillance study in 2014; Spain published an opinion document on CDI [32]; Slovakia was in the process of implementing new diagnostic methods due to an increased interest in CDI; in Cyprus, the central diagnostic laboratory for *C. difficile* implemented a two-step diagnostic algorithm; in Finland, CDI diagnostics were subcontracted to laboratory consortia that applied nucleic acid amplification tests more often; and Hungary relocated its national reference laboratory to expand its laboratory capacity but still had limited resources.

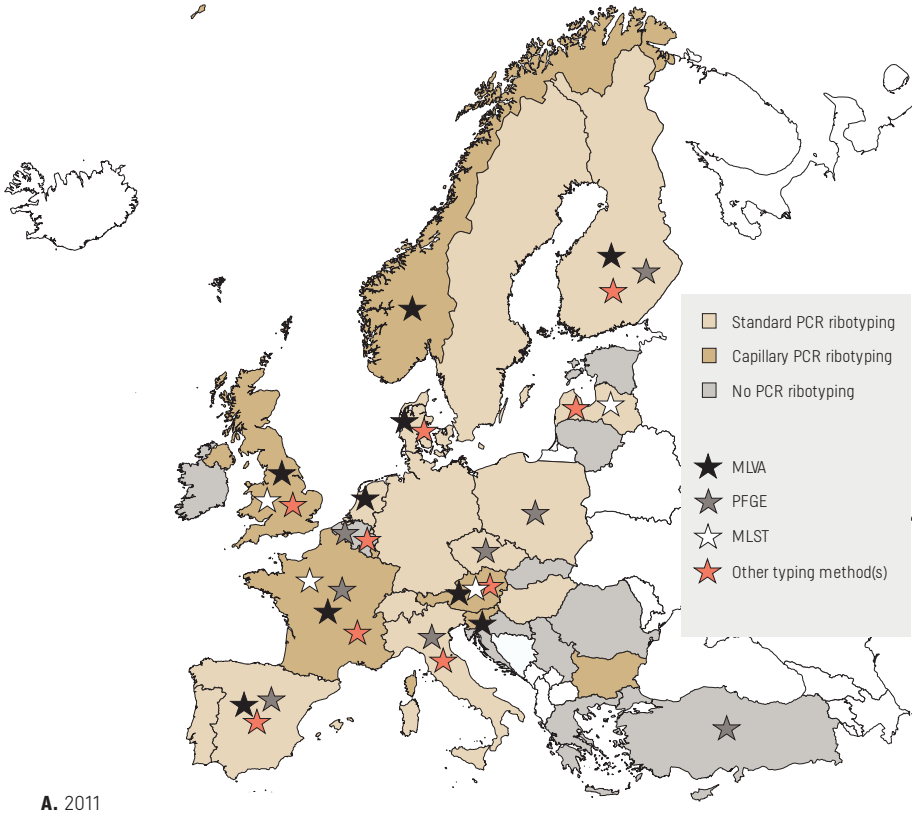
C. DIFFICILE NATIONAL TYPING METHODS

The capacity for various *C. difficile* typing methods in participating countries in 2011 and 2014 is depicted in Figure 3. The number of countries able to perform any method of typing increased from 22/31 countries in 2011 to 26/32 countries in 2014. Only six countries (Croatia, Cyprus, Estonia, Lichtenstein, Lithuania, Serbia) reported that they did not have any national typing capacity in 2014 (none of these countries had typing capacity in 2011); however, Lichtenstein sent samples to another country (Austria) for typing.

Several typing methods were implemented by the countries (Figure 3). PCR ribotyping (either capillary-based or conventional agarose gel-based), the current European standard for *C. difficile* typing, was available in 20/31 countries in 2011 and in 23/32 countries in 2014. Two of the countries that acquired ribotyping capacity (Ireland and Romania) use it for national surveillance. Capillary PCR ribotyping was applied by 7/31 countries in 2011 and by 16/32 countries in 2014. In 2014, nine of the 32 participating countries applied MLVA, six PFGE and seven MLST. In 2014, whole genome sequencing was available in Germany, the Netherlands, Norway, Slovenia and England.

Some countries reported specific changes in national molecular typing capacity between 2011 and 2014. Greece, which previously did not have typing capacity, introduced MLST in January 2014. At the time of the 2014 survey, Estonia was capable of ribotyping for research projects, although there were no such projects. Turkey performed PCR ribotyping but lacked software to analyse the data. Denmark stopped using PCR ribotyping and only applied tandem repeat sequence typing. Hungary reported limited typing capacity for financial reasons although PCR ribotyping remained available at the national reference laboratory. Finland restricted the indications for ribotyping to severe CDI or outbreaks, which unintentionally caused many laboratories to stop all culturing and/or sending isolates for typing.

Figure 3 *Clostridium difficile* typing methods available in countries that participated in the European *Clostridium difficile* Infection Surveillance Network (ECDIS-Net) project in 2011 (n = 31)[a] and 2014 (n = 32)[a]

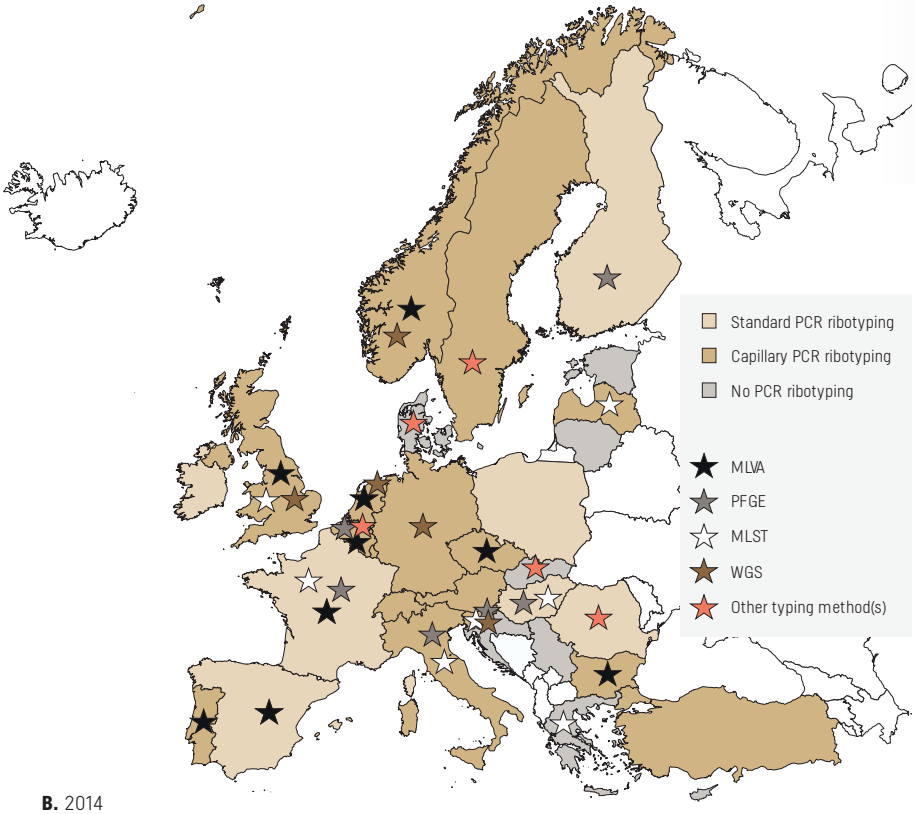


A. 2011

PFGE: pulsed-field gel electrophoresis; MLST: multilocus sequence typing; MLVA: multilocus variable-number tandem repeat analysis; WGS: whole genome sequencing.

Other typing methods used in 2011 were: *tcdC* typing (Austria, Belgium, Finland, France, Italy, Latvia, Luxembourg (not shown), Spain, United Kingdom - Northern Ireland only), repetitive-element PCR (Belgium, Spain), toxinotyping (Italy, Spain), tandem repeat sequence typing (Denmark) and pathogenicity locus (PaLoc) multiplex PCR (Finland).

Other typing methods used in 2014 were: *tcdA/B* (Belgium, Romania, Slovakia), CDT (Belgium, Slovakia), *tcdC* (Belgium), *Δ117TcdC* (Slovakia), and *GyrAΔ* detection (Belgium) detection, tandem repeat sequence typing (Denmark), and high molecular weight typing by MALDI-TOF (Sweden).



B. 2014

[a] In 2011, 31 countries responded: Austria, Belgium, Bulgaria, Croatia, Cyprus, Czech Republic, Denmark, Estonia, Finland, France, Germany, Greece, Hungary, Ireland, Italy, Latvia, Lichtenstein, Lithuania, Luxembourg, the Netherlands, Norway, Poland, Portugal, Romania, Slovakia, Slovenia, Spain, Sweden, Switzerland, Turkey and the United Kingdom (not including Wales). No data were available for Iceland. In 2011, Serbia did not participate in the European *Clostridium difficile* Infection Surveillance Network (ECDIS-Net) project. In 2014, Serbia participated in the ECDIS-Net project and responded to the 2014 questionnaire, as did Wales, and so the number of responding countries in 2014 was 32.

Source of map: FreeVectorMaps.com (http://freevectormaps.com).

Discussion

This study assessed changes in diagnostic testing and typing capacity for CDI in Europe between 2011 and 2014, using surveys of European local laboratories and of national coordinators participating in the ECDIS-Net project. Virtually all participating local laboratories had implemented CDI diagnostics in 2011 and 2014, compared with 88% (186/212) of the local laboratories investigated in eight European countries in 2003 [27]. The percentage of laboratories with optimal CDI diagnostics increased from 19% to 46%, and that with acceptable diagnostics increased from 10% to 15%. Importantly, the ESCMID-recommended two-step diagnostic algorithm [6] became more common. Nevertheless, we still observed a considerable variation in CDI diagnostics within and between European countries, in line with another European study with 482 participating hospitals in 2011–13 [3]. This variation in diagnostics can substantially affect CDI incidence rates obtained by surveillance [15,28]. Our survey showed that suboptimal CDI diagnostics may result from, for example, financial restrictions or limited availability of trained staff. As a consequence of the disagreement by a sizable minority of laboratories with the designation of diagnostic algorithms, the ESGCD undertook to revise its diagnostic guidelines [6] and propose an algorithm that can also be implemented in laboratories with limited numbers of trained staff and limited financial resources. These revised guidelines will be published in 2016 on behalf of ESCMID.

Among countries having national guidelines available, the UK was the only one that had succeeded in harmonising CDI diagnostics, by recommending a single two-test diagnostic algorithm ('comprising a GDH EIA (or NAAT/PCR) followed by a sensitive toxin EIA') [3,26]. The recommendations in the UK Department of Health guidance were supported by local study data and inclusion of frequently asked questions to allay objections of the laboratories to implementing the proposed diagnostic algorithms [26]. Furthermore, the diagnostics guidance was one of many *C. difficile*-related activities in the UK, for example, implementation of mandatory CDI reduction targets with financial penalties for national health services [29]. There probably are two possible ways to optimise testing: either to promote one national diagnostic algorithm or to promote the use of optimal testing strategies by local laboratories. However, the proposed algorithm in the UK was not fully compliant with the designation of diagnostic algorithms as optimal in this survey, highlighting the need for further discussion among experts to reach a consensus. Another example is Spain, where several national studies and meetings were organised [30,31] that resulted in an opinion document to enhance optimal diagnostics for CDI [32]. We hope that the national reference laboratories that participated in the ECDIS-Net project will follow these examples and promote optimal diagnostics for CDI and its implementation in local laboratories.

TYPING CAPACITY

Between 2011 and 2014, PCR ribotyping capacity and capillary PCR ribotyping increased among the participating countries. Capillary PCR ribotyping was validated in 2012–14 by four reference laboratories in England, the Netherlands, the United States and Canada, identifying a 98% consensus (195/200 cases tested) between the laboratories, which indicated the method's suitability for standardised CDI surveillance [17].

We assume that ECDIS-Net activities during 2012–14, including a training programme for *C. difficile* PCR ribotyping, contributed to the increased PCR ribotyping capacity. For example, Romania joined the training programme in 2012 and received a set of reference strains from the ECDIS-Net project and is now able to apply PCR ribotyping in their national surveillance. Poland reported having started their first national surveillance programme, stimulated by ECDIS-Net activities in 2012 [33]. A few countries (Hungary, Italy, Slovenia) had national surveillance under development at time of the 2014 survey. Despite these positive trends, our study also indicates that some European reference and local laboratories are affected by limited resources and budget reductions, which hamper implementation and technical improvements of molecular typing methods.

LIMITATIONS

This study has several limitations including the small, non-random selection of local laboratories for both surveys and the moderate response rate, limiting the degree to which conclusions can be extrapolated to all European microbiological laboratories. The representativeness of the invited and participating laboratories could not be assessed due to the absence of a suitably complete European register. Laboratories with better CDI diagnostics may have been more likely to participate in the original and follow-up surveys, leading to an overestimation of the number of laboratories with optimal CDI diagnostics in Europe. Additionally, the categorisation of CDI diagnostic algorithms into three levels, although made through a series of consultations with a team of international experts from the ECDIS-Net project, was based on expert opinion and some subjectivity cannot be excluded. Also, although the 2014 questionnaire for local laboratories requested quantitative data on the percentage of tests that followed each algorithm on a provided list, as the list had the subheadings 'optimal', 'acceptable' and 'incomplete', it is possible that those responding overestimated the proportion of desirable answers. We estimate that this reporting bias was minimal as for almost all laboratories, just one algorithm was used.

CONCLUSIONS

We conclude that the ECDIS-Net project laid the foundations for Europe-wide surveillance of CDI, although increased use of optimal diagnostic algorithms should be promoted, taking into consideration the limited resources and budget cuts in several European countries. The ESGCD revised the ESCMID diagnostics guidelines for CDI, which, once published, should contribute to standardisation of CDI diagnostics at local and national level in Europe. Typing capacity for CDI in Europe was acceptable overall; however, an internationally standardised capillary PCR ribotyping protocol is now available [17] and requires further implementation in European countries. We would recommend that these important steps are considered as part of the integration of *C. difficile* molecular typing data in The European Surveillance System (TESSy), within the ECDC-coordinated Europe-wide CDI surveillance (since 1 January 2016) [34].

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Chapter 3

Standardised surveillance of Clostridium difficile infection in European acute care hospitals: a pilot study, 2013

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19. Other members of the ECDIS-Net project are listed at the end of the article

Euro Surveill. 2016;21(29).

Clostridium difficile infection (CDI) remains poorly controlled in many European countries, of which several have not yet implemented national CDI surveillance. In 2013, experts from the European CDI Surveillance Network project and from the European Centre for Disease Prevention and Control developed a protocol with three options of CDI surveillance for acute care hospitals: a 'minimal' option (aggregated hospital data), a 'light' option (including patient data for CDI cases) and an 'enhanced' option (including microbiological data on the first 10 CDI episodes per hospital). A total of 37 hospitals in 14 European countries tested these options for a three-month period (between 13 May and 1 November 2013). All 37 hospitals successfully completed the minimal surveillance option (for 1,152 patients). Clinical data were submitted for 94% (1,078/1,152) of the patients in the light option; information on CDI origin and outcome was complete for 94% (1,016/1,078) and 98% (294/300) of the patients in the light and enhanced options, respectively. The workload of the options was 1.1, 2.0 and 3.0 person-days per 10,000 hospital discharges, respectively. Enhanced surveillance was tested and was successful in 32 of the hospitals, showing that *C. difficile* PCR ribotype 027 was predominant (30% (79/267)). This study showed that standardised multicountry surveillance, with the option of integrating clinical and molecular data, is a feasible strategy for monitoring CDI in Europe.

Introduction

After recognition of European outbreaks of *Clostridium difficile* infections (CDIs) associated with the emergence of PCR ribotype 027/NAP1 in 2005, CDI surveillance at country level was encouraged by the European Centre for Disease Prevention and Control (ECDC) [1]. In 2008, an ECDC-supported European CDI survey (ECDIS) identified large intercountry variations in incidence rates and distribution of prevalent PCR ribotypes, with the outbreak-related PCR ribotype 027 being detected in 5% (range: 0–26) of the characterised isolates [2]. The surveillance period was limited to one month and the representation of European hospitals was incomplete; however, this has been the only European (comprising European Union (EU)/European Economic Area (EEA) and EU candidate countries) CDI surveillance study. The authors highlighted the need for national and European surveillance to control CDI. Yet, European countries were found to have limited capacity for diagnostic testing, particularly in terms of standard use of optimal methods and absence of surveillance protocols and a fully validated, standardised and exchangeable typing system for surveillance and/or outbreak investigation.

As of 2011, 14 European countries had implemented national CDI surveillance, with various methodologies [3]. National surveillance systems have since reported a decrease in CDI incidence rate and/or prevalence of PCR ribotype 027 in some European countries [4 – 8]. However, CDI generally remains poorly controlled in Europe [9], and PCR ribotype 027 continues to spread in eastern Europe [10 – 12] and globally [13].

In 2010, ECDC launched a new project, the European *C. difficile* Infection Surveillance Network (ECDIS-Net), to enhance surveillance of CDI and laboratory capacity to test for CDI in Europe. The goal of ECDIS-Net was to establish a standardised CDI surveillance protocol suitable for application all over Europe in order to: (i) estimate the incidence rate and total infection rate of CDI (including recurrent CDI cases) in European acute care hospitals; (ii) provide participating hospitals with a standardised tool to measure and compare their own incidence rates with those observed in other participating hospitals; (iii) assess adverse outcomes of CDI such as complications and death; and (iv) describe the epidemiology of CDI concerning antibiotic susceptibility, PCR ribotypes, presence of tcdA, tcdB and binary toxins and detect new emerging types at local, national and European level.

The primary objectives of the present study were to: (i) test the pilot protocol for the surveillance of CDI in European acute care hospitals developed by ECDIS-Net (methodology, variables and indicators); (ii) assess the feasibility and workload of collecting the required hospital data, case-based epidemiological and microbiological data; and (iii) evaluate the quality of data collected, whether in

the presence or absence of existing national CDI surveillance activities. A secondary aim was to assess the relationship between patient and microbiological characteristics and in-hospital outcome of CDI to confirm the added value of collecting detailed epidemiological and microbiological data on CDI at European level.

Methods

STUDY PROTOCOL AND DEFINITIONS

A pilot protocol for the surveillance of CDI in European acute care hospitals was developed by ECDIS-Net participants (epidemiologists and medical microbiologists from various European countries) and ECDC experts in 2012–13. The pilot protocol version 1.2 specified three options for surveillance: 'minimal', 'light' and 'enhanced' [14]. In the minimal surveillance, aggregated numerator and denominator data were gathered on all CDI cases. In the light surveillance, basic case-based epidemiological data were included (e.g. age, sex, date of hospital admission and of CDI onset, CDI origin, recurrent CDI) on all CDI cases. In the enhanced surveillance, additional epidemiological data (e.g. comorbidities scored by the McCabe score [15] and the Acute Physiology and Chronic Health Evaluation II (APACHE II) chronic health points [16], in-hospital deaths) and *C. difficile* isolates were collected for the first 10 episodes of CDI per hospital. Outcome was not followed up after discharge from the hospital.

The case definitions for CDI (Box) were based on recommendations for CDI surveillance, as proposed by ECDC and the United States Centers for Disease Control and Prevention (CDC) [1,17].

Patients were included as a CDI case if symptom onset occurred within the hospitals' surveillance period, or if the patient was admitted during the surveillance period with symptoms present. Infants (children below two years-old) with 'compelling clinical evidence for CDI' were also included.

PARTICIPANTS AND STUDY PERIOD

A total of 14 countries participated in this pilot study: they were selected by the project leaders given their various levels of ongoing surveillance activities and laboratory and typing capacity for CDI [18]. At the start of the ECDIS-Net project, nine countries (Austria, Belgium, Denmark, Finland, France, Germany, Hungary, the Netherlands and United Kingdom (Scotland only), hereafter referred to as UK-Scotland) had already implemented national surveillance of CDI; five countries (Estonia, Norway, Poland, Romania and Serbia) had not. ECDIS-Net participants identified a convenience sample of two to four acute care hospitals per country to test the pilot protocol for a three-month surveillance period between 13 May and 1 November 2013. Hospitals were encouraged, but not obligated, to test all surveillance options in the protocol and to involve both hospital infection control personnel and microbiology laboratory personnel in data collection. It was agreed that the actual location of participating hospitals would not be disclosed for reasons of confidentiality. We identified the proxy location of participating hospitals by mapping the median healthcare-associated CDI incidence rates obtained in this pilot study using the nomenclature of territorial units for statistics (NUTS) 1 regions [19] that contained at least one participating hospital.

Box. Definitions for surveillance of *Clostridium difficile* infections

CDI case

A patient to whom one or more of the following criteria applies:

1. diarrhoeal stools or toxic megacolon, and a positive laboratory assay for *C. difficile* TcdA and/or TcdB in stools or a toxin-producing *C. difficile* organism detected in stool via culture or other means;
2. pseudomembranous colitis revealed by lower gastrointestinal endoscopy;
3. colonic histopathology characteristic of CDI (with or without diarrhoea) on a specimen obtained during endoscopy, colectomy or autopsy.

Recurrent CDI

An episode of CDI (return of diarrhoeal stools with a positive laboratory test after the end of treatment) > 2 weeks and ≤ 8 weeks following the onset of a previous episode (CDI cases with onset later than 8 weeks after the onset of a previous episode were included as new CDI cases).

Healthcare-associated case

A case of CDI with onset of symptoms at least 48 hours following admission to a healthcare facility or with onset of symptoms in the community within 4 weeks following discharge from a healthcare facility.

Community-associated case

A case of CDI with onset of symptoms outside a healthcare facility or within 48 hours after admission to a healthcare facility, without residence in/discharge from a healthcare facility within the previous 12 weeks.

Complicated course of CDI

CDI leading to any of the following:

1. admission to an intensive-care unit for treatment of CDI or its complications (e.g. for shock requiring vasopressor therapy);
2. surgery (colectomy) for toxic megacolon, perforation or refractory colitis;
3. death within 30 days after diagnosis if CDI is either a primary or contributing cause.

CDI : *Clostridium difficile* infection.

Source: [1,17].

MICROBIOLOGICAL INVESTIGATION

Local laboratories that serviced the participating hospitals used their own diagnostic procedures for CDI. Data on the algorithm used for CDI diagnosis was collected for each patient included in light surveillance. In the enhanced surveillance option, 10 *C. difficile* isolates (or stool samples, if there was no possibility of anaerobic culture at the local laboratory) from samples from the first 10 episodes of CDI per hospital were sent to the national reference laboratory or appointed study laboratory (collectively referred to as NRL) which performed PCR ribotyping and antimicrobial susceptibility testing, performed according to national procedures. Most NRLs used conventional agarose gel-based PCR ribotyping [3] (Finland, France, Hungary, Poland, the Netherlands and UK-Scotland), some used capillary-based PCR ribotyping [3] (Austria, Belgium and Germany). Denmark, Estonia, Romania and Serbia did not perform PCR ribotyping and for Norway, the PCR ribotyping method used was not reported. NRLs were requested to send all *C. difficile* isolates to the coordinating laboratory (Leiden University Medical Centre, the Netherlands), which completed and confirmed microbiological results. The presence of a glutamate dehydrogenase (GDH) gene specific for *C. difficile* was confirmed in the coordinating laboratory by an in-house PCR [20], followed by PCR ribotyping [21]. Toxin genes (*tcdA, tcdB, cdtA, cdtB*) were detected by multiplex PCR [22]. In vitro susceptibility to metronidazole, vancomycin, and moxifloxacin was determined by measuring minimum inhibitory concentrations (MICs) by an agar dilution method [23] and interpreted using epidemiological cut-off values from the European Committee on Antimicrobial Susceptibility Testing (EUCAST). Isolates with a metronidazole MIC>2 mg/L, a vancomycin MIC>2 mg/L and moxifloxacin MIC>4 mg/L were interpreted as resistant [24].

DATA HANDLING

Data were entered in a web-based system developed for the current study (by the Institute of Hygiene and Environmental Medicine, Charité Universitätsmedizin Berlin, Germany, in 2013) and were analysed with SPSS version 20.0 and Stata software version 12.1.

STATISTICAL ANALYSIS AND STUDY ENDPOINTS

PRIMARY ENDPOINTS

Variables and indicators

For all variables in each surveillance option, frequencies and proportions were calculated, as appropriate. Hospital median incidence rates for healthcare-associated (HA) CDI and recurrent CDI were calculated per 10,000 hospital discharges and per 10,000 patient-days using minimal surveillance protocol data. Dispersion around the median was described with the 25th and 75th percentile (interquartile range, IQR). We calculated 95% confidence intervals (CIs) for the incidence rates by Byar's approximation.

Feasibility and workload

Workload, defined as person-days per 10,000 hospital discharges required to complete each surveillance option, and feasibility were measured using a questionnaire distributed to all participants.

Data quality

Epidemiological data quality was primarily assessed by data completeness. This was estimated by comparing each hospital's minimal surveillance numerators (minimal option) with the number of available patient records (light option), and by calculating the proportion of patients for whom origin of the CDI (light option) and course of infection (enhanced option) were recorded, with less than 10% missing data being considered acceptable.

Microbiological data quality was assessed through comparison of each hospital's testing rate per 10,000 patient-days and percentage of positive tests. Additionally, all NRLs' ribotyping results obtained during the pilot study were compared with those of the coordinating laboratory. Additionally, in May 2013 and September 2014, participation in two external quality assessments was offered by Public Health England to all ECDIS-Net NRLs that performed typing. NRLs in nine of the participating countries took part; on each occasion, 10 *C. difficile* strains were sent to the same eight NRLs and the coordinating laboratory of this study.

Secondary endpoints

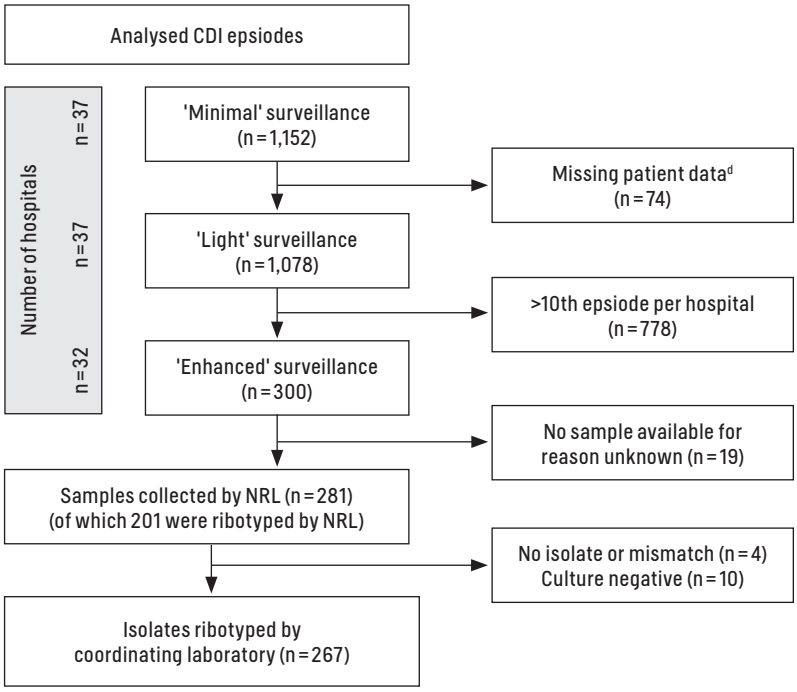
Relationships between the risk of a complicated course of CDI or all-cause in-hospital mortality in CDI cases (of any origin) and patient characteristics and microbiological results (as confirmed by the coordinating laboratory) were analysed by logistic regression. Correlations between incidence rates, testing rates and the proportion of PCR ribotype 027 were analysed by Spearman's rank test.

Results

PARTICIPATING HOSPITALS

A total of 37 acute care hospitals from 14 European countries tested the minimal and light surveillance options for a three-month period between 13 May 2013 and 1 November 2013. Of the 37 acute care hospitals, 21 were tertiary care hospitals, 10 secondary care hospitals, five primary care hospitals and one was a specialised hospital for infectious and tropical diseases. A total of 36 hospitals included all wards; one hospital excluded a neonatal ward. Of the 37 participating hospitals, 32, from 13 countries, tested the enhanced option as well (Figure 1).

Figure 1 Data collection in the pilot study for standardised surveillance[a] of *Clostridium difficile* infection in 37 acute care hospitals in 14 European countries[b], 13 May – 1 November 2013[c]



CDI: *Clostridium difficile* infection.

[a] Three surveillance options were tested: 'minimal' (aggregated hospital data), 'light' (including patient data for CDI cases) and 'enhanced' (including microbiological data on the first 10 CDI episodes per hospital).

[b] Austria, Belgium, Denmark, Estonia, Finland, France, Germany, Hungary, the Netherlands, Norway, Poland, Romania, Serbia, United Kingdom (Scotland only). Enhanced surveillance including PCR ribotyping was carried out by Austria, Belgium, Finland, France, Germany, Hungary, the Netherlands, Norway, Poland and United Kingdom (Scotland only); Denmark, Romania and Serbia participated in enhanced surveillance, but did not perform PCR ribotyping at the national reference laboratory or appointed study laboratory.

[c] Three-month assessment during this time period.

[d] Clincial patient data missing, for reasons unknown.

A total of 1,152 CDI episodes were recorded by minimal surveillance in 37 hospitals (Table 1).

Table 1. Results from 'minimal' surveillancea of *Clostridium difficile* infection in 37 acute care hospitals in 14 European countries, 13 May–1 November 2013[b]

Country (number of hospitals)	Hospital discharges n	Patient- days n	CDI episodes included n	HA-CDIs n (%)	CA-CDIs and CDIs of un-known origin n (%)	Recurrent CDIs n (%)	Median incidence rate of HA-CDI	
							per 10,000 hospital discharges (range)	per 10,000 patient-days (range)
Austria (4)	56,773	307,721	117	88 (75)	16 (14)	13 (11)	15.8 (10.0–35.4)	3.2 (2.0–4.8)
Belgium (3)	20,434	140,603	53	32 (60)	13 (25)	8 (15)	17.7 (6.0–26.6)	2.7 (0.8–3.7)
Denmark (4)	60,572	182,888	171	120 (70)	25 (15)	26 (15)	17.7 (11.0–31.0)	5.3 (4.6–11.0)
Estonia (2)	18,293	133,790	18	16 (89)	1 (6)	1 (6)	8.6 (7.3–10.0)	1.2 (0.8–1.7)
Finland (3)	10,876	39,816	29	17 (59)	9 (31)	3 (10)	14.9 (12.2–20.8)	4.4 (2.6–6.5)
France (2)	9,608	64,203	46	31 (67)	9 (20)	6 (13)	26.7 (9.1–44.3)	3.8 (2.0–5.7)
Germany (3)	66,952	307,791	174	136 (78)	33 (19)	5 (3)	23.1 (16.2–28.2)	3.6 (3.4–6.7)
Hungary (2)	18,207	166,926	254	213 (84)	24 (9)	17 (7)	121.6 (111.5–131.8)	14.9 (11.2–18.5)
Netherlands (3)	20,388	123,507	43	29 (67)	11 (26)	3 (7)	10.5 (10.2–19.4)	1.9 (1.8–2.9)
Norway (2)	35,365	194,204	60	33 (55)	15 (25)	12 (20)	9.6 (8.5–10.8)	1.9 (1.4–2.5)
Poland (2)	15,182	86,771	69	65 (94)	4 (6)	0 (0)	42.6 (40.7–44.6)	7.6 (7.0–8.2)
Romania (2)	19,243	90,582	33	19 (58)	7 (21)	7 (21)	12.1 (8.0–16.5)	6.7 (1.4–12.0)
Serbia (3)	8,930	59,435	49	37 (76)	2 (4)	10 (20)	89.8 (22.0–131.8)	10.0 (3.9–11.3)
UK-Scotland (2)	26,554	94,942	36	16 (44)	13 (36)	7 (19)	5.3 (4.2–6.4)	1.4 (0.6–2.2)
Total (37)	**387,377**	**1,993,179**	**1,152**	**852 (74)**	**182 (16)**	**118 (10)**	**16.4 (4.2–131.8)**	**3.7 (0.6–18.5)**

CA: community-associated; CDI: *Clostridium difficile* infection; HA: healthcare-associated; UK-Scotland: United Kingdom (Scotland only).

The pilot study was based on a non-representative sample, thus the results presented cannot be interpreted as being representative of any participating country or of the European Union/European Economic Area.

[a] The 'minimal' surveillance option comprised aggregated hospital data.
[b] Three-month assessment during this time period.

After exclusion of recurrent episodes, the incidence rate of healthcare-associated CDI by hospital ranged from 4.2 to 131.8 per 10,000 hospital discharges (median: 16.4; IQR: 10.1–29.5) and from 0.6 to 18.5 per 10,000 patient-days (median: 3.7; IQR: 2.0–6.6). The incidence rate of recurrent CDI varied between 0 and 118.6 per 10,000 hospital discharges (median: 2.0; IQR: 0.2–5.2) and between 0 and 9.0 per 10,000 patient-days (median: 0.3; IQR: 0.04–1.2).

LIGHT SURVEILLANCE: PATIENT CHARACTERISTICS AND DIAGNOSTICS

Patient data were submitted for 1,078 CDI episodes in 37 hospitals (Figure 1). Most CDI cases were diagnosed by toxin enzyme immunoassay (EIA), confirmed by toxigenic culture (n = 220) or toxin EIA alone (n = 188). Other cases were diagnosed by GDH detection and confirmed by toxin PCR (n = 101) or toxin EIA (n = 88), by toxin PCR alone (n = 91), toxin PCR and toxigenic culture (n = 72) or other diagnostic algorithms (n = 318).

The median age of patients was 72 years (IQR: 59–80); 38 (4%) CDI episodes were in those younger than 18 years, of whom 13 were younger than two years. The current hospital was reported as being the origin of infection for 66% (n = 673), another hospital for 18% (n = 178), a long-term care facility for 1% (n = 13) and another healthcare facility for 2% (n = 21) of the 1,016 CDI episodes of known origin (for 62 episodes, the origin was unknown). Other patient characteristics are shown in Table 2.

Table 2. Patient characteristics from 'light' (n = 1,078) and 'enhanced' surveillance[a] (n = 300) of *Clostridium difficile* infection in participating acute care hospitals in selected European countries[b], with putative determinants of a complicated course of infection and all-cause in-hospital mortality, 13 May–1 November 2013[c]

Patient characteristics	Light surveillance n[d]/N (%)	Enhanced surveillance n[d]/N (%)	Univariable analysis	
			Complicated course OR (95% CI)	In-hospital mortality OR (95% CI)
Age in years				
< 65	370/1,077 (34)	104/299 (35)	ref.	ref.
65–84	549/1,077 (51)	152/299 (51)	3.4 (1.0–12.2)	1.6 (0.7–3.7)
≥ 85	158/1,077 (15)	43/299 (14)	6.6 (1.6–26.9)	2.1 (0.7–5.9)
Sex				
Female	573/1,078 (53)	157/300 (52)	ref.	ref.
Male	505/1,078 (47)	143/300 (48)	0.8 (0.3–1.8)	1.0 (0.5–2.1)
Recurrent infection				
No	862/978 (88)	240/277 (87)	ref.	ref.
Yes	116/978 (12)	37/277 (13)	0.7 (0.1–3.0)	0.9 (0.3–2.7)
CDI at admission				
No	505/984 (51)	153/276 (55)	ref.	ref.
Yes	479/984 (49)	123/276 (45)	1.7 (0.7–4.2)	0.3 (0.1–0.7)
Days of hospital stay to hospital-onset CDI				
Number (IQR)	11 (IQR: 6–21)	9 (IQR: 6–17)	NA	NA

CDI origin				
HA	885/1,078 (82)	249/300 (83)	ref.	ref.
CA	131/1,078 (12)	37/300 (12)	1.0 (0.3–3.7)	0.4 (0.1–1.6)
Unknown	62/1,078 (6)	14/300 (5)	2.0 (0.4–9.4)	1.1 (0.2–5.1)
Ward speciality				
Medical[e]	NC	194/299 (65)	ref.	ref.
Surgical	NC	53/299 (18)	0.9 (0.3–2.8)	0.8 (0.3–2.3)
ICU	NC	29/299 (10)	1.8 (0.6–5.8)	2.5 (1.0–6.5)
Other	NC	23/299 (8)	NA	0.7 (0.2–3.4)
Healthcare admission <3 months				
No	NC	84/287 (29)	ref.	ref.
Hospital	NC	194/287 (68)	1.0 (0.4–2.5)	1.3 (0.6–2.9)
Other	NC	9/287 (3)	1.6 (0.2–14.5)	1.0 (0.1–9.3)
Antibiotic treatment <3 months[f]				
No	NC	34/254 (13)	ref.	ref.
One course	NC	111/254 (44)	1.4 (0.4–5.2)	1.3 (0.4–4.1)
Multiple courses	NC	109/254 (43)	0.7 (0.2–3.0)	1.0 (0.3–3.3)
Expected survival in years (McCabe score)				
>5	NC	171/285 (60)	ref.	ref.
1–4	NC	83/285 (29)	2.2 (0.9–5.5)	1.8 (0.7–4.5)
<1	NC	31/285 (11)	2.5 (0.7–8.7)	12.0 (4.7–30.5)
Severe comorbidity (APACHE II CHP)[g]				
Liver cirrhosis	NC	16/295 (5)	0.7 (0.1–5.8)	1.7 (0.5–6.1)
NYHA class IV heart failure	NC	29/295 (10)	2.2 (0.7–7.0)	3.4 (1.4–8.3)
Pulmonary disease	NC	38/297 (13)	3.3 (1.2–8.5)	1.7 (0.7–4.3)
Chronic dialysis	NC	18/299 (6)	1.4 (0.3–6.7)	2.2 (0.7–7.2)
Immunocompromised status	NC	92/291 (32)	0.8 (0.3–2.2)	1.3 (0.6–2.7)
***C. difficile* clade**				
Clade 1, 3, 4 and 5	NC	187/267 (70)	ref.	ref.
Clade 2 (ribotype 027/176)	NC	80/267 (30)	0.9 (0.4–2.5)	1.0 (0.4–2.3)
***C. difficile* binary toxin genes**				
No	NC	165/264 (63)	ref.	ref.
Yes	NC	99/264 (38)	0.8 (0.3–2.1)	1.0 (0.4–2.1)

APACHE II CHP: Acute Physiology and Chronic Health Evaluation II chronic health points; CA: community-associated; CDI: *Clostridium difficile* infection; HA: healthcare-associated; ICU: intensive-care unit; IQR: interquartile range; NA: not applicable; NC: not collected; NYHA: New York Heart Association; OR: odds ratio; ref.: reference group.

[a] The 'light' surveillance option included patient data for CDI cases; in the 'enhanced' option, microbiological data on the first 10 CDI episodes per hospital were included.

[b] All 37 hospitals in 14 European countries (Austria, Belgium, Denmark, Estonia, Finland, France, Germany, Hungary, the Netherlands, Norway, Poland, Romania, Serbia and United Kingdom (Scotland only)) tested the light option; 32 hospitals in 13 countries (Austria, Belgium, Denmark, Finland, France, Germany, Hungary, the Netherlands, Norway, Poland, Romania, Serbia and United Kingdom (Scotland only)) tested the enhanced option.

[c] Three-month assessment during this time period.

[d] Number of episodes/total number of episodes for which data were available, unless otherwise indicated.

[e] 'Medical' included several subspecialties of internal medicine (see protocol [14]).

[f] Antibiotic treatment in past 3 months was the only variable with >10% missing data.

[g] The reference group consisted of patients without the comorbidity listed.

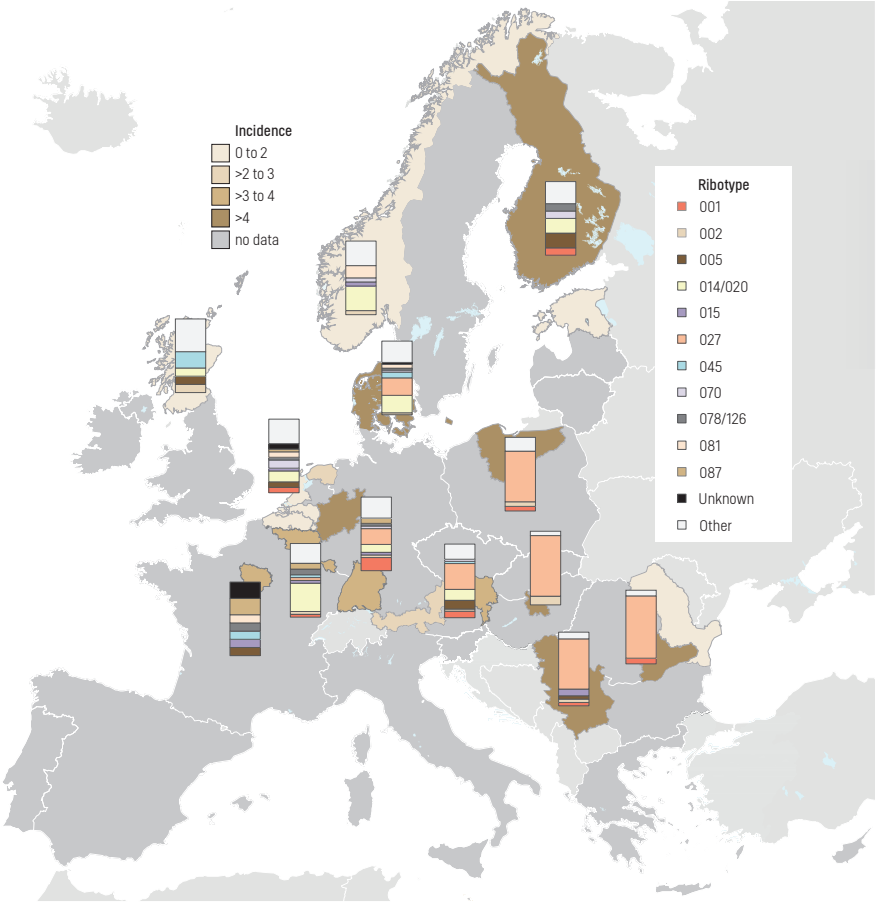
ENHANCED SURVEILLANCE: COMPLICATED CDI AND IN-HOSPITAL MORTALITY

For 300 CDI episodes in 32 hospitals, enhanced surveillance data were also submitted (Table 2). The course of CDI was known for 98% (n = 294) of cases; 8% (n = 24) experienced a complicated course of infection (as defined in the Box). In univariable analysis, a complicated course was associated with age of 85 years or older and severe pulmonary disease, but not with CDI origin, presence of PCR ribotypes 027 or 176, or of binary toxin genes (Table 2). A total of 12% (n = 37) of CDI cases died during hospitalisation. Six deaths (2% of all CDI episodes) were related to CDI, 23 deaths (8% of all CDI episodes) were unrelated to CDI, and the relationship between CDI and death was unknown for the remaining eight episodes (3% of all CDI episodes). Patients with a complicated course had a 42% risk of in-hospital death (of which 25% were CDI-related) compared with 9% among patients with an uncomplicated course. All-cause in-hospital mortality was associated with a lower number of years of expected survival (a high McCabe score), healthcare-onset CDI and severe heart failure, but not with CDI origin, presence of PCR ribotypes 027 or 176, or of binary toxin genes (Table 2).

ENHANCED SURVEILLANCE: MICROBIOLOGICAL DATA

C. difficile was cultured and characterised in the coordinating laboratory for 267 (89%) of the 300 CDI episodes registered during enhanced surveillance. The presence of toxin A and B genes was confirmed in 99% (263/265) of the cultured isolates; binary toxin genes were present in 38% (99/264) of the isolates. A total of 51 different PCR ribotypes were characterised. The predominant PCR ribotype was 027 (30%; n = 79), followed by the highly related PCR ribotypes 014 and 020 (15%; n = 40), and PCR ribotype 001 (6%; n = 15). PCR ribotype 027 was identified in isolates from eight European countries in 4–85% of all characterised samples, depending on the country (Figure 2).

Figure 2 Incidence rate of healthcare-associated *Clostridium difficile* infection using 'minimal' surveillance[a], by region (n = 22)[b] and distribution of PCR ribotypes identified using enhanced surveillance, by European country (n = 13)[c], 13 May–1 November 2013[d]



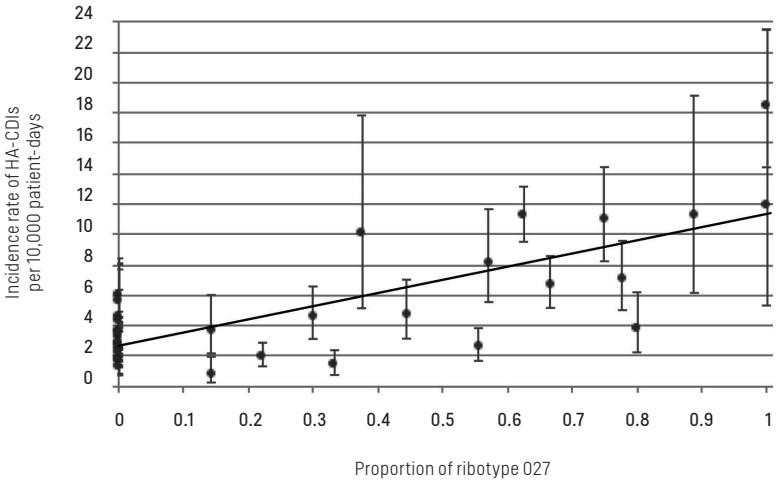
CDI: *Clostridium difficile* infection; NUTS: nomenclature of territorial units for statistics.
The pilot study was based on a non-representative sample, thus the rates and distributions presented in this figure cannot be interpreted as being representative of any NUTS region.

[a] The 'minimal' surveillance option comprised aggregated hospital data; the 'enhanced' option included microbiological data on the first 10 CDI episodes per hospital.
[b] The NUTS 1 region indicates the geographical location of each participating hospital, rather than that of the hospital's catchment area. The incidence rate per 10,000 patient-days in each NUTS 1 region is the median for all hospitals that participated within that same region.
[c] The number of PCR ribotyped strains varied by country: Austria (34), Belgium (26), Denmark (38), Finland (10), France (9), Germany (28), Hungary (17), the Netherlands (27), Norway (18), Poland (16), Romania (13), Serbia (22) and United Kingdom (Scotland only) (9).
[d] Three-month assessment during this time period.

Source of map: FreeVectorMaps.com (http://freevectormaps.com).

PCR ribotype 176, which is highly related to 027, was found in one CDI case in a country where no PCR ribotype 027 isolates were identified. The proportion of PCR ribotype 027 isolates correlated with the incidence rate of HA-CDI per 10,000 patient-days (Spearman's rho: 0.64; 95% CI: 0.36–0.81) (Figure 3).

Figure 3 Incidence rate of healthcare-associated *Clostridium difficile* infection in relation to the proportion of PCR ribotype 027 isolates, from 'enhanced' surveillance[a] in acute care hospitals in 13 European countries[b], 13 May–1 November 2013[c]



CA: community-associated; CDI: *Clostridium difficile* infection; CI: confidence interval; HA: healthcare-associated.

Whiskers indicate the 95% CI around the incidence rate of HA-CDI per 10,000 patient-days per hospital. The proportion of PCR ribotype 027 isolates correlated with the incidence rate (Spearman's rho: 0.64; 95% CI: 0.36–0.81).

[a] The 'enhanced' surveillance option included microbiological data on the first 10 CDI episodes per hospital.
[b] Austria, Belgium, Denmark, Finland, France, Germany, Hungary, the Netherlands, Norway, Poland, Romania, Serbia and United Kingdom (Scotland only).
[c] Three-month assessment during this time period.

All isolates that were investigated for antimicrobial susceptibility (n=251) were susceptible in vitro to metronidazole. Eight PCR ribotype 027 isolates from Austria, Germany and Hungary showed reduced susceptibility to metronidazole, with a MIC just below the EUCAST epidemiological cut-off value [24]. Two PCR ribotype 027 isolates from Denmark showed reduced susceptibility to vancomycin, with a MIC just below the EUCAST epidemiological cut-off value [24]; however, resistance to vancomycin was not detected. In vitro moxifloxacin resistance was identified in 37% (n=92) isolates, of which 77% (n=71) belonged to PCR ribotype 027.

FEASIBILITY AND WORKLOAD

Participating hospitals reported a median of seven CDI episodes (IQR: 4–12) per month through both minimal and light surveillance. The feasibility questionnaire was completed by 26 of the 37 participating hospitals. Completion of the light and enhanced options were found to be 'not difficult' for 23/26 and 21/24 respondents, respectively. The remaining respondents found them 'quite difficult'.

The median workload for the 'minimal', 'light' and 'enhanced' surveillance options was 1.1, 2.0 and 3.0 person-days per 10,000 hospital discharges, respectively (Table 3).

The highest workload was reported by countries with the highest aggregated CDI incidence rates during the pilot (Serbia and Hungary). There were no differences in surveillance indicators by pre-existing surveillance activities, or when considering laboratory or typing capacity for CDI in the pilot study (Table 3).

DATA QUALITY

Completeness of data was 94% (1,078/1,152) for patient data in the light option and 98% (294/300) for data on the course of CDI in the enhanced option. Testing frequency (range: 17–308 tests per 10,000 patient days) and the proportion of positive tests (range: 2–46%) varied between countries (Table 3). The testing frequency correlated with the overall CDI incidence rate per 10,000 patient days (Spearman's rho: 0.45; 95% CI: 0.15–0.68). PCR ribotyping results from the NRLs obtained during enhanced surveillance were concordant with the coordinating laboratory's results for 77% (128/166) of the isolates. Discordant results were either due to a mismatch in the identified PCR ribotype (n = 19; 11%), or because a PCR ribotype pattern result was not recognised by a NRL (n = 17; 10%) or by the coordinating laboratory (n = 2; 1%). External quality assessment demonstrated 75% and 86% accuracy of PCR ribotype allocation by the NRLs in 2013 and 2014, respectively.

Table 3. Surveillance indicators used to evaluate the ability to collect data and workload for the three surveillance options[a] for *Clostridium difficile* infection in 37 acute care hospitals in 14 European countries[b], 13 May–1 November 2013[c]

Country (number of hospitals in light/enhanced surveillance)	Surveillance option								
	Minimal			Light			Enhanced		
	Testing frequency	Proportion of positive tests	Workload	Patient data available[f]	Data on CDI origin	Workload	Data on CDI outcome[g]	Matching PCR ribotype[h]	Workload
	Median number of tests per 10,000 patient days[d] (range)	n/N (%)	Median number of person-days per 10,000 hospital discharges[e] (range)	n/N (%)	n/N (%)	Median number of person-days per 10,000 hospital discharges[e] (range)	n/N (%)	n/N (%)	Median number of person-days per 10,000 hospital discharges[e] (range)
Austria (4/4)	31 (21–66)	111/1,117 (10)	0.7 (0.1–2.1)	111/117 (95)	109/111 (98)	2.1 (0.5–10.3)	40/40 (100)	26/34 (76)	2.8 (1.0–3.0)
Belgium (3/3)	55 (50–85)	60/833 (7)	0.3 (0.1–0.8)	53/53 (100)	52/53 (98)	1.6 (1.5–2.2)	26/28 (93)	16/26 (62)	1.6 (0.8–4.4)
Denmark (4/4)	71 (43–105)	202/1,360 (15)	0.5 (0.3–0.9)	168/171 (98)	163/168 (97)	1.0 (0.9–2.0)	37/39 (95)	NA	1.7 (1.3–2.5)
Estonia (2/0)[i]	17 (10–24)	17/218 (8)	NA	17/18 (94)	17/17 (100)	NA	NA	NA	NA
Finland (3/1)	129 (33–151)	48/448 (11)	1.2 (0.8–4.2)	23/29 (79)	23/23 (100)	3.3 (1.2–4.2)	10/10 (100)	9/10 (90)	5.0[j]
France (2/1)	72 (63–81)	35/493 (7)	NA	40/46 (87)	39/40 (98)	NA	10/10 (100)	5/9 (56)	NA
Germany (3/3)	82 (70–111)	174/2,656 (7)	1.0 (0.1–1.8)	171/174 (98)	153/171 (89)	1.2 (0.5–1.8)	30/30 (100)	21/27 (78)	2.1 (1.2–3.0)
Hungary (2/2)	77 (67–86)	237/1,192 (20)	2.5 (2.0–3.0)	251/254 (99)	236/251 (94)	38.7 (28.4–49.0)	19/20 (95)	14/17 (82)	9.6 (4.1–15.1)
Netherlands (3/3)	45 (7–262)	79/1,124 (7)	1.7 (0.6–1.8)	43/43 (100)	38/43 (88)	1.8 (1.7–5.1)	29/29 (100)	NA	5.3 (4.0–13.6)
Norway (2/2)[i]	38 (23–52)	60/614 (10)	0.8[j]	60/60 (100)	55/60 (92)	1.5[j]	20/20 (100)	12/18 (67)	2.3[j]
Poland (2/2)[i]	20 (18–21)	79/173 (46)	NA	34/69 (49)	34/34 (100)	NA	19/19 (100)	16/16 (100)	NA
Romania (2/2)[i]	308 (9–607)	26/427 (6)	NA	26/33 (79)	24/26 (92)	NA	12/13 (92)	NA	NA
Serbia (3/3)[i]	40 (7–184)	49/253 (19)	15.0 (2.9–26.4)	49/49 (100)	49/49 (100)	15.0 (2.9–26.4)	30/30 (100)	NA	37.4 (5.9–92.2)
UK-Scotland (2/2)	179 (142–216)	33/1,813 (2)	2.2 (2.1–2.3)	32/36 (89)	24/32 (75)	4.7 (2.1–7.3)	12/12 (100)	9/9 (100)	3.7 (1.2–6.3)
Total (37/32)	**58 (7–607)**	**1,210/12,721 (10)**	**1.1 (0.1–26.4)**	**1,078/1,152 (94)**	**1,016/1,078 (94)**	**2.0 (0.5–49.0)**	**294/300 (98)**	**128/166 (77)**	**3.0 (0.8–92.2)**

CDI: *Clostridium difficile* infection; NA: not available; UK-Scotland: United Kingdom (Scotland only).
The pilot study was based on a non-representative sample, thus the results presented in this table cannot be interpreted as being representative of any participating country or of the European Union/European Economic Area. Missing values indicate that hospitals did not participate in enhanced surveillance and/or did not reply to the feasibility questionnaire.

[a] Three surveillance options were tested: 'minimal' (aggregated hospital data), 'light' (including patient data for CDI cases) and 'enhanced' (including microbiological data on the first 10 CDI episodes per hospital).
[b] Austria, Belgium, Denmark, Estonia, Finland, France, Germany, Hungary, the Netherlands, Norway, Poland, Romania, Serbia, United Kingdom (Scotland only) carried out minimal and light surveillance. Austria, Belgium, Denmark, Finland, France, Germany, Hungary, the Netherlands, Norway, Poland, Romania, Serbia and United Kingdom (Scotland only) also carried out enhanced surveillance.
[c] Three-month assessment during this time period.
[d] Median testing of the country's participating hospitals.
[e] Workload needed to complete the surveillance option, as reported by 26 respondents who completed the feasibility questionnaire.
[f] Number of patients with clinical data available, divided by the number of patients reported by minimal surveillance, expressed as a percentage.
[g] Percentage of patients for whom the presence or absence of a complicated in-hospital outcome (as defined in the Box) was identified.
[h] Percentage of isolates of which the reported ribotype matched the results of the coordinating laboratory.
[i] Countries without an implemented national surveillance of CDI at the start of the European *Clostridium difficile* Infection Surveillance Network (ECDIS-Net) project.
[j] One hospital provided a response to this question, therefore no range was calculable.

Discussion

CDIs are a major concern for hospitals in Europe. The first ECDC point prevalence survey in 2011–12 estimated that 123,997 patients (95% CI: 107,697–441,969) developed a HA-CDI within the European Union each year [9]. In the United States, CDI has been declared an 'urgent threat' [25], with an estimated 80,400 HA-CDI cases in 2011 [26]. Establishing Europe-wide surveillance of CDIs is a pre-requisite to controlling these infections in Europe. In 2011, 14 European countries had national CDI surveillance, but methodologies varied, and only four countries regularly linked *C. difficile* microbiological results to epidemiological data [3]. Therefore, a standardised protocol was proposed for periodical or continuous CDI surveillance in European acute care hospitals, allowing direct interhospital and intercountry comparison of surveillance results.

FEASIBILITY

Results of our study in which we piloted a standardised surveillance protocol for CDI for European acute care hospitals suggests that all three surveillance options were manageable in participating countries, regardless of the countries' pre-established level of CDI surveillance and microbiological typing capacity. Completeness of data was high, and hospital participants reported that the workload was manageable. Nevertheless, modifications were made on the surveillance methodology and forms to further optimise data collection. The finalised protocol version 2.2 is now available on the website of ECDC [27].

EPIDEMIOLOGICAL AND MICROBIOLOGICAL FINDINGS

Using the pilot protocol, participating hospitals could obtain detailed information on the local epidemiology of CDI at their respective facilities that could be used to target and reinforce infection prevention and control measures and resources. This pilot study had an important impact on certain national CDI-related activities as well: three of five participating countries that did not have national CDI surveillance at start of the ECDIS-Net project reported a high percentage of PCR ribotype 027 isolates in this study, and two of these countries (Poland [28] and Romania) decided to continue with intensified CDI surveillance. Interest in the surveillance and completeness of results also suggests that wide-scale implementation at national and European level would be successful in acute care hospitals.

Although the non-representative selection of hospitals does not allow for interhospital or intercountry comparisons in the pilot study, patients enrolled in the enhanced option permitted a more in-depth analysis of the pilot data collected, allowing us to assess the relationship between patient and microbiological characteristics and in-hospital outcome of CDI, our secondary objective. Similar to the

findings of a European study performed in 2008 [2], the majority of the patients in our pilot study had risk factors for CDI (e.g. median age of 72 years and 87% had used antibiotics in the previous three months). We found plausible associations between certain comorbidity variables and a complicated course of CDI or all-cause in-hospital mortality of CDI cases; however, the presence of PCR ribotypes 027 and 176 was not associated with a higher risk of all-cause in-hospital death, as found in a larger study in the United Kingdom in 2006–11 [29]. In contrast, the proportion of PCR ribotypes 027 isolates correlated with a higher incidence rate of HA-CDI, thus corroborating existing evidence on the high potential of this *C. difficile* PCR ribotype to spread. Indeed, this fluoroquinolone-resistant strain that emerged in Europe in 2004 [13] was the most frequently isolated ribotype, particularly in participating hospitals of eastern European countries. This finding is in line with the 'European, multicentre, prospective, biannual, point-prevalence study of *C. difficile* infection in patients admitted with diarrhoea' (EUCLID) study (2011–13) that found PCR ribotype 027 to be most prevalent, clustering in Germany, Hungary, Poland and Romania [12].

Resistance to antibiotics that are routinely used to treat CDIs such as metronidazole and vancomycin was not detected in our study. Two PCR ribotype 027 isolates from one hospital showed a decreased susceptibility to vancomycin (MIC = 2 mg/L), but the clinical relevance of this finding is uncertain.

DATA QUALITY

We found varying frequencies of testing for CDI and percentages of positive tests in participating hospitals and countries, primarily indicating the need for an update of the European diagnostic guideline [30] and for promotion of optimal ascertainment of CDI. In addition, there is a need to address local or national variations in CDI case finding, ascertainment and reporting, which may be substantial across Europe, due to probable differences in clinical and laboratory awareness, practices of specimen collection from diarrhoeic patients and specimen transport, clinical and laboratory indications, requests from physicians and CDI testing methods, local epidemiology (e.g. intensified testing during outbreaks), financial resources to test for CDI, data sources for surveillance, and reporting incentives or disincentives. Therefore, we suggest that in CDI surveillance programmes the possibility of adjusting CDI incidence rates at least for key factors related to sampling and testing methods should be investigated. We recommend that validation studies accompany national surveillance to estimate sensitivity and specificity, in order to correct national and European CDI infection rate estimates.

Furthermore, standardisation of PCR ribotyping is essential for implementation of the enhanced surveillance option, as results show suboptimal concordance between results of national and external laboratories. Agarose-based ribotyping

results are more difficult to interpret and to exchange between laboratories than capillary-based results [31]. The increase, from 23% in 2011 to 50% in 2014, in the percentage of ECDIS-Net participating countries that use capillary-based PCR ribotyping [18] was the most likely explanation for the better performance in the external quality control exercise in 2014 [31]. Further standardisation of PCR ribotyping will likely be achieved by regular exchange of new *C. difficile* strains and build-up of a consistent reference database. The first steps have already been taken by concerted action of ECDIS-Net members with reference laboratories from CDC and the Public Health Agency of Canada [31]. At the same time, new developments in DNA sequence analyses should be monitored closely for application in ribotyping modifications and considered for implementation in surveillance activities of *C. difficile* [32]. In our pilot study, PCR ribotyping of the first 10 strains per hospital in the enhanced option was performed to balance effort, costs and benefits, such as in the national surveillance programme of Belgium [5]. Despite these positive experiences, further evidence for this approach should be obtained and evaluated at European level.

OTHER LIMITATIONS

The results of our pilot study are not generalisable to all European acute care hospitals as it was based on a non-representative convenience sample, as also indicated by the disproportionally high number of tertiary care hospitals (21/37) in our sample. Similarly, our analytical epidemiological results and country-specific results are based on very small numbers of hospitals and should not be considered as representative. Specifically, the number of events allowed for univariable analysis only when exploring associations between covariables and outcome of CDI. Assessing the local context in more details (e.g. gathering information on clinical practices and/or policies related to specimen collection and CDI testing in the participating hospitals) or covering all CDC surveillance evaluation attributes [33] was beyond the scope of this pilot study. Local audits to determine surveillance sensitivity, in both case finding and collection of denominator data, could have helped to elucidate some of the larger observed variations.

CONCLUSIONS

We conclude that continuous or periodical surveillance with collection of different levels of epidemiological and microbiological data following a standardised protocol is a feasible strategy to monitor CDIs in European acute care hospitals. Ideally, national and international validation studies, regular and comprehensive evaluation of the surveillance protocol, as well as CDI case finding, ascertainment and reporting should complement the surveillance activity.

ECDC has used the final protocol version 2.2 to initiate CDI surveillance in EU/EEA countries in 2016, and will gradually incorporate enhanced surveillance data in The European Surveillance System (TESSy) [27,34]. Importantly, the surveillance of CDI in European acute care hospitals will be the first Europe-wide, hospital-based surveillance of a primarily healthcare-associated infection with a distinct microbiological component. The protocol can be used as a tool to guide local CDI surveillance and ultimately contribute to reducing CDI incidence rates in acute care hospitals. Finally, aggregated data from nationally representative samples should allow an estimation of the true incidence rate of CDIs in Europe.

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Chapter 4

Clinical and microbiological characteristics of *Clostridium difficile* infection among hospitalized children in the Netherlands

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Background. Little is known about paediatric *Clostridium difficile* infection (CDI) epidemiology. We describe the clinical and microbiological characteristics of CDI among hospitalized children in the Netherlands.

Methods. Between May 2009 and May 2015, 26 hospitals registered characteristics of paediatric (aged 2–18 years) and adult (aged ≥18 years) CDI in a national sentinel surveillance study. Routine polymerase chain reaction (PCR) ribotyping and multiple-locus variable-number tandem-repeat analysis (MLVA) of selected strains was performed. Paediatric and adult results were compared using proportion and 95% confidence interval (CI). Time trend of paediatric CDI was evaluated using a mixed-effect Poisson model.

Results. Paediatric CDIs were reported in 17 of the 26 participating hospitals (n = 135; 3% of all CDIs); the monthly number was constant over time. The median age of paediatric cases was 10 years (interquartile range, 4.7–14.5 years). Fifty-five percent of the children had community onset and 31% had severe CDI. Compared with adults (n = 4,556), complication and mortality rates were lower. *Clostridium difficile* PCR ribotype 265 (toxin A negative, B positive) was most prevalent in children (15%; 95% CI, 8.8%–24.0%) but rarely found in adults (1%; 95% CI, 0.9%–1.6%). This strain was rarely found in other countries, except for Belgium. MLVA showed genetic relatedness between three-fourths of paediatric and adult ribotype 265 strains, without a clear epidemiological link.

Conclusions. Paediatric CDI in hospitals has remained stable over the last 6 years and resulted in fewer complications than for adult CDI. Further studies are needed to elucidate the source and epidemiology of PCR ribotype 265, primarily found in children.

Introduction

Clostridium difficile is a commensal bacterium in newborns (30%–35% positive) and infants aged up to 2 years (10%–15% positive) [1 – 3]. Infants are considered insensitive to free *C. difficile* toxins in the intestinal tract for several reasons, including lack of mature toxin-binding receptors on epithelial cells and incomplete cellular uptake of the toxins and/or protecting microbiome composition [1, 4]; however, clear scientific evidence is lacking.

Clostridium difficile is potentially harmful for children aged >2 years [5], though the presence of toxin-producing *C. difficile* in stools is not conclusive for diagnoses [6 – 8]. Children with a presumed *C. difficile* infection (CDI) have a 1.2 – 2 times higher in-hospital mortality risk and prolonged hospitalizations compared with matched controls [9, 10]. However, the absolute mortality risk, primarily ranging from 0% to 1.5% [10 – 16] and from 3% to 5% in certain studies [9, 17, 18], is much lower than for adults [11].

In the last decade, the reported incidence of paediatric CDI in both community and hospitals settings in the United States increased [10, 14, 17, 19]. Most children who develop CDI have underlying comorbidities [9, 13, 17]. Recent use of anti-biotic drugs, the presence of gastrointestinal feeding devices, prior hospitalization, and comorbidities, such as malignancies, inflammatory bowel disease, and organ transplantation, are the main risk factors [10, 20, 21].

Here, we describe the clinical and microbiological characteristics of CDI in hospitalized children in the Netherlands as part of a CDI national sentinel surveillance study and compare these results to those of adult cases. Our aim is to determine the burden of paediatric CDI and to determine if additional strategies to prevent, diagnose, and treat CDI in children are needed.

Methods

STUDY DESIGN

We included all CDIs reported between May 2009 and May 2015 by 26 Dutch hospitals that participate in a national sentinel surveillance study (no ethical approval needed). Hospitals were requested to register all hospitalized patients who fulfilled the clinical CDI definition listed below. Children aged <2 years were excluded. For each CDI patient, information on the history of CDI <8 weeks prior to the current infection, the location of onset (community or hospital), and CDI severity was collected. Antibiotic use prior to CDI onset (for treatment of infections other than CDI) at time of or during admission was registered. Polymerase chain reaction (PCR) ribotyping was used to characterize *C. difficile* isolates. Thirty days after diagnosis, complications, including surgery and admission to an intensive care unit due to CDI and mortality, were assessed. Additionally, hospitals reported the primary CDI diagnostic test applied during participation (possibly limiting surveillance sensitivity).

DEFINITIONS

A paediatric CDI was defined as the occurrence of diarrhoea (3 or more loose stools per day for 2 subsequent days) or a toxic megacolon and a positive test for a toxin-producing *C. difficile* or presence of pseudomembranous colitis in children aged 2–18 years. Other causes of diarrhoea were excluded (by chart review and/or results of diagnostic tests for other enteropathogens). Patients aged ≥18 years who fulfilled the same criteria were categorized as "adults" with CDI. A case was considered as healthcare-onset CDI (HO-CDI) if symptoms started in a hospital or long-term care facility and as community-onset CDI (CO-CDI) if symptoms started at home. Severe CDI (for both children and adults) was defined as either the presence of bloody diarrhoea and/or pseudomembranous colitis, and/or diarrhoea accompanied by dehydration (as judged by the treating physician) and/or hypoalbuminemia (<20 mg), and/or fever (≥38.0°C) with leucocytosis (>15.0 × 10^9/L). Mortality was considered as "contributable to CDI" if other comorbidities would not have caused death or "partly contributable to CDI" if both CDI and other comorbidities caused death.

LABORATORY METHODS

The primary (first) diagnostic test of each participating hospital was variable for many hospitals (Supplementary Table 1). Positive samples were sent to the central laboratory (Leiden University Medical Centre, the Netherlands) for typing. Real-time PCR was used to identify the GDH (glutamate dehydrogenase) gene specific to *C. difficile* [22], and PCR ribotyping was used to characterize the isolates [23].

To study relatedness of the predominant paediatric PCR ribotype 265 in more detail, multiple-locus variable-number tandem-repeat analysis (MLVA) was applied [24]. We selected all ribotype 265 strains from children with CDI included in this

study and from 2 preceding and 2 successive adult ribotype 265 cases in the same hospital (also from cases typed apart from the sentinel surveillance study). MLVA results were visualized in a minimum spanning tree using BioNumerics software, version 7.1 (Applied Maths, Saint-Martens-Latem, Belgium). All ribotype 265 isolates were tested for deletions in the *TcdA* gene using PCR [25]. The genetic relatedness of PCR ribotype 265/ sequence type 88 to other *C. difficile* types was visualized in a phylogenetic tree, using multilocus sequence typing (MLST) results of the *Clostridium difficile* MLST Databases. Further, we requested international collaborators (eg, the ESCMID Study Group for *Clostridium difficile*, founders of the *Clostridium difficile* MLST Databases, Webribo, and the US Centers for Disease Prevention and Control) to verify the presence of ribotype 265 in their databases by sharing the capillary electrophoresis PCR ribotyping peak file.

STATISTICAL ANALYSES

Categorical or binary variables were reported as frequencies and percentages and compared based on their 95% confidence intervals (CIs). Continuous variables were reported as medians and the interquartile ranges (IQRs). Quantile regression was used to assess associations between age and covariables. To evaluate a time trend of paediatric CDI, the monthly number of reports was analysed using a mixed-effect Poisson model, allowing random effects per hospital, to account for clustered data. Additionally, we corrected for the type of primary diagnostic test applied (categorized in free toxin detection, tests including GDH detection, or Nucleic acid amplification test (NAAT)). Complete case analysis was performed, except for 1 sensitivity analysis on CO-CDI vs HO-CDI and prior antibiotic usage. Data were analysed using Stata software, version 12.1 (StataCorp LP, College Station, Texas).

Results

PAEDIATRIC CDI REPORTING AND TIME TRENDS

Between May 2009 and May 2015, 4691 CDIs were reported. A total of 135 paediatric CDIs (3% of all CDIs) were reported by 17 hospitals (65%). There were large interhospital differences in the proportion of paediatric CDIs, especially when university hospitals (range, 1.8%–14.3%) were compared with nonuniversity hospitals (range, 0%–7.7%; Supplementary Table 1). No CDI outbreaks on paediatric wards were reported. The number of paediatric CDIs per month was stable according to mixed-effect Poisson modelling ($P = .578$), also when correcting for the type of primary diagnostic test applied ($P = .145$).

CLINICAL CHARACTERISTICS AND 30-DAY OUTCOME

The median age of children with CDI was 10 years (IQR, 4.7–14.5 years) and similar for children with CO-CDI and HO-CDI (11 vs 10 years [$P = .84$]). Fifty-three percent of the children with CO-CDI received non-CDI antibiotics prior to CDI onset (28/53 [95% CI, 39.1%–66.6%]) compared with 81% of the children with HO-CDI (43/53 [95% CI, 70.4– 91.9%]), though information on antibiotic use was missing for 29 cases. If we assumed that all children with missing information either used or did not use non-CDI antibiotics prior to CDI onset, the difference persisted (66% vs 84% and 38% vs 70%, respectively).

A total of 39 children (31%, data for 8 children missing) met the criteria for having severe CDI. Eighteen of these children were dehydrated or had hypoalbuminemia (46%), 16 had bloody diarrhea (41%), 10 had fever and leucocytosis (26%), and 5 had pseudomembranous colitis (13%). Females had severe CDI more often than males (45% [95% CI, 31.8%–58.2%] vs 19% [95% CI, 9.5%–28.2%]).

A complicated course within 30 days after diagnosis was reported for at least 3 children (3%, data of 37 children missing). One 6-year-old female was admitted to the intensive care unit suffering from CDI with dehydration/hypoalbuminemia and fever with leucocytosis. Two males died within 30 days after diagnosis due to causes other than CDI.

PAEDIATRIC CDI COMPARED WITH ADULT CDI

In Table 1, clinical data of children are compared with those of adult CDI patients (n = 4556). CO-CDI was more com-mon in children than in adults (55% [95% CI, 46.4%–63.2%] vs 33% [95% CI, 31.8%–34.6%]). Severe CDI was more frequently observed in children than in adults (31% [95% CI, 22.7%–38.8%] vs 23% [95% CI, 22.1%–24.6%]. In contrast to adults, CDI-related mortality did not occur in children (0% vs 4% [95% CI, 3.0%–4.2%].

Table 1. Clinical Characteristics and 30-Day Outcome of Children With *Clostridium difficile* Infection Compared With Adults

Patient demographics and course of CDI	Paediatric cases, n = 135 (%)	Adult cases, n = 4556 (%)
Age category, y		
2–5	41 (30.4)	-
6–9	26 (19.3)	-
10–13	30 (22.2)	-
14–17	38 (28.1)	-
Male gender	74 (54.8)	2209 (48.5)
Previous CDI		
Yes, clinical presentation only	1[a] (1.2)	173 (6.5)
Yes, clinical presentation and positive test	19[a] (23.2)	474 (17.8)
No, no clinical presentation	42[a] (51.2)	1408 (52.9)
No, negative test	20[a] (24.4)	608 (22.8)
Days to diagnosis of hospital-onset CDI[b]	2 (1–8)	2 (1–6)
Community onset of symptoms	74 (54.8)	1480 (33.2)
Prior antibiotic use[c]	71[d] (67.0)	2674 (70.2)
Severe CDI	39[e] (30.7)	968 (23.3)
30-day outcome		
Complicated course	3[f] (3.1)	568 (15.4)
Overall mortality	2[f] (2.0)	507 (13.7)
CDI-related mortality	0[f] (0)	133 3.6)

Abbreviation: CDI, *Clostridium difficile* infection.

[a] Data missing for 53 children.
[b] Expressed as median number of days and the interquartile range.
[c] At time of diagnosis or during admission for treatment of infections other than CDI.
[d] Data missing for 29 children.
[e] Data missing for 8 children.
[f] Data missing for 37 children.

PCR RIBOTYPING DISTRIBUTION

For 113 of 135 paediatric CDIs (84%), a stool sample or *C. difficile* isolate was sent to the reference laboratory for PCR ribotyping. *Clostridium difficile* was detected in 98 samples (n = 5 culture negative; n = 10 *Clostridium* species but not *C. difficile*). In total, 36 different *C. difficile* PCR ribotypes were identified. Table 2 illustrates the ribotyping distribution of paediatric CO-CDI and HO-CDI compared with adults (information on CDI onset and typing results for 3573/4556 adults available). Ribotype 265 was most prevalent in children (15% [95% CI, 8.8%–24.0%]) but rarely found in adults (1% [95% CI, 0.9%–1.6%]). Ribotype 014/020 was commonly found in both children and adults (12% [95% CI, 6.5%–20.4%] and 15% [95% CI, 13.9%–16.3%], respectively). Ribotypes 001 and 078/126 were less frequently isolated from children than from adults (5% [95% CI, 1.7%–11.5%] vs 15% [95% CI, 13.7%–16.1%]

and 5% [95% CI, 1.7%–11.5 %] vs 13% [95% CI, 12.3%–14.5%], respectively). Ribotype 027 strain was not found in the paediatric population but was present in 3% of the adult population (n=94 [95% CI, 2.1%–3.2%]). The differences in ribotype distribution were larger when HO-CDI was compared with CO-CDI (Table 2).

PCR RIBOTYPE 265

Paediatric ribotype 265 cases occurred in 7 hospitals located in different regions of the Netherlands (Figure 1). Two-thirds of the cases occurred between March 2012 and March 2013 (n=10), dispersed over several months. Ribotype 265–infected children were younger than those infected by other ribotypes (median age of 4 vs 11 years). Children and adults with a ribotype 265 CDI (n=15 and n=45) did not have more severe CDI com-pared with those infected by other ribotypes (29% vs 31% and 28% vs 24%, respectively) and did not have a higher 30-day mortality risk (0% vs 4% and 14% vs 14%, respectively). None of the ribotype 265–infected adults deaths were partly related to CDI.

MLVA showed that three-fourths of the ribotype 265 strains isolated from children and adults were genetically related (defined as ≤10 summed tandem-repeat difference [24]; Figure 1). Three clonal complexes (defined as ≤2 summed tandem-repeat difference on ≤2 loci [24]) were found, of which 2 complexes included adult and paediatric isolates from different hospitals. Three children had a recurrent ribotype 265 infection (Figure 1). Three ribotype 265 strains that were isolated in 1 university hospital (U3) in October 2012–November 2012 were genetically, but not clonally, related.

Ribotype 265 had an intact toxin B production but lacked toxin A due to a *TcdA* 1.8 kb deletion as ribotype 017 [25] and was negative for binary toxin genes. A phylogenetic tree of multilocus sequence types submitted to the *Clostridium difficile* MLST Databases illustrated the genetic relatedness of ribotype 265 (assigned as sequence type 88) and ribotype 017 (assigned as sequence type 37; Supplementary Figure 2). According to the survey in our international network, ribotype 265 appears to be very uncommon or absent in other countries, except for Belgium (Supplementary Table 2). In Belgium, ribotype 265 was primarily found isolated from children aged <2 years.

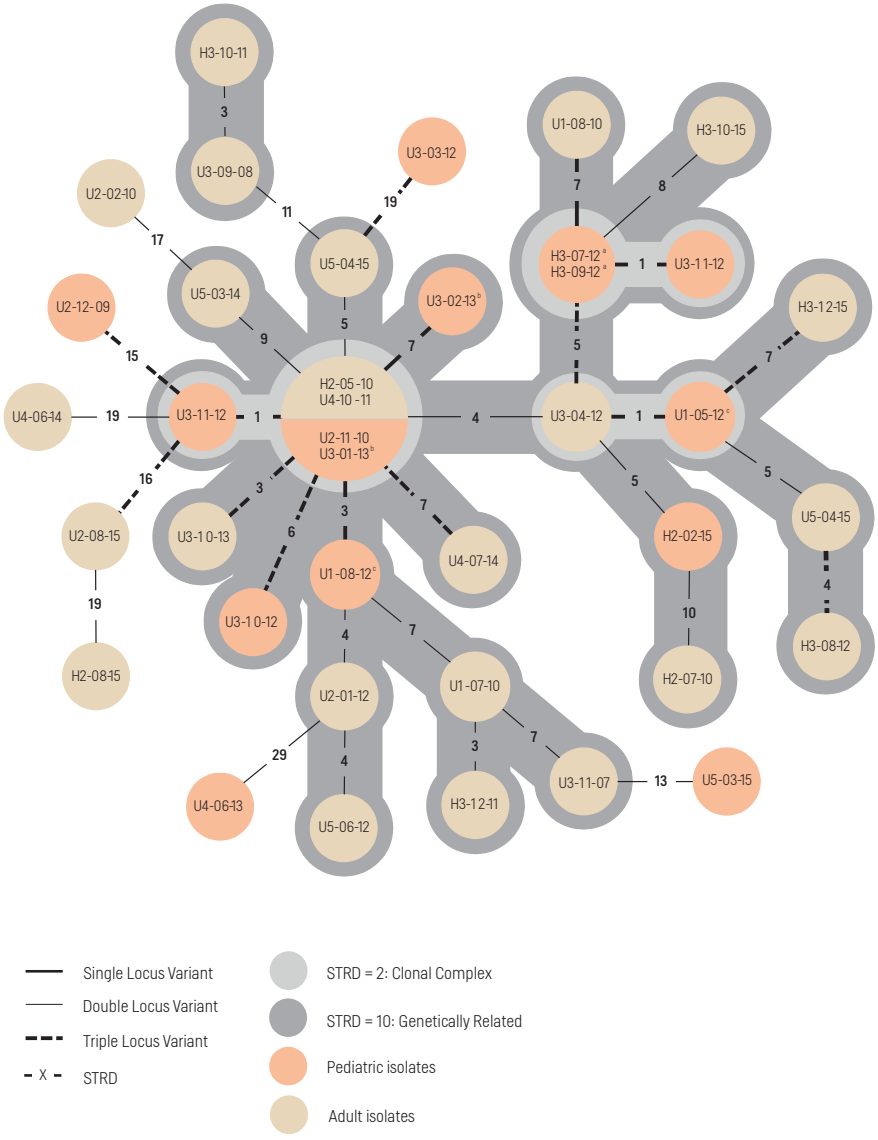
Table 2. Polymerase Chain Reaction Ribotype Distribution of Community Onset and Healthcare Onset *Clostridium difficile* Infection in Hospitalized Children Compared With Hospitalized Adults in the Netherlands

Polymerase chain reaction ribotype	Children		Adults[a]	
	CO, n = 53 (%)	HO, n = 45 (%)	CO, n = 1182 (%)	HO, n = 2391 (%)
001	2 (3.8)	3 (6.7)	105 (8.9)	427 (17.9)
002	6 (11.3)	2 (4.4)	76 (6.4)	148 (6.2)
005	6 (11.3)	2 (4.4)	54 (4.6)	105 (4.4)
014/020	8 (15.1)	4 (8.9)	179 (15.1)	359 (15.0)
027	-	-	24 (2.0)	70 (2.9)
078/126	4 (7.5)	1 (2.2)	176 (14.9)	302 (12.6)
087	2 (3.8)	2 (4.4)	18 (1.5)	34 (1.4)
106	1 (1.9)	2 (4.4)	6 (0.5)	6 (0.3)
265	4 (7.5)	11 (24.4)	14 (1.1)	31 (1.3)
Other types	20 (37.7)	15 (33.3)	405 (34.3)	748 (31.3)
Unknown	-	3 (6.7)	125 (11.2)	161 (6.7)

The 8 most common ribotypes (n ≥3 isolates) in children were taken as a reference, and ribotype 027 was included for its clinical relevance. A dash indicates a zero.
Abbreviations: CO, community onset; HO, healthcare onset.

[a] Two adults had a mixed infection, the polymerase chain reaction ribotype with the lowest number is reported in the table.

Figure 1 Minimum spanning tree of *Clostridium difficile* polymerase chain reaction ribotype 265 strains isolated from children (in blue; n = 15) and adults (in green; n = 24), according to multiple-locus variable-number tandem-repeat analysis. The STRD is shown between the circles. Dark gray areas represent a cluster of genetically related strains (defined as ≤10 summed tandem-repeat difference), and light gray areas represent clonal complexes (defined as ≤2 summed tandem-repeat difference on ≤2 loci). Each circle specifies the code for the hospital where the patient was located and the month and year of isolation. Superscripts indicate samples that were isolated from identical patients (a, b, and c). Abbreviations: STRD, summed tandem-repeat difference; U, H (U indicates University hospitals and H primary and secondary care hospitals, see Supplementary Table 1).



Discussion

We report the clinical characteristics and PCR ribotypes of paediatric CDI in a large sentinel surveillance study in the Netherlands during a 6-year period.

In contrast with several studies performed in the United States [10, 14, 17, 19] and Italy [26] but in agreement with a recent study in the United Kingdom [24], we did not find an increase in paediatric CDIs over time. The increase in the United States could be related to the high prevalence of NAP1/ribotype 027 or to an increased awareness of the need to test children for CDI, though this is opposed by 1 study [17]. The recent implementation of NAAT in many laboratories may have contributed to this increase [27] by possibly including *C. difficile* carriers as CDIs. It did have a significant effect on paediatric CDI reporting in our analysis and was incorporated into our model. However, both the unadjusted and adjusted models did not indicate an increase in paediatric CDIs.

We found large interhospital differences in the proportion of reported paediatric CDIs, especially between university hospitals and nonuniversity hospitals (5% vs 0.3%). University hospitals indeed treat children with a higher CDI risk, such as those with cancer, as well as organ and bone marrow trans-plantation patients [21]. However, we questioned whether non-university hospitals actually did test for *C. difficile* in children. We contacted 9 hospitals that do not or rarely report paediatric CDI; 1 did not test for CDI in children, but 7 tested on clinical request in children of all ages, and 1 only in patients aged >2 years. There is no guideline for CDI testing in children in the Netherlands (or in Europe) in contrast to the United States [4, 28]. We assume that differing views among both paediatricians and microbiologists on CDI testing in children contributed to interhospital differences.

The clinical characteristics of paediatric CDI found in our study resemble findings of other studies, despite the higher median age of patients in our study [9, 13, 17, 18, 20]. Approximately half of the paediatric cases had CO-CDI, which is in line with 2 studies that used different criteria (29% and 54%) [9, 18], while others found 26% and 71% of paediatric CDI cases to be acquired in the community [13, 15]. Antibiotics are considered to be a risk factor but not a prerequisite for developing paediatric CDI [21]. In our study, 67% of the children received non-CDI antibiotics prior to CDI onset, which is similar to previous studies that showed rates of 61%–74%, depending on the measured time period of exposure [14, 18]. As expected, the percentage exposed to non-CDI antibiotics was lower in paediatric CO-CDI than in HO-CDI.

Compared with adults, we found a high proportion of severe CDI (31%) in children but a lower complication and mortality rate 30 days after diagnosis. Possibly, children are more capable than adults of recovering from severe CDI [21] or possibly

the current severity criteria do not fit the paediatric population [29]. This is supported by a study that revealed that 50% of children with CDI did not require specific CDI treatment, while 76% were classified as "severe CDI" [24]. However, the severity rate in that study was much higher than in our study, and as described in literature [13 – 15].

PCR ribotype 265 (toxin A negative due to a *TcdA* 1.8 kb deletion, toxin B positive, and negative for binary toxin genes) was most prevalent in children and rarely found in adults. Epidemiological and molecular typing data could not elucidate ribotype 265 transmission routes. MLVA showed that three-fourths of the ribotype 265 strains isolated from children and adults were genetically related. There was no clustering according to host (children vs adults), date, or place of isolation. These results suggest ongoing (regional) transmission between the 2 populations, whereas this ribotype may favour younger hosts in particular. In Europe, ribotype 265 was initially observed in Leiden in 2006 and determined to be a new ribotype at the Anaerobe Reference Laboratory in Cardiff (personal communication, Dr Michael Perry) in 2010. In the United States, it was first isolated in 1988 (personal communication, Dr Jane Marsh) using MLST and belongs to the same lineage as ribotypes 017, 047, 088, 130, and 172 (Supplementary Figure 2) [30]. Our international survey showed that this ribotype was rarely found in North America and Europe, except for the neighbouring Belgium where most were isolated from children aged <2 years. Although we did not include children aged <2 years in our study (as many other studies), these findings support our hypothesis that the ribotype favours younger hosts and that transmission may be restricted to certain countries.

After ribotype 265, ribotype 014/020 was most often detected in children (12%), consistent with previous studies where NAP4/ PCR ribotype 014 was predominant in 26% and 24% [13, 31]. Another study showed PCR ribotype 014 to be predominant in infants (aged <2 years) in 25% in Spain [32]. Our study, as well as a German study [31], indicated an absence of ribotype 027 in children in contrast to adults, though both studies typed a limited number of children. In contrast, NAP1/ ribotype 027 was found in 11% and 23% of the paediatric CDI cases in the United States and Canada [13, 15], including community-acquired CDI [15]. In a different single-center US cohort, NAP1/ribotype 027 was found in <1% of the children, opposed to 31% of adults in the same area [33]. These geographical differences may be due to diverse antibiotic exposure (e.g., fluoroquinolones), infection prevention measures, or infection pressure in the community.

Our study has several limitations. CDI testing of children was not as well standardized as for adults and may have resulted in a general underestimation of the burden of paediatric CDI. Our definition of community-onset CDI, which was introduced in 2009 for feasibility reasons, differs from international guide-lines and hampers benchmarking. Our sample size was relatively small, hampering

multivariate analysis, and we had high levels of missing data for some variables. Age-specific denominator data to calculate age-stratified incidence rates were not available, though we evaluated the monthly number of paediatric CDIs using a mixed-effect Poisson model, corrected for the primary diagnostic method applied, and showed that no variation in time was present. In addition, the number of paediatric admissions in the Netherlands [34] did not change over time. The absence of epidemiological links between ribotype 265 cases may be caused by incomplete sampling (eg, nondiagnosed children and asymptomatic carriers) and the lack of detailed patient data, although a dispersion in time and location was evident.

In conclusion, we did not observe an increase in the monthly number of reported paediatric CDIs over a 6-year period. *Clostridium difficile* ribotype 265 was more prevalent in children than in adults, without a clear explanation. Future prospective paediatric studies are needed to obtain more detailed information on CDI risk factors, transmission, and treatment in children and to confirm and elucidate why some PCR ribotypes are more or less abundant compared with adults.

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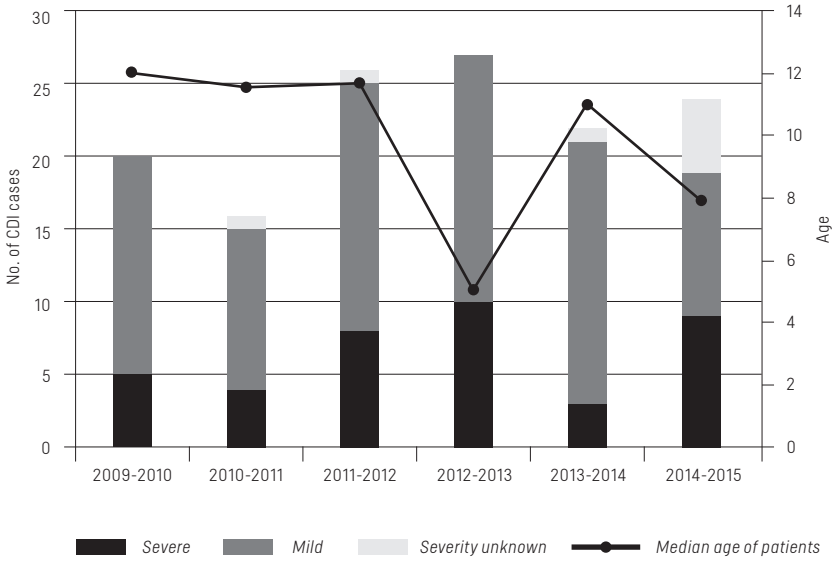
Supplementary figures

Supplementary Figure 1 Number of reported paediatric CDIs per 12-month time period, stratified by CDI severity. The line illustrates the median age of the patients for each time period.

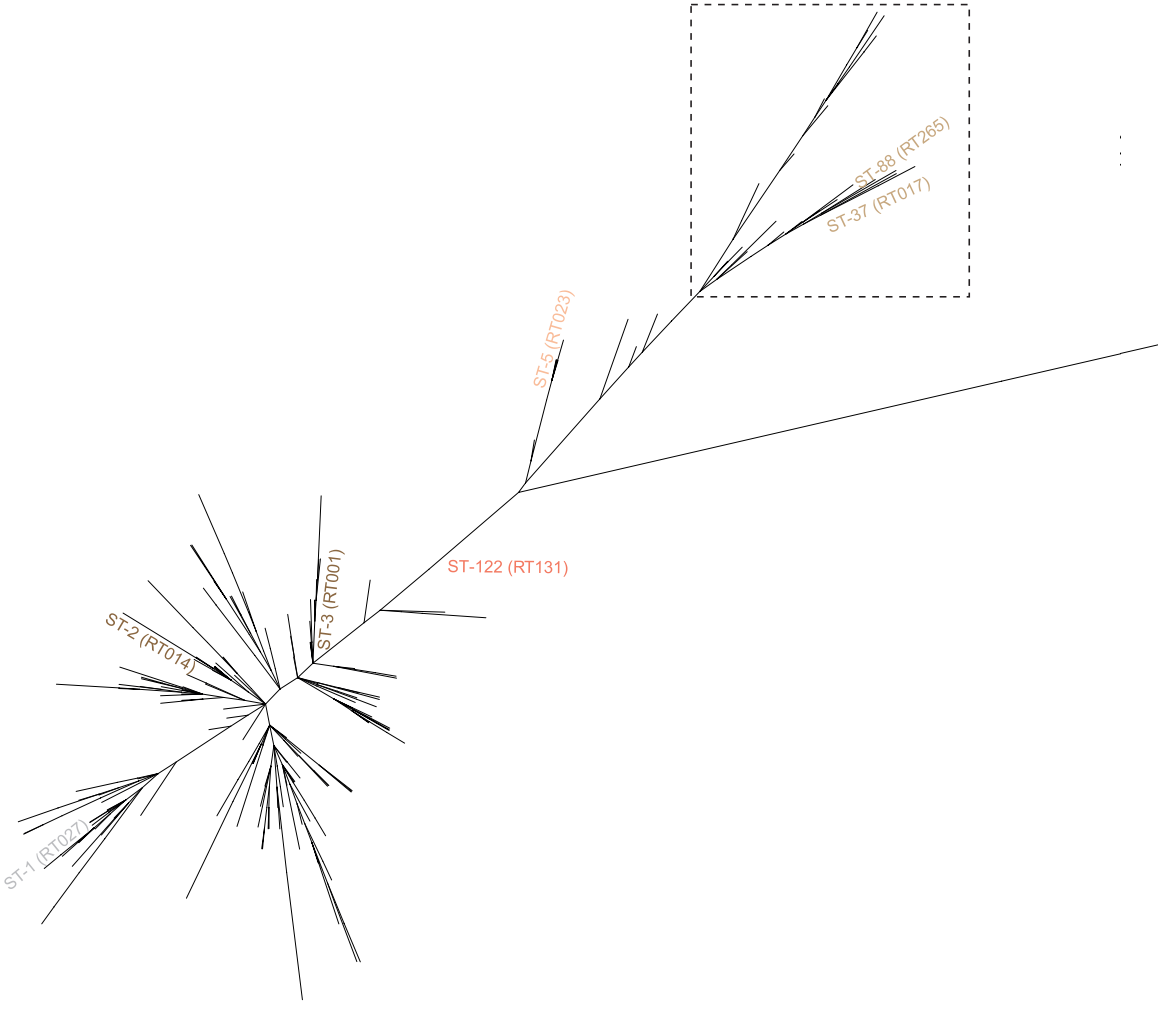


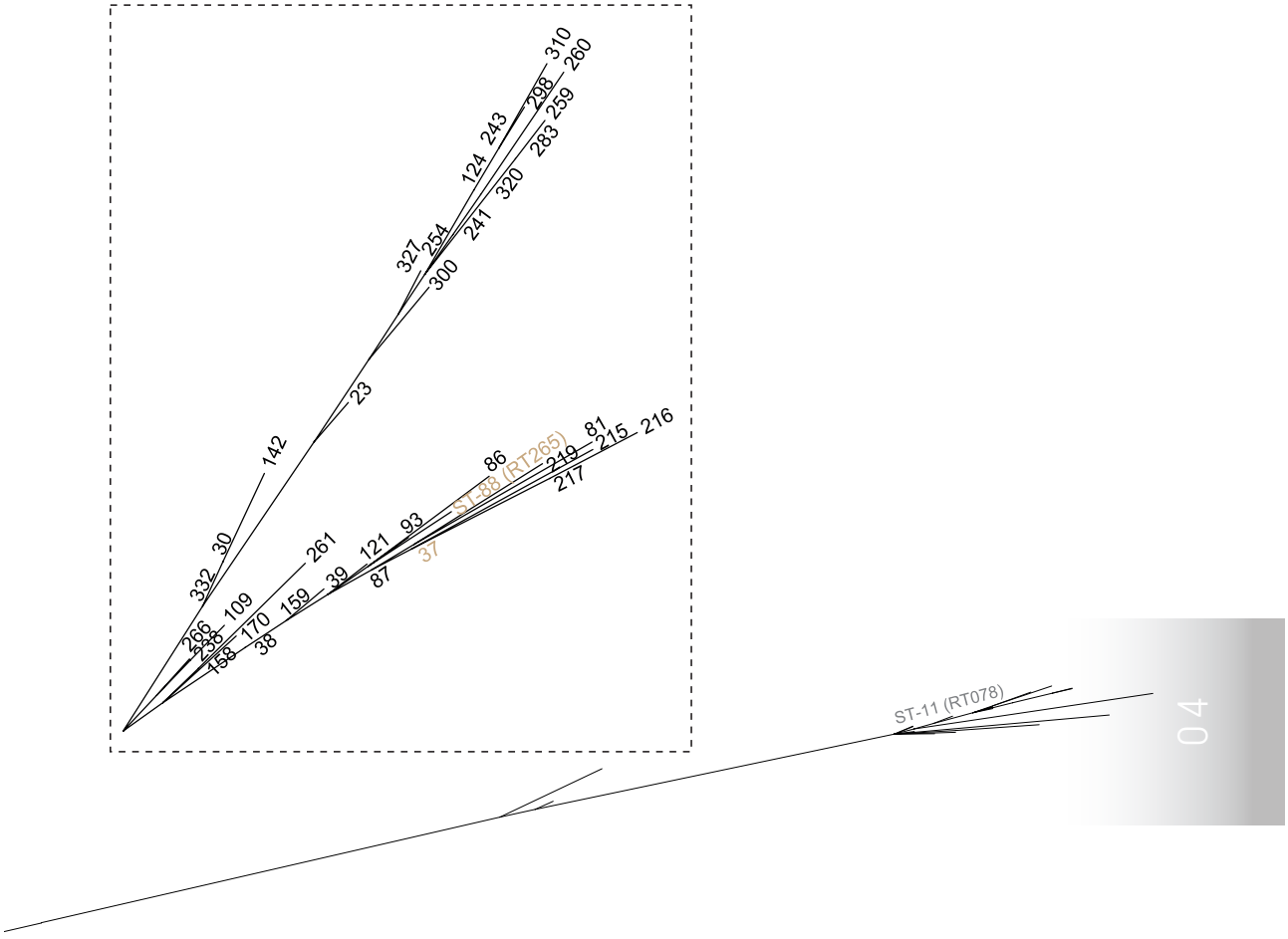
Supplementary Table 1. Numbers of reported paediatric CDIs per total number of CDIs, stratified by 12-month time period (May 2009-April 2015) and hospital, and primary CDI diagnostic test(s) applied.

Hospital type	Primary CDI diagnostic test(s)[a]	No. of reported children of all CDI cases (%)						
		2009-2010	2010-2011	2011-2012	2012-2013	2013-2014	2014-2015	Total
University		20/677 (3)	16/750 (2)	26/748 (4)	27/750 (4)	22/825 (3)	24/941 (3)	135/4,691 (3)
U1	Toxin EIA, 3/15 NAAT	7/27 (26)	4/25 (16)	10/63 (16)	8/58 (14)	9/56 (16)	1/43 (2)	39/272 (14.3)
U2	Toxin EIA	2/30 (7)	4/38 (11)	2/18 (11)	2/32 (6)	2/37 (5)	1/27 (4)	13/182 (7.1)
U3	Toxin EIA, 1/11 NAAT, 2/15 GDH/toxin EIA	5/72 (7)	3/91 (3)	6/76 (8)	10/76 (13)	2/48 (4)	3/57 (5)	29/420 (6.9)
U4	Toxin EIA, 1/14 NAAT	0/25 (0)	1/32 (3)	1/45 (2)	1/24 (4)	1/51 (2)	4/78 (5)	8/255 (3.1)
U5	Toxin EIA, 11/13 NAAT	0/41 (0)	0/43 (0)	1/45 (2)	1/13 (8)	1/55 (2)	5/92 (5)	8/289 (2.8)
U6	GDH/toxin EIA					1/20 (5)	0/37 (0)	1/57 (1.8)
Primary and secondary care								
H1	CCA	3/29 (10)	0/10 (0)					3/39 (7.7)
H2	Toxin EIA, 4/14 GDH/toxin EIA	0/67 (0)	0/50 (0)	1/47 (2)	1/40 (3)	1/36 (3)	4/61 (7)	7/301 (2.3)
H3	Toxin EIA, 1/12 GDH/toxin EIA and NAAT	0/79 (0)	3/124 (2)	3/111 (3)	3/79 (4)	0/68 (0)	3/64 (5)	12/525 (2.3)
H4	Toxin EIA, 5/11 GDH/toxin EIA	1/28 (3.6)	1/7 (14)	0/19 (0)	0/32 (0)	0/13 (0)	0/28 (0)	2/127 (1.6)
H5	NAAT					1/36 (3)	0/48 (0)	1/84 (1.2)
H6	Toxin EIA, 1/14 GDH EIA	1/13 (8)	0/10 (0)	0/22 (0)	0/17 (0)	0/17 (0)	0/7 (0)	1/86 (1.2)
H7	GDH/toxin test, 8/10 NAAT	1/29 (3)	0/54 (0)	1/52 (2)	0/64 (0)	1/42 (2)	0/58 (0)	3/299 (1.0)
H8	Toxin EIA, 1/12 NAAT	0/53 (0)	0/55 (0)	0/13 (0)	0/29 (0)	2/26 (8)	0/21 (0)	2/197 (1.0)
H9	Toxin EIA, 3/12 NAAT	0/85 (0)	0/95 (0)	0/86 (0)	0/83 (0)	1/64 (2)	3/60 (5)	4/473 (0.8)
H10	Toxin EIA		0/19 (0)	1/66 (2)	0/87 (0)	0/61 (0)	0/52 (0)	1/285 (0.3)
H11	NAAT	0/48 (0)	0/46 (0)	0/43 (0)	1/52 (2)	0/70 (0)	0/37 (0)	1/296 (0.3)
H12	NAAT					0/33 (0)	0/52 (0)	0/85 (0.0)
H13	Toxin EIA, 11/14 GDH/toxin EIA	0/4 (0)	0/7 (0)	0/4 (0)	0/9 (0)	0/4 (0)	0/8 (0)	0/36 (0.0)
H14	Toxin EIA, 8/13 GDH/toxin EIA, 9/14 NAAT				0/18 (0)	0/54 (0)	0/38 (0)	0/110 (0.0)
H15	Unknown	0/7 (0)	0/5 (0)					0/12 (0.0)
H16	Toxin EIA	0/14 (0)	0/17 (0)	0/19 (0)	0/4 (0)			0/54 (0.0)
H17	Unknown	0/24 (0)	0/15 (0)					0/39 (0.0)
H18	Toxin EIA, 5/13 NAAT	0/2 (0)	0/7 (0)	0/8 (0)	0/12 (0)	0/6 (0)	0/10 (0)	0/45 (0.0)
H19	Toxin EIA			0/11 (0)	0/21 (0)	0/28 (0)	0/30 (0)	0/90 (0.0)
H20	NAAT						0/33 (0)	0/33 (0.0)

[a] If a change of the primary diagnostic test occurred, the month and year of implementation is reported.

Supplementary Figure 2 Phylogenetic tree of the genetic relatedness of PCR ribotype 265 (sequence type 88) to other *C. difficile* types. To include as many sequence types (STs) for the phylogenetic analysis as currently described, we downloaded (20-04-2016) the STs from the online *C. difficile* multilocus sequence typing (MLST) database (http://pubmlst.org/cdifficile/). In total, 319 STs were included in the phylogenetic analysis (Last updated: 2016-04-04). Nucleotide sequences of the seven housekeeping genes used for *C. difficile* MLST (Griffiths et al.) were concatenated; 219 SNPs were identified for the 319 STs. A maximum likelihood phylogeny was reconstructed using RaxML (ref: Stamatakis) with a general time reversible (GTR) model and gamma correction for among-site rate variation combined with 100 random bootstrap replicates (default). The STs numbers were removed to improve readability, only STs and corresponding PCR ribotypes (RTs) were kept for commonly found *C. difficile* types. The dashed window is enlarged to improve resolution for the lineage in which RT265 (ST-88) was located.

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ST-53 (RT265)
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ST-11 (RT078)

Chapter 5

Ribotype 078 *Clostridium difficile* infection incidence in Dutch hospitals is not associated with provincial pig farming: results from a national sentinel surveillance, 2009-2015

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Background. It has been suggested that the high incidence of ribotype 078 *Clostridium difficile* infections (CDI) in the Netherlands is related to pig farming.

Methods. We used data of hospitalised CDI patients (>2yrs of age) diagnosed between May 2009 and May 2015 in 26 hospitals participating in a national sentinel surveillance. We compared clinical and geographical characteristics of 078 CDI to other CDI. We investigated the association between 078 CDI incidence and four indicators of pig farming (piglet, pig, piglet farm and pig farm density) by mixed-effects Poisson regression. We used a space-time permutation model to search for community-onset 078 CDI clusters (using SaTScan).

Results. A total of 4,691 CDI were identified. Ribotype 078 was isolated in 493 of 3,756 patients (13.1%) including a typing result. These patients had slightly higher community-onset disease and a 35% increase of 30-day mortality compared to non-078 CDI patients. The pooled overall and 078 incidence rates were 2.82 (95% CI, 2.42-3.29) and 0.26 (95% CI, 0.21-0.31) CDI per 10,000 patients-days respectively. Hospital 078 CDI incidence was not associated with provincial pig (IRR, 0.98; 95% CI, 0.89-1.08), piglet (IRR, 0.95; 95% CI, 0.75-1.19), pig farm (IRR, 1.08; 95% CI, 0.84-1.39), or piglet farm density (IRR, 1.00; 95% CI, 0.56-1.79). No clusters of community-onset ribotype 078 CDI were found.

Conclusions. Our results do not indicate that the ribotype 078 CDI incidence in hospitals is related to pig (farm) or piglet (farm) density. However, transmission beyond provincial borders or in non-hospitalised patients cannot be excluded.

Introduction

The Gram-positive spore-forming bacterium *Clostridium difficile* emerged as an important cause of infectious diarrhoea and diarrhoeic outbreaks in hospitals in the Netherlands [1]. Hospitalised patients are considered to be primarily infected by other *C. difficile* infection (CDI) patients, possibly mediated through healthcare personnel or the hospital environment [2]. Yet, in at least 45% of the cases the source of infection is unknown [3]. Animals might be an alternative source of *C. difficile* infection [4, 5]. Several animal species are colonized by similar *C. difficile* subtypes as found in humans [4]. More in-depth genomic studies have suggested *C. difficile* transmission between pigs and humans of ribotype 014 in Australia [6] and ribotype 078 in the Netherlands [7]. Ribotype 078 is the predominant ribotype in pigs and piglets in the Netherlands [5, 8]. About 50%-80% of piglets are colonised by *C. difficile* and can develop disease [8 – 10]. At slaughter age, 1%-9% of pigs are positive [10, 11]. *C. difficile* spores can subsequently contaminate farm environments and pig-derived manure [12, 13] and can be found in meat [4, 5]. Considering the fact that 12 million pigs coexist with nearly 17 million inhabitants in the Netherlands [14], the impact of pig-farming on ribotype 078 transmission can be significant. Pig farming is concentrated in specific provinces in the Netherlands.

Since mid-2006, ribotype 078 is one of the most common ribotypes to cause CDI in hospitalised patients in the Netherlands [1]. Ribotype 078 appeared to be associated to community-acquired disease [15, 16] and more abundant in areas of concentrated pig farming in 2005-2008 [16]. In other European regions with high ribotype 078 rates, such as Northern Ireland and Scotland, the link between ribotype 078 and pig-farming was not extensively investigated [17, 18].

We hypothesise that if *C. difficile* ribotype 078 shedding by pigs and/or piglets leads to enhanced regional human exposure, we would find a relation between pig farming and the ribotype 078 CDI incidence in our country. In the present study, we use data of a national *C. difficile* infection sentinel surveillance in the Netherlands (May 2009 – May 2015). We investigate the association between hospital incidence rates of ribotype 078 CDI and pig(let) density and pig(let) farm density at a provincial level. Second, we compare clinical characteristics of ribotype 078 compared to other CDI. Third, we use a space-time permutation model to identify the location of clusters of community-onset 078 CDI followed by hospitalisation.

Methods

SENTINEL SURVEILLANCE SYSTEM

Prospective national *C. difficile* infection sentinel surveillance (SeS) was initiated in the Netherlands in May 2009 by the National Reference Laboratory for *C. difficile* (Leiden University Medical Centre, Leiden and the National Institute for Public Health and the Environment (RIVM), Bilthoven). Hospitals were included in SeS according to their geographical location, with the aim to obtain a geographically representative sample of all hospitals in the Netherlands (Figure 1a). SeS hospitals prospectively submitted anonymous patient forms of all included CDI episodes to a web-based system (ethics approval not required). Subsequently, the National Reference Laboratory for *C. difficile* received the samples (faeces, or *C. difficile* isolates) of CDI episodes included in SeS for PCR ribotyping [19], and performed data analysis. For the current study, CDI SeS data from May 2009 to May 2015 was used. In that time period, surveillance was conducted at 26 hospitals (8/75 primary care hospitals, 12/23 secondary care hospitals, and 6/8 university hospitals), representing 25% of all hospitals in the Netherlands [14].

Figure 1 Location of SeS hospitals and pooled CDI incidence rates in relation to average provincial pig density (no. of pigs per hectare) in 2009-2015.

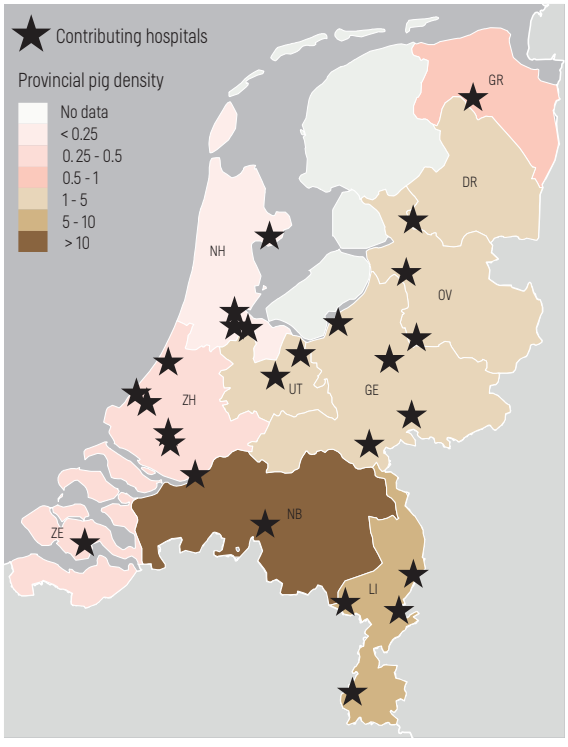


Figure 1A Locations of SeS hospitals (n=26) and the average provincial pig density per hectare (Source of map: ArcGis, Environmental Systems Research Institutes, Inc. Redlands CA).

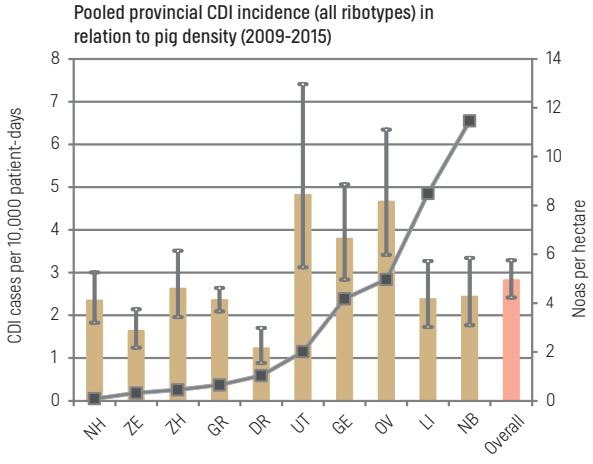


Figure 1B Pooled provincial CDI (all ribotypes) incidence rates and 95% CI (bars) in relation to average provincial pig density per hectare (line).

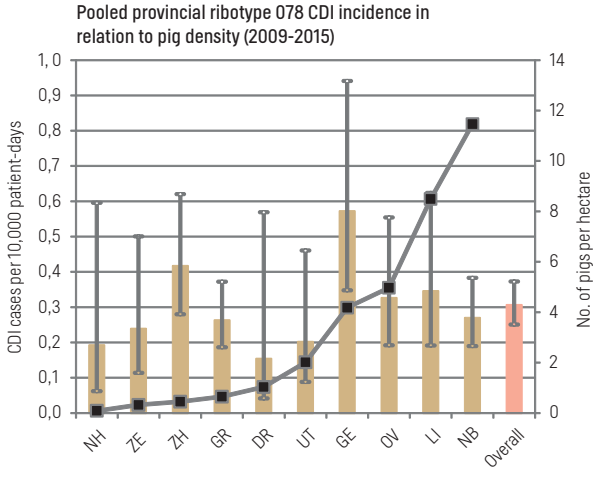


Figure 1C Pooled provincial ribotype 078 CDI incidence rate and 95% CI (bars) in relation to average provincial pig density per hectare (line).

DR: Drenthe, FL: Flevoland, FR: Friesland, GE: Gelderland, GR: Groningen, LI: Limburg, NB: North Brabant, NH: North Holland, OV: Overijssel, UT: Utrecht, ZE: Zeeland, ZH: South Holland.

DATA COLLECTION AND DEFINITIONS

Hospitalised CDI patients (>2yrs of age) were eligible to be included in SeS. Children aged <2 yrs were excluded for reasons described earlier [20]. A case was defined as the presence of clinical symptoms (an abnormal stool frequency, >3 times per day diarrhoea during two subsequent days, or radiological or clinical signs of a toxic megacolon), and either the presence of a toxin-producing *C. difficile* in the faeces, or a confirmed pseudomembranous colitis (by endoscopy or by histopathology after colectomy or autopsy) [20, 21]. Participating hospitals applied their own diagnostic procedures to test for the presence of toxin-producing *C. difficile* in the faeces.

The patient form included information on the location of CDI onset (i.e. community or healthcare), the presence of CDI in the prior 8 weeks ('recurrence'), CDI severity, CDI-related and all-cause 30-day mortality, as has been described previously [20]. Community-onset CDI included all cases with a reported onset of symptoms in the community, whereas healthcare-onset CDI related to cases with a reported onset of symptoms in a hospital, nursing home or other healthcare institution (e.g. care home). Four-digit postal code data was requested from patients with community-onset CDI. Severe CDI was defined by the presence of bloody diarrhoea and/or pseudomembranous colitis and/or diarrhoea with either dehydration and/or hypoalbuminemia, and/or fever (>38 ºC) and leucocytosis (>15.0 X 109/L). 30-day mortality was considered 'CDI-related' if other comorbidities would not have caused death.

HOSPITAL INCIDENCE RATES

Yearly hospital incidence rates of CDI were calculated per 10,000 inpatient-days [22]. Numbers of inpatient-days of participating hospitals were extracted from a website of the CIBG of the ministry of Health, Welfare and Sport, to ensure standardised data collection [23]. Children below the age of two could not be excluded from these denominator data, which was not considered to have a large impact on incidence rates of CDI. We calculated Poisson rate 95% CIs for all incidence rates. Overall and provincial incidence rate of CDI were generated by inverse-variance weighting.

PROVINCIAL PIG AND PIGLET DENSITY

The StatLine database of the Dutch National Bureau of Statistics [14] was used to calculate four indicators of provincial pig-farming; (i) the number of pigs per hectare ('pig density'), (ii) the number of piglets per hectare ('piglet density'), (iii) the number of pig farms per 1000 hectare ('pig farm density'), and (iv) the number of piglet farms per 1000 hectare ('piglet farm density') for 2009-2015.

STATISTICAL ANALYSIS

Risk ratios (RR) and 95% confidence intervals (CI) were calculated to compare patient characteristics of ribotype 078 CDI, with CDI caused by other ribotypes. Highly genetically related ribotypes that were difficult to discriminate by PCR ribotyping e.g. ribotypes 078/126 (as of now referred as 'ribotype 078') were clustered.

The association between four indicators of pig-farming (see Provincial pig and piglet density) and ribotype 078 hospital incidence rates at a provincial level was analysed by a multilevel mixed-effects Poisson regression model. We considered the outcome (incidence rate) to follow a Poisson distribution, and included 'hospital'

as a random effect to account for clustered data. We calculated incidence rate ratios (IRR) for each of the four indicators of provincial pig-farming, and adjusted for diagnostic testing and year. Diagnostic testing was categorised into algorithms with 'free toxin detection', 'PCR' or 'glutamate dehydrogenase detection' since a variety of diagnostic algorithms were applied, and these categories are indicative for the sensitivity diagnostic testing for CDI. A similar analysis was performed for the hospital incidence of community-onset ribotype 078 infections. We performed two sensitivity analyses by (1) excluding university hospitals, where relatively more patients are treated that originate from a different province to receive highly specialised care, and (2) excluding ribotype 126 CDI from the ribotype 078 subgroup, to avoid potential misclassification. STATA software version 12.1 (StataCorp, College Station, USA) was used for data analysis.

SPATIAL CLUSTER ANALYSIS OF COMMUNITY-ONSET CDI

To identify clusters of community-onset CDI that might be missed while investigating overall hospital incidence rates of CDI, we studied SeS postal code data of community-onset CDI by a retrospective space-time permutation model (SaTScan, M Kulldorff, Boston, MA, USA) [24]. This model does not require population-at-risk data, but data should derive from a stable population [24]. Therefore, we analysed data of two time periods (period I: September 2009-December 2013 and period II: December 2013-May 2015) of hospitals that continually participated in surveillance. To select an appropriate spatial window setting, we initially used 50% population-at-risk as maximal spatial cluster size, and repeated the analysis with a maximal cluster size of 25 km radius, and compared our results [25]. We searched for clusters of community-onset CDI in general, as well as for ribotype 078 specific clusters, and assessed if they were located in provinces with a high pig and piglet density. We performed a sensitivity analyses by excluding ribotype 126 CDI from the ribotype 078 subgroup, to avoid potential misclassification.

Results

REPORTED CDI EPISODES

In total, 4,691 CDI cases were reported by 26 hospitals in a period of six years. A third (n=1,554) was designated as community-onset and two-thirds as healthcare-onset CDI (n=3,038). Of all healthcare-onset CDI episodes, 2,751 (90.6%) were reported to have started in a hospital, 148 in a long-term care facility (4.9%) and 139 (4.6%) in other healthcare facilities. Healthcare-onset CDI was severe in 17.6% (n=489) versus community-onset CDI in 34.9% (n=517). Of patients with healthcare-onset CDI, 17.0% experienced a complicated course; 1 (0.04%) needed surgery for CDI, 37 (1.5%) had to be admitted to an intensive care unit for CDI, and 381 (15.5%) died within 30 days. Eight of these patients died due to CDI (0.3% of all HO-CDI), another 82 patients died from factors contributed to by CDI (3.3% of all HO-CDI). Of patients with community-onset CDI 11.3% experienced a complicated course; 8 (0.6%) needed surgery, 16 (1.2%) were admitted to an intensive care unit for CDI, and 126 (9.5%) died within 30 days. Two of these patients died due to CDI (0.15% of all CO-CDI) and 41 (3.1% of all CO-CDI) patients died from factors contributed to by CDI. Of the remaining patients cause of death was indeterminate, not related to CDI or unknown. Supplementary Table 1 illustrates time trends of the characteristics and the outcome of all reported CDI episodes.

MOLECULAR TYPING OF REPORTED CDI EPISODES

For 3,755 CDI cases (80.0%) a PCR ribotyping result could be obtained and linked to the clinical data. Ribotype 014 (including ribotypes 020/295) was the most frequently isolated ribotype (n=570; 15.2%), followed by ribotype 001 (n=547; 14.6%). The occurrence of ribotype 001 declined in time (Table 1). Ribotype 078 (including ribotype 126) was the third most commonly found ribotype (n=493; 13.1%). Its prevalence was constant over the study period. Ribotype 027 was occasionally found (n=97; 2.6%).

Table 1. Changes in the number of participating hospitals, the number of reported episodes, and the clinical characteristics and PCR ribotypes of all CDIs reported in *C. difficile* infection sentinel surveillance between May 2009 and May 2015.

	2009/10		2010/11		2011/12		2012/13		2013/14		2014/15		Total	
	No./%	(95% CI)	No./%	(95% CI)	No./%	(95% CI)	No./%	(95% CI)	No./%	(95% CI)	No./%	(95% CI)	No./%	(95% CI)
No. of participating hospitals	19	-	20	-	18	-	19	-	21	-	22	-	26	
No. of reported CDI episodes	677	-	750	-	748	-	750	-	825	-	941	-	4691	
Mean age in years	68	(66.5-69.3)	67	(65.5-68.2)	66	(64.2-67.1)	67	(65.5-68.2)	67	(65.7-68.3)	67	(66.1-68.5)	66.9	(66.4-67.5)
Females	51%	(47.3-54.9%)	49%	(45.8-52.9)	52%	(48.5-55.7)	52%	(48.8-56.0)	51%	(47.9-54.7)	51%	(48.2-54.6)	51.3%	(49.8-52.7)
Community-onset CDI	30%	(26.1-33.0)	27%	(23.4-29.9)	31%	(27.4-34.1)	36%	(32.9-39.9)	36%	(33.0-39.7)	41%	(37.7-44.1)	33.8%	(32.5-35.2)
Recurrent CDI	33%	(27.9-38.2)	22%	(18.0-25.5)	24%	(19.8-27.6)	23%	(19.1-27.6)	24%	(20.4-27.8)	23%	(19.5-26.2)	24.3%	(22.7-25.9)
Severe CDI	24%	(21.1-27.5)	20%	(17.3-23.2)	27%	(24.0-30.7)	26%	(22.8-29.4)	20%	(17.3-23.2)	23%	(20.6-26.4)	23.6%	(22.3-24.8)
Complicated course within 30 days	20%	(17.1-23.7)	15%	(12.3-18.0)	15%	(11.9-17.6)	14%	(11.2-16.7)	13%	(10.6-15.7)	14%	(11.3-16.2)	15.1%	(13.9-16.2)
All-cause 30-day mortality	18%	(15.1-21.5)	14%	(10.8-16.2)	14%	(10.9-16.4)	12%	(9.8-15.1)	11%	(8.5-13.3)	13%	(10.2-15.0)	13.4%	(12.3-14.5)
CDI-related 30-day mortality	4%	(2.7-6.1)	4%	(2.1-5.1)	3%	(1.8-4.6)	3%	(1.6-4.3)	3%	(1.5-4.0)	4%	(2.7-5.5)	3.5%	(2.9-4.1)
Ribotype														
001	27%	(22.8-30.5)	21%	(17.6-24.2)	15%	(12.3-17.9)	14%	(11.3-16.8)	9%	(7.1-11.6)	6%	(4.2-7.6)	14.6%	(13.4-15.7)
014/020/295	12%	(9.0-14.6)	14%	(11.5-17.1)	16%	(12.7-18.5)	17%	(13.6-19.4)	15%	(12.3-17.8)	17%	(14.2-19.6)	15.2%	(14.0-16.3)
078/126	11%	(8.7-14.1)	14%	(10.8-16.4)	15%	(12.0-17.6)	13%	(10.7-16.1)	12%	(9.9-15.0)	13%	(10.6-15.4)	13.1%	(12.0-14.2)
027	4%	(2.5-6.0)	2%	(1.1-3.6)	2%	(1.1-3.5)	3%	(2.0-4.8)	3%	(1.9-4.6)	1%	(0.1-1.2)	2.6%	(2.1-3.1)
Other	46%	(41.7-50.3)	49%	(44.8-52.9)	52%	(48.3-56.2)	53%	(48.6-56.5)	60%	(56.2-63.7)	64%	(60.1-66.9)	54.5%	(52.9-56.1)

CDI DUE TO RIBOTYPE 078 COMPARED TO OTHER RIBOTYPES

The mean age of patients with a ribotype 078 infection was similar to those infected by other ribotypes (69 vs.67 years), but the age distribution was marginally different (*P*=0.039; Table 2). Community-onset CDI was slightly more common in 078 patients than in non-078 patients (RR, 1.13; 95% CI, 0.99-1.28). CDI severity was higher in 078 cases compared to cases caused by other ribotypes (RR, 1.28; 95% CI, 1.10-1.49). Further, patients with a ribotype 078 infection more often had a complicated course of disease (18.6% vs. 14.9%; RR, 1.25; 95% CI 1.00-1.56), a higher mortality (17.6 vs. 13.0%; RR, 1.35; 95% CI, 1.07-1.71), and higher CDI-related mortality (5.6 vs. 3.4%; RR, 1.65; 95% CI, 1.05-2.60) compared to those with CDI caused by other ribotypes.

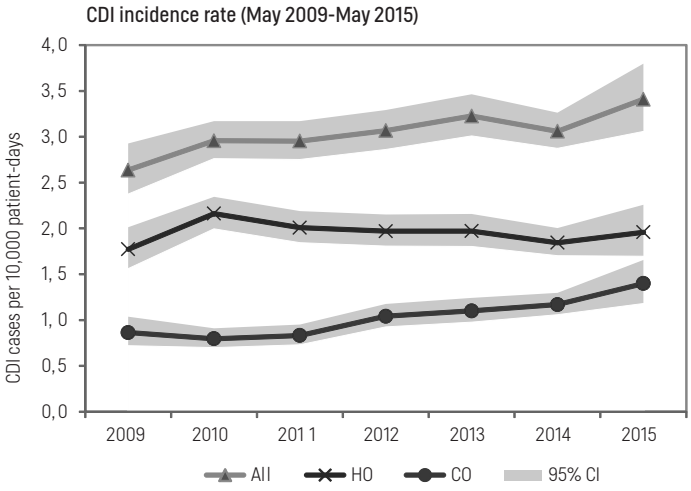
	078 CDI (n=493)		Non-078 CDI (n=3,263)		Risk ratio or P-value	
	%	(95% CI)	%	(95% CI)	RR	(95% CI)
Age						
< 18 years	1.2%	(0.2-2.2)	2.9%	(2.3-3.4)	P=.039	
18-65 years	30.8%	(26.7-34.8)	32.0%	(30.4-33.6)		
65-85 years	56.4%	(52.0-60.8)	51.2%	(49.5-53.0)		
> 85 years	11.6%	(8.8-14.4)	13.9%	(12.7-15.1)		
Female gender	50.7%	(46.3-55.1)	51.2%	(49.4-52.9)	0.99	(0.90-1.09)
Hospital service						
Medical	68.8%	(63.2-74.3)	71.6%	(69.6-73.7)	P=.517	
ICU	5.5%	(2.8-8.2)	4.3%	(3.4-5.2)		
Surgery	25.7%	(20.5-30.9)	24.1%	(22.1-26.0)		
Previous CDI (>8 weeks)	29.6%	(24.3-35.0)	23.1%	(21.2-25.0)	1.28	(1.05-1.57)
Community-onset of symptoms	37.3%	(32.9-41.6)	33.1%	(31.5-34.7)	1.13	(0.99-1.28)
Antibiotic therapy prior to CDI	71.1%	(66.7-75.5)	71.2%	(69.5-72.9)	1.00	(0.93-1.07)
Severe CDI	30.3%	(26.1-34.6)	23.7%	(22.2-25.3)	1.28	(1.10-1.49)

Table 2. Patient characteristics of PCR ribotype 078 CDIs compared to non-078 CDIs reported in *C. difficile* infection sentinel surveillance between May 2009 and May 2015.

CDI INCIDENCE RATES

The pooled overall and ribotype 078 specific CDI incidence rates were 2.82 (95% CI, 2.42-3.29) and 0.26 (95% CI, 0.21-0.31) cases per 10,000 patients-days respectively. There was no significant increase in time for all CDI (IRR, 1.00; 95% CI, 0.94-1.07) or ribotype 078 CDI (IRR, 1.04; 95% CI, 0.84-1.29) adjusted for diagnostic category. Community-onset CDI increased in time (IRR, 1.03; 95% CI 0.93-1.15) while hospital-onset CDI remained stable (IRR 0.98; 95% CI 0.90-1.06) when adjusting for diagnostic category (Figure 2). The overall CDI incidence rates were higher in secondary care hospitals (IRR, 1.44; 95% CI, 1.09-1.92) compared to primary care and university hospitals, as well as for ribotype 078 (IRR 1.44; 95% CI, 0.78-2.67) when adjusting for year and diagnostic category.

Figure 2 Incidence rate and 95% confidence interval of community-onset, hospital-onset and all CDI in participating hospitals (n = 26, 2009-2015). HO: hospital-onset, CO: community-onset.



CDI RIBOTYPE 078 INCIDENCE RATES IN RELATION TO PIG DENSITY

Fig 1b illustrates the pooled CDI incidence for each province with the corresponding average provincial pig density and Fig 1c the pooled provincial 078 incidence with the corresponding average pig density. Hospital ribotype 078 incidence was not associated pig density (IRR, 0.98; 95% CI, 0.88-1.09) or piglet density (IRR, 0.95; 95% CI, 0.75-1.21) of the province where the hospital was located. Further, ribotype 078 incidence was not associated with the number of pig farms per 1000 hectare (IRR, 1.08; 95% CI, 0.84-1.39) and the number of piglet farms per 1000 hectare (IRR, 1.00; 95% CI, 0.56-1.79). The incidence of community-onset ribotype 078 CDI was not related to the annual pig density (IRR, 0.98; 95% CI, 0.82-1.16) as well. The first sensitivity analysis excluding university hospitals (IRR, 0.98; 95% CI, 0.87-1.10) confirmed our primary results. The second sensitivity analysis excluding ribotype 126 from the ribotype 078 subgroup (IRR, 0.99; 95% CI, 0.89-1.10) also supported our primary findings.

SPATIAL ANALYSIS OF COMMUNITY-ONSET 078 CLUSTERS

Of patients with community-onset CDI (n = 1,554) the postal code was registered. For period I postal codes of 792 CO-CDIs (of which n = 90 ribotype 078) were analysed. For period II postal codes of 490 CO-CDIs (of which n = 52 ribotype 078) were included. In the analysis restricted to 25 km radius three large clusters (28, 37 and 49 km radius) of community-onset CDI were missed and five extra small clusters were detected (2, 5, 7, 8 and 20 km radius). None of these clusters were found

to be statistically significant. We continued with the restricted model to detect smaller clusters that might be overlapped or non-significant in non-restricted settings [25]. In both time periods no clusters of community-onset CDI or community-onset ribotype 078 CDI were found. Also, in our sensitivity analysis (excluding ribotype 126 infections from the 078 subgroup) no significant clusters were detected.

Discussion

C. difficile ribotype 078 persists as one of the most common ribotypes in hospitalised patients in the Netherlands, causing 13.1% of the cases in the present study. CDI due to type 078 was found to be associated with a worse clinical outcome (35% increase of 30-day mortality and 65% increase of CDI-related mortality) as in other studies [26]. We investigated the association between the hospital incidence of ribotype 078 CDI and pig farming at a provincial level as suggested before [16]. However, our results did not indicate any association of ribotype 078 CDI incidence in hospitals with provincial pig (farm) or piglet (farm) density. Besides, ribotype 078 did not cause spatial clusters of community-onset CDI followed by hospitalisation. Consequently we presume that *C. difficile* ribotype 078 shedding by pigs and/or piglets in our country does not lead to provincial excesses or localised clusters of hospital ribotype 078 CDI.

Earlier reports showed an association of ribotype 078 with community-onset or community-acquired disease [15 – 18] as one would expect if transmission was driven by pig or other animal contact in the community. We found ribotype 078 patients to have slightly higher community-onset disease compared to other patients. This could have been a result of the relatively low abundance of other endemic CDI strains (such as the healthcare-associated ribotype 027) taken as a reference to study ribotype 078 characteristics. Moreover, widespread transmission in the population may result in a change in clinical manifestation like for livestock-associated MRSA [27]. Previous studies in the Netherlands show that ribotype 078 was found in 11% of the CDI patients visiting their general practitioner with diarrhoea (absolute prevalence 0.09%) [28] and in 13% of the healthy community residents living in the proximity of livestock farms (absolute prevalence 0.16%) [29] similar to our results of hospitalised patients. In asymptomatic *C. difficile* carriers admitted to three Dutch hospitals (one hospital located in the province with the highest pig density, North Brabant), ribotype 078 was not one of the foremost ribotypes found [30]. These findings challenge the hypothesis that ribotype 078 primarily is a community-related disease.

This study is the first to search for clusters of community-onset ribotype 078 followed by hospitalisation in relation to animal density, as has been done for other stock-related pathogens such as Q-fever [25]. No significant clusters were found, in line with the absence of community outbreak reports of ribotype 078 CDI [5]. Remarkably, ribotype 078 rarely causes outbreaks in healthcare facilities (including those participating in the current study) despite its high virulence in infected patients.

LIMITATIONS

Our study has several limitations. This surveillance study targeted a geographically representative sample of hospitals, but selection bias may nonetheless have occurred. Not all pig farming areas were included in our data. Our analysis was based on the assumption that *C. difficile* ribotype 078 shedding by pigs and piglets leads to enhanced human exposure in the province [13]. Exposure may occur more localised, but we did not have data on individual exposure to pig-farming in this study. Ribotype 078 CDI not associated or followed by hospitalisation were not included. Besides, our provincial indicators for pig-farming did not differentiate between areas of low and high intensive pig-farming. We only differentiated between community-onset and healthcare-onset CDI for feasibility, but community-onset CDI could be related to previous healthcare exposure. Therefore, our results probably reflect an overestimation of CDI that is actually acquired in the community. Diagnostics for CDI were not standardised in the Netherlands during this study, which could bias incidence rates. We adjusted for three diagnostic categories in our models, but not for the specific testing strategy and diagnostic algorithm. Currently, 87% of the participants in the sentinel surveillance study apply a two-step algorithm to diagnose CDI [31]. CDI incidence rates were slightly underestimated due to the fact that children below the age of two could not be excluded from denominator data. The space-time permutation model was hampered by a variable number of participating hospitals which would induce population shift bias. To avoid this, we split the data in two time periods and selected hospitals that continually participated in surveillance (~80% all data) and thus might have missed clusters in the residual ~20% of the data.

CONCLUSIONS

According to the results of this study hospital incidence rates of ribotype 078 CDI in the Netherlands were not associated with pig-farming at a provincial level. Transmission beyond provincial borders (e.g. meat consumption) or in non-hospitalised patients cannot be excluded. No clusters of community-onset ribotype 078 followed by hospitalisation were detected in provinces with higher pig densities, although we might be scratching the surface of the burden of CDI in the community. For prospective studies on the zoonotic potential of CDI, we suggest that multiple reservoir hosts and 'sinks' of CDI are considered and advanced molecular methods are used to prove transmission.

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Chapter 6

Spatial clustering and livestock exposure as risk factor for community-acquired *Clostridium difficile* infection

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Objectives. *Clostridium difficile* infections (CDI) account for 1.5% of diarrhoeic episodes in patients attending a general practitioner in the Netherlands, but its sources are unknown. We searched for community clusters to recognise localised point sources of CDI.

Methods. Between October 2010 and February 2012, a community-based prospective nested case-control study was performed in three laboratories in the Netherlands with a study population of 2,810,830 patients. Bernoulli spatial scan and space-time permutation models were used to detect spatial and/or temporal clusters of CDI. In addition, a multivariate conditional logistic regression model was constructed to test livestock exposure as a supposed risk factor in CDI patients without hospital admission within the previous 12 weeks ('community-acquired CDI').

Results. In laboratory A, B and C, 1.3%, 1.8%, and 2.1% of patients with diarrhoea tested positive for CDI respectively. The mean age of community-acquired CDI patients (n=124) was 49 years (SD, 22.6); 64.5% were female. No spatial or temporal clusters of CDI cases were detected compared to *C. difficile* negative diarrhoeic controls. Except for one false-positive signal, no spatio-temporal interaction amongst CDI cases was found. Livestock exposure was not related to community-acquired CDI (OR, 0.99; 95% confidence interval, 0.44-2.24). Ten percent of community-acquired CDIs was caused by PCR ribotype 078, spatially dispersed throughout the study area.

Conclusions. The absence of clusters of CDI cases in a community cohort of diarrhoeic patients suggests a lack of localised point sources of CDI in the living environment of these patients.

Introduction

Clostridium difficile infection (CDI) occurs when its spores germinate in the intestinal tract, and bacterial growth and toxin production surpass the host resistance. *C. difficile* toxins damage the intestinal epithelium causing symptoms ranging from diarrhoea to life-threatening colitis [1]. Although hospitalised patients have the greatest risk for CDI, the infection has been increasingly recognised in patients living outside healthcare facilities [2, 3].

The risk profile and transmission of community-acquired CDI (CA-CDI) are not fully understood. Fourteen to 17% of patients with CA-CDI have no evident risk factors that predispose for CDI, such as medication use, prior hospitalisation and underlying diseases [4, 5]. Transmission from infants, asymptomatic carriers, household members and pets has been suggested, but never thoroughly investigated [6 – 8]. Livestock animals can acquire *C. difficile* as well, and may contribute to transmission of certain subtypes of *C. difficile* in the community [9]. In the Netherlands, piglets are typically infected by *C. difficile* ribotype 078 [10] and its spores contaminate the farm environment [11]. In-depth genomic studies indicate that *C. difficile* transmission between pigs and humans is likely [12, 13]. A publication from North Carolina, one of the largest pig producing states in the United States, suggested that environmental exposure to livestock farms increases the risk for CA-CDI, but calls for further spatial analysis that includes data of molecular strain typing [14].

The main objectives of the present study were to investigate i.) the spatial and/or temporal clustering of patients with CDI compared to *difficile* negative diarrhoeic controls in the community, ii.) the association of community-acquired CDI with livestock exposure, and iii.) the *C. difficile* PCR ribotypes and risk factors associated with spatial clustering of CDI in the community.

Methods

STUDY DESIGN

We used data from a prospective community-based prospective nested case-control study on CDI, performed between October 2010 - February 2012 in the Netherlands. Details on the study design have been published previously [4]. In summary, three medical microbiological laboratories (A, B and C) tested all unformed stool samples of patients >2 yrs submitted by 832 general practitioners (with a population of 2,810,830 patients) for the presence of free *C. difficile* toxins in the faeces. Diagnostics of other enteropathogens were performed on request of the physician. The study area encompassed areas of varying levels of pig farming. Questionnaire data (e.g. on CDI risk factors and several environmental exposures, e.g. contact with livestock) were requested from all positive patients and a matched control group (on age, sex, and calendar time). PCR ribotyping was used to characterize all *C. difficile* isolates [15]. The study was approved by the LUMC Medical Review Ethics Committee.

GEOCODING AND MAPPING

Full residential postal codes were requested from both *C. difficile* positives (n = 194) and *C. difficile* negative patients with diarrhoea (n = 12,520). If the exact residential postal code was unknown (all patients of laboratory A, four patients of laboratory B, and two patients of laboratory C), the location of the general practitioners' practice was used. Locations were obtained for 6,882 patients for laboratory A (83%), 3,009 patients for laboratory B (100%) and 1,367 patients for laboratory C (100%). Locations were geocoded to X- and Y-coordinates of the centroid of the full postal code. ArcGIS version 10.5 was used for mapping (Environmental Systems Research Institutes, Inc. Redlands CA).

SPATIAL CLUSTERING ANALYSIS AND SPACE-TIME PERMUTATION MODEL

Scan statistics were used to detect clusters of CDI in the community in temporal, spatial and space-time settings [16 – 18]. Likelihood ratio tests were used to detect the most likely clusters, while Monte Carlo simulation was used to correct for multiple testing [16, 18]. Bernoulli models were applied to assess if CDI cases were non-randomly distributed in space and time compared to *C. difficile* negative diarrhoeic controls [18]. Subsequently, we searched for space-time interaction of CDI and non-CDI diarrhoeic events by space-time permutation models [17]. For both models, we aggregated the data per week, used a standard maximal temporal cluster size of 50% of the population at risk, and a maximal cluster size of 25km, and scanned for high rates. As the three participating laboratories had slightly different study periods, we performed separate space-time permutation analyses for three laboratories. SaTscan (version 9.4.4, M Kulldorf, Boston, MA, USA) was used to perform all geospatial analysis.

LIVESTOCK EXPOSURE AS A RISK FACTOR FOR COMMUNITY-ACQUIRED CDI

We tested livestock exposure as a risk factor for CA-CDI by multivariate conditional logistic regression analysis. Patients were excluded if they were admitted to a hospital <12 weeks prior to onset of diarrhoea [19]. Livestock exposure included professional contact and/or recreational contact with farm animals (e.g. visiting a children's farm) <30 days before the onset of diarrhoea. Using data from the literature, putative confounders (other than matching variables) were antibiotic use [5, 20 – 23], hospital visits [22, 23], PPI use [24], CDI household contacts [8], and contact with infants [25] and comorbidities [23]. Antibiotic exposure was categorized into 4C antibiotics (cephalosporins, clindamycin, ciprofloxacin and amoxicillin/clavulanic acid), non-4C antibiotics and antibiotics of unknown type [26]. We created a comorbidity score adapted from the chronic disease score for infectious diseases (CDS-ID), but scored reported illnesses in <1 year before diarrhoea [27]. Putative confounders were visualized in a directed acyclic graph (Supplementary Figure 1) [28] and incorporated in the multivariate model accordingly. For the total effect of animal exposure we adjusted for age and gender (matching variables), comorbidities, and contact with infants. Odd ratios (ORs) were presented with a 95% confidence interval (95% CI). We used STATA version 14.1 (StataCorp, College Station, TX, USA) for our analyses.

Results

C. DIFFICILE PREVALENCE

The study covered a population of 2,810,830 patients inhabiting 3,848 postal code areas. In total, 194 of 12,714 patients (1.5%) tested positive for *C. difficile* toxins. Laboratory A tested 111 of the 8,338 patients positive for *C. difficile* (1.3%) between October 4, 2010 and October 28, 2011. Laboratory B tested 54 of the 3,009 patients positive for *C. difficile* (1.8%) between November 16, 2010 and January 31, 2012. Laboratory C tested 29 out of 1,367 patients positive for *C. difficile* (2.1%) between September 30, 2010 and September 30, 2011. The distribution of *C. difficile* positive and negative patients of all three laboratories is depicted in Figure 1. Of the 194 *C. difficile* positive patients, 152 completed the questionnaire and 124 complied with the definition of CA-CDI. The mean age of CA-CDI patients was 49 years (SD, 22.6), and 64.5% were female.

SPATIAL AND TEMPORAL CLUSTERS OF CDI

According to Bernoulli modelling, no significant clusters of CDI (n = 179) were detected compared to *C. difficile* negative diarrhoeic controls (n = 11,258) for laboratory A, B and C. Furthermore, testing for purely spatial or temporal clusters did not yield significant results. One non-significant temporal cluster of 45 CDI cases (RR, 2.06; $p = 0.051$) was found between the October 23, 2010 and April 1, 2011 for laboratory A, and one of 11 cases (RR, 2.10; $p = 0.89$) between the November 30, 2011 and January 24, 2012 for laboratory B.

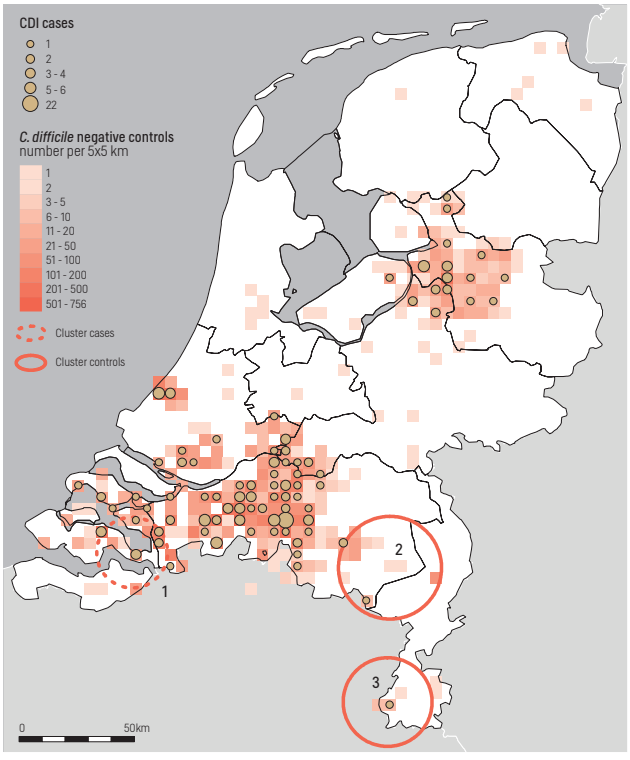
SPACE-TIME INTERACTION OF CDI

One significant spatio-temporal cluster of six CDI cases (not caused by one ribotype) between December 11, 2010 - February 4, 2011 was found (15.31 km radius; $p = 0.0066$; Figure 1). However, this cluster was located in an area surrounded by large water surfaces (Figure 1), not found by Bernoulli modelling, and interpreted as false positive. CDI patients were not clustered according to a space-time permutation model for laboratory B and C.

SPACE-TIME INTERACTION OF *C. DIFFICILE* NEGATIVE PATIENTS WITH DIARRHOEA

Two clusters of *C. difficile* negative diarrhoeic controls were detected for laboratory A (Figure 1). The first consisted of 159 patients diagnosed between October 16, 2010 – April 22, 2011 (22.25 km radius, $p < 0.0001$) of whom 9% were tested positive for a combination of infectious pathogens causing diarrhoea. The second cluster included 15 diarrhoeal patients without infectious pathogens detected between November 20, 2010 and January 21, 2011 (19.19 km radius, $p = 0.0012$; Figure 1). For laboratory B and C, *C. difficile* negative diarrhoeic controls were not clustered according to a space-time permutation modelling.

Figure 1 Distribution of 179 community CDI cases (yellow circles) in contrast to density of *C. difficile* negative diarrhoeic controls (number per 5x5 km; blue) as detected by three medical microbiology laboratories in the Netherlands. Red circles indicate spatial-temporal clusters of community CDI cases (dotted line; false-positive) and *C. difficile* negative diarrhoeic controls (solid line in three regions of the Netherlands.



RISK FACTORS FOR CA-CDI

Table 1 shows the putative risk factors for community-acquired CDI cases (n = 124) and controls (n = 232). Comorbidities and antibiotic exposure were associated with CA-CDI, while there was no apparent association with environmental exposures to livestock. In multivariate analysis, livestock exposure was not related to CA-CDI (OR, 0.99; 95% confidence interval, 0.44-2.24).

PCR RIBOTYPES OF CA-CDI

C. difficile isolates were available for PCR ribotyping of 120 patients with community-acquired CDI. Of 25 different PCR ribotypes found, ribotypes 002 (11.4%), 015 (10.1%), and 078 (10.1%) were most common. Since no CDI clustering was found, associations to specific PCR ribotypes could not be investigated. PCR ribotype 078 cases and those caused by the highly related ribotype 126 were spatially dispersed throughout the study area.

Table 1. Putative risk-factors of community-acquired CDI, and multivariate conditional logistic regression analysis of livestock exposure as a risk factor for community-acquired CDI. aAdjusted for age and gender, bmatching variables. CA-CDI; community-acquired CDI; OR: Odds ratio; CI: confidence interval; MVA: multivariate analysis; sd: standard deviation.

	CA-CDI (N=124)			Controls (N=232)								
	n	N	%	n	N	%	OR[a]	(95% CI)		MVA	OR	(95% CI)
Age, mean (±sd)[b]	**49.0**	**22.6**		**48.4**	**22.4**							
Female[b]	**80 / 124**	**64.5**		**151 / 232**	**65.1**							
Comorbidities <1yr before diagnosis												
Diabetes	12 / 124	9.7		18 / 230	7.8		1.34	0.62	2.91			
Respiratory illness	20 / 124	16.1		20 / 230	8.7		2.03	1.06	3.87			
Kidney disease	5 / 124	4.0		4 / 230	1.7		2.49	0.58	10.70			
Transplant	1 / 124	0.8		0 / 230	0.0							
Cancer	3 / 124	2.4		3 / 230	1.3		1.76	0.24	12.73			
Gastro-intestinal illness	12 / 124	9.7		17 / 230	7.4		1.35	0.60	3.03			
Comorbidity score, mean (±sd)	0.78	1.17		0.49	1.04		1.29	1.05	1.58	1.27	1.03	1.56
Antibiotic use <90 days before diarrhoea												
4C antibiotics	19 / 122	15.6		3 / 228	1.3		43.42	8.43	223.64			
Non-4C antibiotics	27 / 122	22.1		6 / 228	2.6		24.33	7.08	83.55			
Unknown type of antibiotics	18 / 122	14.8		23 / 228	10.1		4.32	1.78	10.48			
Overall antibiotic use	64 / 122	52.5		32 / 228	14.0		10.87	5.14	22.96			
Hospital visits <30 days	39 / 119	32.8		64 / 216	29.6		1.16	0.71	1.89			
CDI household contacts	1 / 121	0.8		2 / 221	0.9		1.01	0.09	11.20			
Diarrhoeic household contacts	6 / 121	5.0		18 / 222	8.1		0.62	0.24	1.56			
Contact with infants <2 year old	35 / 121	28.9		89 / 246	36.2		0.76	0.46	1.28	0.87	0.51	1.49
Animal, manure, and meat exposure												
Livestock	10 / 121	8.3		22 / 231	9.5		0.94	0.42	2.07	0.99	0.44	2.24
Pet(s)	79 / 119	66.4		137 / 228	60.1		1.36	0.82	2.23			
Gardening	34 / 119	28.6		85 / 219	38.8		0.59	0.35	0.99			
Working in food and beverage	4 / 123	3.3		4 / 229	1.7		1.84	0.46	7.40			
Eating meat												
never	2 / 121	1.7		4 / 231	1.7		ref.					
1-2 times a week	16 / 121	13.2		35 / 231	15.2		0.88	0.15	5.17			
3-6 times a week	54 / 121	44.6		113 / 231	48.9		0.94	0.17	5.24			
daily	49 / 121	40.5		79 / 231	34.2		1.32	0.23	7.49			

Discussion

The incidence of CDI in the community in the Netherlands was estimated at 0.67 per 10,000 persons per years (95% confidence interval, 0.58-0.78), comparable to Salmonella infections [4]. Our multicentre study of community CDI is the first to assess both spatial clustering and environmental risk factors for CA-CDI in combination with molecular typing data. We did not find spatial clusters of CDI in a large community cohort of diarrhoeic patients. Correspondingly, there was no space-time interaction indicative for unusual increases of CDI in the community except for one false-positive signal. Our results support the hypothesis that CDI transmission in the community derives from widespread sources and not from localised environmental point sources, such as livestock farms [11].

Concerns that livestock farms –piglet and pig farms in particular– contribute to transmission of CDI to humans occurred for several reasons in the Netherlands [9]. High rates of *C. difficile* ribotype 078 have been found among piglets, farmers and the farm environments [10, 11, 29]. One out of four persons with daily contact with pigs was positive for intestinal carriage with *C. difficile* of which virtually all were ribotype 078 [29]. Application of whole-genome sequencing confirmed the presence of 100% identical ribotype 078 strains in pigs and humans [13]. In the current study, ribotype 078 cases accounted for 10% of CA-CDI, but its occurrence was not spatially clustered in areas of livestock farming. Other studies indicated that livestock farming does not lead to regional increases of CDI associated with PCR ribotype 078, or to a higher risk of *C. difficile* colonization in neighbouring residents [30, 31].

Our study incorporated spatial scan statistics. To our knowledge, two other studies assessed spatial clustering of CDI in the community in Australia [32] and North Carolina [14] respectively. In Australia, no spatial clusters were found among 1,792 *C. difficile* cases deriving from 392 postal code areas in Queensland [32]. In contrast, clustering was found in 21% of the 1,895 CA-CDI cases analysed in 10 counties of North Carolina comprising an area of approximately 1.94 million residents [14]. CA-CDI was associated with living in proximity to a livestock farm, farming raw materials service and nursing home [14]. The latter finding may result from the fact that long term care facility residents were eligible for inclusion as CA-CDI patients. Both studies included several demographic and environmental factors in the constructed spatial models, but molecular typing data and detailed patient information on CDI risk factors and animal exposure was lacking. A comparison between these findings and our case-control data is not straightforward. Interestingly, *C. difficile* PCR ribotype 014 was the most prevalent type in pigs in Australia, whereas ribotype 078/Toxinotype V was most prevalent in pigs in North Carolina [33] and the Netherlands [10]. In Australia, transmission of genetically identical ribotype 014 isolates was demonstrated between pigs and humans [12], similarly as for ribotype 078 in the Netherlands [13].

Our study has several limitations. We were not able to use exact residential data for all patients and experienced overall 10% missing location data. In the spatial analyses of laboratory A, we used locations of the general practitioners' clinics which may have caused a bias towards more clustering in this region. Second, our control group consisted of patients with diarrhoea due to other causes than *C. difficile* and not the total population-at-risk. Therefore we might have overlooked CDI clusters that occurred at the same time and place as non-CDI diarrhoeic clusters. We assumed that CDI result in a different pattern of clustering than other (also non-infectious) causes of diarrhoea, but there is no literature to support this assumption. We compensated by use of a second spatial model (space-time permutation model), not requiring population-at-risk data. However, further exploration of the scale and spatial patterns to be incorporated in spatial models for CDI are needed. Finally, we have not included cases (and controls) that did not visit their general practitioner or did not submit a stool sample [34].

CONCLUSIONS

Our study using spatial scan statistics did not find clusters of CDI in the community. The lack of geographical and temporal clustering in the present study in combination with a lack of environmental risk factors (e.g. livestock exposure) suggest that widespread sources most likely are key in CDI infection and transmission in the community.

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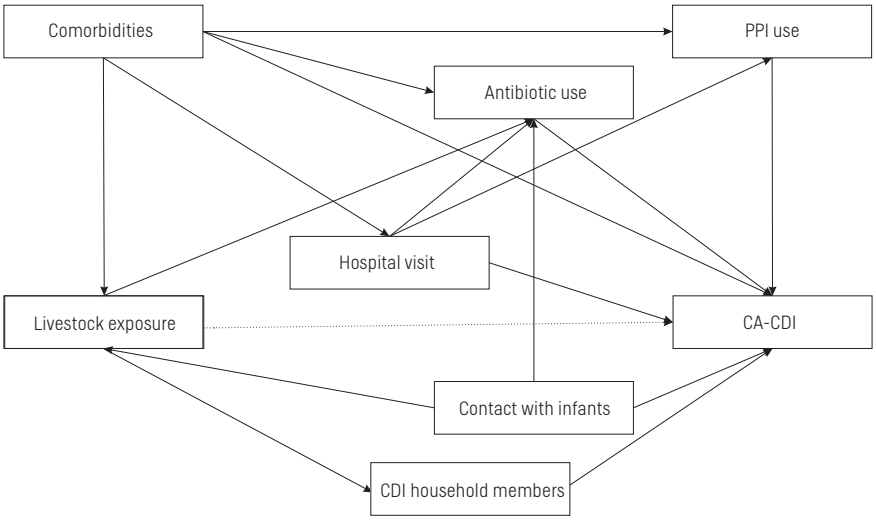
Supplementary figures



Supplementary Figure 1 Directed acyclic graph of the supposed relation of livestock exposure on evelopment of community-acquired CDI (CA-CDI).

Chapter 7

Discussion

Summary

CDI became the most common healthcare-associated infection (HAI) in Northern-America and Europe during the antibiotic era, especially after global transmission of a fluoroquinolone-resistant ribotype 027 strain originating from Canada, 2003 [1, 2]. The rise of CDI in Northern-America and Europe urged the introduction of molecular typing services and epidemiological surveillance systems to monitor disease dynamics and to detect outbreaks [2]. Surveillance data are used to direct and monitor the effect of local and national infection prevention controls interventions, facilitate education of hospital staff, setting priorities for research projects, and persuade national authorities to provide sufficient resources to combat CDI – as recommended by the WHO [3]. In the Netherlands, a national reference laboratory for molecular typing and ad-hoc surveillance of *C. difficile* has been set up to control transmission of ribotype 027 and other CDI in 2005 [4]. The Dutch national reference laboratory for *C. difficile* implemented a national Sentinel Surveillance for CDI for ongoing monitoring of the incidence of CDI and detection of new outbreaks in 2009. The Sentinel Surveillance helps to improve local infection prevention in participating hospitals, controls the transmission of highly virulent types such as ribotype 027 and generates new hypotheses to understand and optimise the control of CDI [5].

The Dutch national reference laboratory achieved corporation with many other national or central reference laboratories in Europe. However, the heterogeneity, insufficiency and/or unavailability of diagnostic, typing and surveillance methodologies across Europe impeded combined efforts to control CDI. Hence, the European Centers for Disease Control and Prevention (ECDC) supported a 4-year project named 'the European CDI Surveillance Network' (ECDIS-Net) to enhance CDI surveillance and laboratory capacity to test for CDI in Europe in 2010.

This thesis aims to describe trends in diagnostic capacity and epidemiology of CDI in the Netherlands and Europe and determines requirements to optimise surveillance and control of CDI. The thesis incorporated two ECDIS-net studies **(CHAPTER 2 AND 3)** as well two studies using data of Dutch Sentinel Surveillance for CDI **(CHAPTER 4 AND 5)** complemented by a community-based case-control study in the Netherlands **(CHAPTER 6)**. The studies' implications for surveillance and control are discussed in the current chapter.

In **CHAPTER 2** of this thesis, we showed that 46% of the surveyed ECDIS-Net laboratories had optimal diagnostics for CDI in 2014. This was in line with a larger study, illustrating that 48% of the European laboratories used optimal diagnostics for CDI in 2014 [6]. Our survey provided more insight in the barriers for optimal CDI laboratory diagnostics (e.g. limited resources, budget cuts, and disagreement on optimal CDI algorithms) informing the ESCMID study group for *C. difficile* throughout revision of the European diagnostic guidance document

for CDI [7]. We proposed strategies to promote optimal diagnostics for CDI at national level in Europe. These strategies included advocating a single national two-step algorithm for CDI like in the United Kingdom [8], or encouraging the use of one of the optimal two-step algorithms of the revised ESCMID diagnostic guideline for CDI [7]. Surveillance programs can stimulate the use of optimal diagnostics for CDI and explore statistical strategies to remove residual bias caused by diagnostic variability. Still, one should note that many other factors (e.g. clinical awareness and criteria for selection of faecal samples tested for CDI) bias detection of CDI besides laboratory diagnostics.

Molecular typing of *C. difficile* can enhance the potency of surveillance systems by early detection and confirmation of outbreaks, monitoring the prevalence of endemic strains and source tracking, and complements investigation of *C. difficile* antibiotic resistance [9, 10]. Yet, the variety of applied molecular typing methodologies by (national) reference laboratories for *C. difficile* hampers data pooling to obtain an overall view of the European and worldwide molecular epidemiology of CDI. In **CHAPTER 2** of this thesis we demonstrated that the typing capacity of CDI in Europe increased from 71 to 81% in the course of ECDIS-Net. PCR ribotyping – exploiting the variable 16S-23S rRNA interspacic region of *C. difficile* – is the standard typing method in Europe for its relative simplicity and low costs [10]. Capacity for PCR ribotyping (standard or capillary) increased from 65 to 72%, and capillary PCR ribotyping from 23 to 50%. Capillary PCR ribotyping has been preferred for its enhanced discriminatory power compared to standard PCR ribotyping and has been standardised by four reference laboratories in England, the Netherlands, the United States and Canada [11]. Despite these technical developments, use of PCR ribotyping has been restricted by limited resources and budget reductions in several countries. This underlines the need for more targeted application of molecular typing in epidemiological surveillance for outbreak detection and source tracking of CDI, and will also affect the implementation of more discriminatory typing methodologies (e.g. whole-genome sequencing).

Implementation of national epidemiological surveillance systems for CDI has been recommended after the rise of CDI in Europe in 2003 to control its transmission [2, 10, 12]. Only fourteen of the 31 European countries (45%) had adopted surveillance for CDI in 2011 and methodologies were heterogeneous. Continued integration of microbiological data was limited [13]. A multistate CDI surveillance system, such as implemented in the United States [14], was considered the only viable option to monitor and control the burden of CDI in Europe. In **CHAPTER 3** of this thesis, we tested a standardised European surveillance protocol aimed to monitor and estimate the burden of CDI in Europe in 14 countries with varying levels of implemented surveillance activities in 2013. This surveillance system had the option of integrating clinical and molecular data, and obtained important information on the high number of patients having CDI symptoms at

admission (49%) and the presence of PCR ribotype 027 (30%). Results of this study were used by ECDC to optimise and initiate European Surveillance of CDI in 2016 [15]. A recent European survey illustrated that 20 out of 33 European countries had surveillance systems for CDI in place in 2017 and hospitals in at least 21 countries applied the European Surveillance protocol [16]. Standardisation of CDI diagnostics and molecular typing as described in **CHAPTER 2** was considered part of integration of *C. difficile* data in The European Surveillance System (TESSy) within this project. Other challenges relate to representative sampling of healthcare facilities participating in surveillance, optimisation of CDI surveillance definitions and validation of surveillance data.

In the Netherlands, the national Sentinel Surveillance for CDI aims to describe trends of the incidence of CDI in participating hospitals, to estimate the total burden of CDI Dutch hospitals, and detect circulating and new emerging PCR ribotypes in relation to their clinical outcome for targeted control of highly virulent ribotypes affecting patient safety. Participating hospitals get direct notification after isolation of *C. difficile* ribotype 027 or when an outbreak is suspected. The main results of Sentinel Surveillance are annually published on the website of the National Institute of Public Health and the Environment (RIVM) and included in national reports on the '*Consumption of antimicrobial agents and antimicrobial resistance among medically important bacteria in the Netherlands*' (Nethmap), and presented to hospital infection control personnel and microbiologists in the Netherlands [17, 18]. In this thesis, data of Sentinel Surveillance was used to study (spatial) trends in the epidemiology of CDI in the Netherlands, in particular for children and the potentially zoonotic *C. difficile* ribotype 078.

During recent years, there have been concerns about the rise of CDI in children as observed in the United States [19, 20]. Although *C. difficile* rarely causes infection in children up to 2 years, the bacterium is potentially hazardous in older children [21]. Children above the age of two years have been included in Sentinel Surveillance since its start in 2009. In **CHAPTER 4** of this thesis, we analysed clinical and microbiological data of hospitalised children included in Sentinel Surveillance to investigate the time trend of CDI among children in our country. We did not find an increase of the number of CDI reports (n= 135, 3% of all CDIs) amongst children in 2009-2015 in twenty-six Dutch hospitals. There were no outbreaks on paediatric wards. Approximately one-third of the children met the criteria for severe CDI, but only 3% experience serious complications and none died related to CDI. PCR ribotyping showed a high prevalence of ribotype 265 (toxin A negative due to a *TcdA* 1.8 kb deletion, toxin B positive) compared to adults (15 versus 1%). Multiple-locus variable-number tandem-repeat analysis (MLVA) showed that three-fourths of the ribotype 265 strains isolated from children were genetically related to those found in adults. According to our international survey, ribotype 265 was uncommon or absent in many countries except for neighbouring Belgium. Transmission routes could not be elucidated. Notably,

ribotype 027 was not isolated from children. We underlined the need for reconsideration of diagnostic testing and surveillance definitions for children, as well as studies to elucidate the observed differences in the prevalence of specific ribotypes of *C. difficile* compared to adults.

In the Netherlands, there have been concerns about the high burden of the potentially zoonotic *C. difficile* PCR ribotype 078 and its link to pig farming [5, 22]. In our country, 12 million pigs coexist with nearly 17 million humans and pig farming is unevenly geographically distributed [23, 24]. Pigs and pig farm environments are commonly contaminated with *C. difficile* ribotype 078 [25]. A quarter of Dutch farmers tested positive for *C. difficile* ribotype 078 in a small study [26] alike livestock-associated MRSA [27] and clonal relatedness of ribotype 078 strains with those isolated from pigs and clinical patients has been confirmed by whole-genome sequencing [28]. In **CHAPTER 5** of this thesis, we hypothesised that *C. difficile* ribotype 078 shedding by pigs (including piglets) leads to increased human exposure at a provincial level and consequently higher incidences of ribotype 078 CDI in hospitals there situated. However, we found that the hospital incidence of ribotype 078 CDI was not related to the pig farming density of the province where the hospital was located. Nevertheless, the burden of PCR ribotype in the Netherlands was substantial (13.1% of all CDI), and these patients had an increased mortality risk compared to other CDI patients (Risk Ratio 1.35). We introduced the use of space-time statistics [29] to search for clusters of community-onset CDI followed by hospitalisation, but found no evidence for the presence of clustering. Our analyses was limited by the fact that not all hospitals in provinces with the highest farm density were included due to our sentinel approach and transmission beyond provincial borders or in outpatients could not be excluded. We suggested molecular investigation of multiple reservoir populations and sinks (e.g. One Health approach) to study zoonotic transmission of CDI in the absence of observed epidemiologic associations.

In **CHAPTER 6** of this thesis, we extended the use of spatial scan statistics to search for clustering of CDI in the community, using data of a prospective nested case-control study of three laboratories in the Netherlands from 2010-2012 [30] in the absence of community-based CDI surveillance. CDI is a common cause of diarrhoea in the community with an incidence rate of 0.67 cases per 10,000 patient years comparable to Salmonella infection in the Netherlands [30]. CDI clusters in the community could potentially co-localise with undetected sources of CDI, that may also contribute to CDI in the hospital setting [31]. We did not detect any spatial or temporal clusters of CDI cases compared to *C. difficile* negative diarrhoeic controls and no evident space-time interaction amongst CDI cases. Livestock exposure was not related to community-acquired CDI (Odds Ratio 0.99). Ten percent of community-acquired CDI was caused by PCR ribotype 078, but its occurrence was spatially dispersed throughout the study area. Our findings supported the hypothesis that CDI transmission in the community derives from widespread

sources [32] and not from localised environmental community point reservoirs, such as livestock farms [25].

Lately, the combat on the increasing threat of antibiotic resistance was brought into national focus, in line with the worldwide action plan of the WHO [33]. Goals set are e.g. to reduce preventable HAI by 50% in 2015-2019 and to enhance and improve regional surveillance using a 'One Health' approach. In order to achieve a reduction of CDI additional efforts are needed to e.g.; i) Improve evidence-based infection prevention in hospitals, ii) evaluate the role of asymptomatic carriers in the transmission of CDI iii) provide full reporting of hospital incidence rates for CDI linked to indicators of antibiotic stewardship and patient safety, iv) application of more discriminatory molecular typing methodologies and model-based (spatial) studies for source tracking within and beyond the healthcare setting.

In conclusion, this thesis provided several options for improving surveillance and control of CDI at a national and European level.

Methodological and analytical challenges for CDI surveillance

Compelling evidence how to design (inter)national surveillance systems is lacking [34], but several studies including those incorporated in this thesis indicated the strengths and weaknesses of existing epidemiological surveillance systems for CDI. The following paragraphs discuss the main methodological and analytical challenges for CDI surveillance encountered during the realisation of this thesis and provide several options to improve future surveillance of CDI.

CASE DEFINITIONS FOR CDI SURVEILLANCE

Soon after recognition of the emergence of ribotype 027 outbreaks in Europe, 'interim' surveillance definitions for CDI were publised by the ESCMID study group for *C. difficile* in collaboration with ECDC in 2006 [2]. Interim surveillance definitions included a case definition for CDI, recurrent CDI, healthare-associated and community-associated CDI, severe CDI and a CDI outbreak. The proposed surveillance defininitions were mostly based on clinical experience and considered to be subjected to evidence-based revisions. Most European countries implemented the interim ESCMID definitions for national surveillance of CDI [13]. The ESCMID definitions were also adopted by the *Clostridium difficile* Surveillance Working Group in the United States in 2007, whom underlined the need for standardised CDI definitions for benchmarking of incidence rates among healthcare facilities and to direct surveillance activities [35]. The ESCMID surveillance definitions are now used for over one decade and have only slightly been adapted –presumably due to their usability, the shortage of validation studies and the need of stable parameters.

CLINICAL CASE-DEFINITION

Both Dutch Sentinel Surveillance and European Surveillance of CDI use the ESCMID case-definition for CDI as published in 2006 [2]. In contrast to the surveillance module of the National Healthcare Safety Network in the United States [14], this definition includes clinical criteria (i.e CDI symptomatology) in addition to laboratory confirmation of toxin-producing *C. difficile* in the stools of the patient. The Dutch Sentinel Surveillance protocol additionally defines that other causes of diarrhoea should be excluded to be eligible for inclusion as CDI case. The use of clinical criteria contributes to the specificity of the case-definition compared to using laboratory criteria/events only, but goes with an increased surveillance workload [36] due to the need of chart review before patient inclusion. However, this process may be supported by use of sophisticated computer algorithms [36, 37]. The added value of verifying clinical criteria in addition to laboratory events for case finding depends on the local criteria for selection of faecal samples and applied diagnostic algorithms for CDI. In Chapter 2 of this

thesis, we have illustrated the variety of criteria for selection of faecal samples tested for CDI in Europe as also illustrated by others [6]. Besides, the increased use of highly sensitive but potentially less specific diagnostics algorithms for CDI (see *How to standardise laboratory diagnostics for CDI*) emphasises the need to preserve clinical criteria in the case-definition of CDI [38]. Whether or not to use a similar case-definition for children and as for adults is unclear; a more stringent case-definition for children seems plausible because of high rates of CDI colonization [39] as discussed in Chapter 4. The use of risk-factors (i.e. hospitalisation, comorbidities and antibiotic use) as previously proposed diagnostic criteria [40] hampers diagnosis of CDI in children in the community. Yet, compelling clinical evidence (e.g. exclusion of other causes of diarrhoea) and laboratory confirmation of the presence of free toxins in the faeces should at least be considered for inclusion of children in CDI surveillance.

RECURRENT CDI

In Chapter 3 of this thesis, we demonstrated that 12% of all patients suffered from recurrent CDI. The surveillance definition of recurrent CDI *("≤8 weeks following the onset of a previous episode of CDI"*) is based on the time interval between two episodes and not on microbiological molecular typing results [2]. The Dutch Sentinel Surveillance protocol specifies if the prior CDI episode was verified by laboratory diagnostic results or clinical symptomatology solely, possibly leading to higher rates of reported recurrent CDI (Chapter 4 and 5). Recurrent infection within 8 weeks can occur due to a relapse of the same or reinfection by another *C. difficile* strain [2]. The ESCMID definition for recurrent CDI has several advantages. The simplicity of the definion allows its widespread and consistent use (not depending on the availibity and methodology of molecular typing for CDI). Further, there is no bias due to mixed infections present in 3-7% of the CDI patients troubling the confirmation of relapsing strains of *C. difficile* in one patient [41]. Reported recurrence rates of CDI underlined the high cumulative burden of CDI and urged the need for improved treatments stategies, such as fecal microbiota transfer [42]. The disadvantage of not distuiginging CDI relapse and reinfection by the current ESCMID definition is missing out the opportunity to investigate the separate contributions of reexposure and decreased host resistance to *C. difficile* to direct infection prevention control measures. However, the question is to what degree these aspects can be dissociated from each other. Small studies using whole-genome sequencing showed that the majority of infections (within and beyond the 8-week cutoff) were caused by identical *C. difficile* strains, suggestive of persistance and outgrowth of *C. difficile* spores during and after antibiotic therapy for CDI [43, 44]. In a validation study, the cutoff of 8 weeks had a low sensitivity (56%) and specificity (74%) to indicate patients with a relapse due to a same PCR ribotype, and a 20-week cutoff was suggested [45]. Extension of the 8-week cutoff was also suggested by a whole-genome sequencing study on ribotype 027 tracking [46]. Overall, application of the ESCMID definion for recurrent CDI is important to highlight the cumulative burden of CDI for patients and

to monitor the overall effect of treatment and infection prevention control strategies for recurrent CDI, but should not imply any conclusions on relapse and reinfection of CDI. The latter requires well-performed molecular typing (See *Use of molecular typing for surveillance*) that should be part of efficacy trials of CDI treatment modalities and enhanced surveillance. In the Netherlands, one should consider to simplify reporting of recurrent CDI in Dutch Sentinel Surveillance, or at least validate the discrimination of two levels of case-ascertainment of the prior CDI.

HEALTHCARE-ASSOCIATED AND COMMUNITY-ASSOCIATED CDI

As CDI has a variable 'incubation' time, not all CDI occurring during admission in healthcare facilities derives from transmission within healthcare facilities. Besides, a part of healthcare-acquired CDI have its onset after discharge. Clear cutoff levels are lacking. Discrimination of local transmission from imported CDI has important consequences for hospital infection control [47]. Whereas local transmission requires intensified measures to reduce the infection load in the hospital (i.e. cleaning) and interruption of transmission paths (e.g. hand washing and patient isolation), imported CDI requires investigation of community sources and may be an argument to screen for *C. difficile* at admission. In Chapter 3, we specified 82% of CDI to be healthcare-associated CDI using the ESCMID surveillance definition (*"CDI with onset of symptoms at least 48 hours following admission to a healthcare facility or with onset of symptoms in the community within 4 weeks following discharge from a healthcare facility"*). Notably, half (49%) of the patients had symptoms present at admission, but 68% had been hospitalised in the prior 3 months. Another hospital than the one where the patient was located at time of diagnosis was reported to be origin of infection for 18% of CDI. The Dutch Sentinel Surveillance does not practise the ESCMID definitions of healthcare-associated and community-associated CDI, but registers the location of onset of CDI. In the Netherlands, 65% of CDI in hospitalised patients started in a healthcare facility, in 91% of the cases an acute care hospital (Chapter 5). Hospital-onset CDI was less common in children (45%) than in adults (67%, Chapter 4).

Proportions of reported healthcare-associated CDI contrast the findings of a benchmark whole-genome sequence study illustrating that only 55% of the hospital cases in Oxfordshire were considered to be locally transmitted by other symptomatic CDI patients (≤10 SNVs difference) [31]. In another study performed in the United Kingdom, 20% of CDI was linked (≤1-2 SNVs difference to a CDI case in the prior 90 days (inter-hospital range 7-24%) [48]. Yet, these studies did not investigate all potential transmission paths of CDI within healthcare facilities and criteria for 'direct' transmission by other CDI patients (SNVs difference and time interval) need further analysis. One of the studies indicated large inter-hospital variations of hospital transmission [48], emphasising the need of confirmation studies in other countries and settings before global extrapolation. These studies should also focus on validation of the ESCMID definition of healthcare-associated CDI, as suggested by a smaller study [49].

SEVERE CDI

Severity criteria are used for choosing the best treatment strategy for individual patients and prediction of unfavourable outcomes of CDI [50]. Patients that were treated according to a guideline basing treatment decisions on CDI severity criteria had a lower 90-day mortality (Risk Difference 31%) compared to other patients, although the study was limited by its observational design [51]. In surveillance systems, the use of severity criteria aims to detect signals indicative of increased virulence (as observed for PCR ribotype 027) or treatment failure, and data can be applied for burden estimations to guide local and national policy. Ribotypes that pose an increased threat for patient safety due to enhanced virulence need stronger, more timely and potentially tailored responses to prevent transmission [52, 53]. Although surveillance definitions are generally well-standardised across Europe, the largest variation has been observed for definitions of severe CDI [13]. There is some muddling of concepts in surveillance systems as some severity definitions include symptoms indicative of severe colitis of the gut at time of diagnosis, while other definitions include outcomes of CDI (e.g. mortality). The Dutch Sentinel Surveillance uses both severity criteria and distinguishes 'severe' CDI (i.e. bloody diarrhoea and/or pseudomembranous colitis, and/or diarrhoea accompanied by dehydration and/or hypoalbuminemia, and/or fever with leucocytosis) from 'complicated' CDI (leading to IC admission and/or surgery for CDI, or death). The ESCMID definition for 'severe' CDI [2] was adopted by The European Surveillance of CDI but labelled as 'complicated' CDI. Reporting complication rates of CDI conceivably contributes to the readiness to prioritise and improve CDI treatment and infection prevention control at a local and (inter)national level. However, use of an additional definition for the severity of CDI at diagnosis as in the Netherlands provides the opportunity to use the same definition for treatment decisions improving patient care.

OUTBREAK DETECTION

Detection of CDI clustering in space and time can curtail transmission of CDI e.g. by indicating shortcomings in infection prevention control and is one of the purposes of surveillance for CDI at an institutional level [35, 54]. There is no clear definition for a CDI outbreak. Outbreak definitions are usually based on the detection of ≥2 related CDI in a locally defined time period [2], or a relative or absolute increase of the CDI incidence rate [44]. In the Netherlands, we suspect an outbreak if ≥2 cases of the same PCR ribotype occur on one ward of a healthcare facility or if the incidence rate is higher than the background incidence (no clear cutoff level) [55]. Ward-based outbreak definitions are practicable but probably insufficient due to the fact that hospitals have a high flow of patient transfers between wards and procedural diagnostic locations. A recent paper from the United States illustrated the usefulness of 'network graph displaying' of such patient transfers registered by electronic health records tracking patients in space and time during admission [56]. On average, CDI patients moved to four different hospital locations between CDI diagnosis and discharge. The analysis

revealed the hospital location linked to increased risk of CDI and urged improvement of cleaning practices for that particular location [56]. Yet, the model assumptions on contamination of the hospital environment of *C. difficile* need local confirmation and molecular typing should confirm the transmission paths as found by spatial analysis. A Dutch study describing the largest reported ribotype 027 outbreak so far, showed that 25% of the CDI patients were transferred ≥3 times and the number of ward transfers was associated with the risk of CDI [57]. Hence, ward-based outbreak detection will therefore miss out other ways of CDI transmission important for infection prevention control. Monitoring hospital-wide incidence rates of CDI to detect outbreaks does not have this disadvantage but requires valid cutoff levels with sufficient sensitivity to detect smaller outbreaks [54]. Reliance on manual review of microbiological records by infection control personnel and epidemiologists may lead to errors [58]. In the recent years, spatial scan statistics [29, 59] were developed for automated outbreak detection not requiring pre-set cutoff levels. In Chapter 5 and 6 we introduced these models to search for spatial clustering beyond the hospital setting, but these models have been applied for hospital outbreak detection by others – using the same freely available software [58]. The models have several advantages, e.g. correction for random variability, time-trends, seasonality, and multiple testing [29, 54, 60]. Use of the models is feasible and some of its software is freely available, which enables its global application (also in countries with limited resources). To conclude, CDI outbreak detection should not be restricted to onward transmission but should be generated by locally validated models with clear thresholds, confirmed by molecular typing.

SAMPLING POPULATION FOR SURVEILLANCE

ACUTE CARE HOSPITALS

The ECDC European Surveillance of CDI (Chapter 3) as well as the Dutch Sentinel Surveillance system (Chapter 4 and 5) target acute care hospitals. Ideally, for optimal outbreak detection, all hospitals participate in surveillance. However, a representative sample of institutions in surveillance suffice to monitor trends and reduce the efforts and costs of surveillance. The ECDC European Surveillance of CDI has no requirements of hospital sampling at a national level and encourages participation of every willing hospital. The Dutch Sentinel Surveillance system targets a geographically representative subset of participating hospitals and de-links initiation of surveillance from outbreak control. Sampling of healthcare facilities for sentinel surveillance has been described by the WHO [61]. Yet, both methodologies are prone to selection bias. In general, the 'best' sampling strategy depends on the main aim of the surveillance system. For valid national estimates on the burden of disease, regular epidemiological methodologies can be applied (aiming for high representativeness of the obtained data). However, if the main goal of surveillance is rapid detection of outbreaks or other deviations from the

background epidemiology for inciting additional infection control interventions, healthcare network modelling may be preferable to sample hospitals for participating in surveillance [34]. This model uses a 'movement matrix' describing direct and indirect transfers between hospitals and subsequently simulates transmission of an infection within that network. Hospitals with the lowest detection time of the first infection are prioritised for participation in surveillance [34]. Yet, since both the Dutch Sentinel Surveillance and European Surveillance of CDI primarily aim to estimate the incidence and burden of CDI in the Netherlands and Europe respectively, regular epidemiological methodologies can be used to sample a subset of hospitals with a balanced population in relation to general patient characteristics, CDI risk factors, clinical course and outcome, and medical practices compared to all hospitals [62].

OTHER POPULATIONS

Expanding CDI surveillance to long-term care facilities (LTCFs) as in the United States [63] should be considered for several reasons. LTCF residents have a high risk for CDI and liaise *C. difficile* transmission [54, 63]. In the Netherlands, several persisting ribotype 027 outbreaks occurred in the past. One of these outbreak occurred in one hospital and several LTCFs in the Southern part of the Netherlands between 2008-2011 [64]. Of LTCF residents, 23% of the patients tested positive for CDI and ribotype 027 was isolated from 58% of all samples were submitted for PCR ribotyping. MLVA proved clonal genetic relatedness of 027 strains in LTCF and hospital patients. Modelling of transmission of antimicrobial-resistant pathogens within healthcare networks of hospitals and LTCFs in the Netherlands illustrated that extension of surveillance to LTCFs could accelerate detection of outbreaks and support national infection control [65]. CDI surveillance in LTCFs could urge improved antibiotic stewardship and infection control in these settings, improving patient safety. The number of CDI in LTCF residents is significant in the United Sates, about a quarter of all CDIs is found in LTCF patients [63]. The high burden of CDI in LTCFs in the United States was already suggested by the high number of LTCF-onset CDIs in hospital patients (36%) [14]. Results of Dutch Sentinel Surveillance indicate that only 5% of the healthcare-onset CDIs are related to LTCFs in the Netherlands (Chapter 5). In the European CDI surveillance pilot, LTCFs were reported to be the origin of infection for 1% of the patients (Chapter 3). These numbers suggest that the burden of CDI in LTCF in European countries might be much lower than in the United States and CDI surveillance is these settings would be excessive. In our experience, collection of CDI surveillance data in LTCFs in the Netherlands is challenging. Data collection of resident information and institutional parameters, as well as laboratory testing of diarrhoeic residents, is not part of daily routine (except for outbreaks) due to a lack of time and resources. Therefore, CDI surveillance data of LTCFs is very likely to be prone to bias due to underdiagnosis (much more than in hospitals) and missing data. Further research (e.g. large point-prevalence studies) is needed to indicate the burden of CDI in LTCFs and to indicate if implementation of CDI surveillance

in LTCFs outweighs the additional efforts and costs. In the Netherlands, we have initiated an ongoing repeated point-prevalence study on CDI and multidrug-resistant organisms in 2016-2017 in collaboration with the national reference laboratory from Ireland. Moreover, the National Institute of Public Health (RIVM) incorporated microbiological detection of colonization of toxin-producing *C. difficile* in a cross-sectional prevalence survey of the surveillance network for HAIs in nursing homes (SNIV) once [66].

Continued CDI surveillance in the community setting is less often suggested, although being common [30, 67, 68]. According to the 'One health' approach further efforts are needed to link human and animal surveillance systems (see *Control of CDI beyond healthcare-facilities; a one health approach*) [69]. Community-based laboratory surveillance of CDI could indicate the currently unidentified time-trend of CA-CDI in the Netherlands, and would complement hospital-based surveillance. Yet more awareness of general practitioners and revision of the national guideline for 'Acute diarrhoea' is needed before introducing community-based laboratory surveillance in the Netherlands, since only 40% of the CDI patients in the community setting are diagnosed [30]. Surveillance of environmental sources is not recommended at present, but would be very useful to detect the attributable portion of these sources for human CDI [54].

MICROBIOLOGICAL ASPECTS

HOW TO STANDARDISE LABORATORY DIAGNOSTICS FOR CDI

In Chapter 2 of this thesis, we have demonstrated the variety of laboratory diagnostics for CDI in Europe. In total, 19% and 46% of the ECDIS-Net laboratories used optimal diagnostics in 2011 and 2014, respectively. These percentages are probably an overestimation of the use of optimal diagnostics in all European laboratories caused by selection bias. Still, our findings accorded with a large European study indicating that 32% and 48% of the European laboratories used optimal diagnostics in 2010-11 and 2012-13, respectively [6]. Of all European hospitals (n=117) that participated in start-up ECDC European Surveillance of CDI in 2016, 70% used an optimal diagnostic algorithm [16]. In the Netherlands, 54% of the hospitals participating in Sentinel Surveillance used optimal CDI diagnostics in 2016-17 [70]. In Chapter 2 and 3, we stressed the need for further optimisation of laboratory diagnostics for CDI to improve clinical care, and s sensitivity and specificity of surveillance. Results of ECDIS-Net informed the ESCMID Study Group for *C. difficile* to revise their diagnostic guideline for CDI contributing to optimisation and standardisation of CDI diagnostics [71]. We anticipated that the updated guideline would end the debate on optimal laboratory diagnostics engaged in ECDIS-Net. Yet after publication of the updated guideline in 2016 [7] ongoing debate focussed on the importance of detecting free *C. difficile* toxins in the faeces compared to toxigenic *C. difficile* [72]. The ECSMID diagnostic guideline

provides the option of performing a third test (toxigenic culture or NAAT) of GDH EIA positive (step 1) but toxin A/B EIA negative (step 2) samples [7]. Patients with toxigenic *C. difficile* but without detected toxins in the faeces (potentially excretors and not CDI) appear to have a similar 30-day mortality risk compared to *C. difficile* negative patients, whereas patients with *C. difficile* toxins in the faeces have an increased mortality risk [73]. Yet, symptomatic patients with toxigenic *C. difficile* are as infectious as patients with *C. difficile* toxins in the faeces [48]. Besides the clinical consequences, whether or not to approve patients without detected toxins in the faeces as having CDI will have an impact on surveillance outcomes. Hospitals using algorithms that detect toxigenic *C. difficile* have higher incidence rates that those detecting free *C. difficile* toxins in the faeces (adjusted Incidence Rate Ratio 1.09) [72]. As the current European guideline did apparently not end the debate on optimal laboratory diagnostics for CDI, additional efforts are needed for standardisation. Probably the most viable option is to adopt one of the algorithms recommended by ESCMID on a regional or national level (incorporated in national guidelines and surveillance protocols), and to explore statistical strategies to remove residual bias caused by diagnostic variability for benchmarking [72]. Additionally, the role of free toxin detection in the faeces in CDI diagnostics needs further investigation, including the option to use of NAAT cycle thresholds predictive for the presence of free toxins [74].

HOW TO ADJUST CDI INCIDENCE RATES FOR DIAGNOSTIC VARIABILITY

Several statistical strategies could be explored to remove bias caused by diagnostic variability for benchmarking of CDI incidence rates. Illustrating the need of these strategies, some hospitals participating in Dutch Sentinel Surveillance consider their high CDI incidence rate compared to other hospitals to result from their highly sensitive diagnostic algorithm. Hospitals with a higher burden of CDI might be more aware and motivated to optimise their diagnostic capability exemplifying the chicken and egg situation. In Chapter 3 of this thesis, we stated that incidence rates derived from CDI surveillance systems should be adjusted for '*key factors related to sampling and testing methods*', i.e. by use of a standardized infection ratio (SIR) [75]. A SIR is a summary statistic to express the relative incidence of CDI compared to a reference population (e.g. national or European estimate) adjusted for biasing factors. For clarification, the proposed biasing factors in Chapter 3 do not relate to the patient population at risk (like used for Standardized Mortality Rates in Dutch hospitals) but to the chance of a CDI to be accurately detected and included for surveillance estimates. So how should such a SIR be calculated? The simplest approach would be to use a formula that replaces the sensitivity and specificity of the applied algorithm by the sensitivity and specificity of the 'reference' algorithm [76]. However, other factors (e.g. clinical awareness) that bias estimations of the CDI incidence cannot be incorporated in this formula. One multi-country study in Europe illustrated that three factors most significantly bias CDI incidence rates, i.e. diarrhoea sampling frequency, testing rate and the laboratory diagnostic algorithm [77]. Except for the number of all

tested diarrhoeal samples, these data are incorporated in European Surveillance of CDI (Chapter 3) and Dutch Sentinel Surveillance [70]. A hindrance to include the three factors in a formula is the variety of applied laboratory diagnostic algorithms and uncertainty how to classify all these. In Chapter 3 and 4 while using data of Dutch Sentinel Surveillance, we adjusted for three categories of diagnostic algorithms that we considered to have the most contrast in terms of sensitivity. We found that use of NAAT as a primary test for CDI did influence the incidence of CDI in children, but their might be room for improvement. Another elegant approach is the use of place-specific risk-adjustment factors to adjust for diagnostic intensity, found by Finkelstein et al. for 'balanced' reimbursement of health care providers in the United States [78]. However, their approach requires data of migrating patients across states, and could be applicable within European countries or cross-border regions, but not for Europe. The use of a risk adjustment formulas seems more feasible though not fully accounting for all variations in diagnostic testing [79]. Further studies should validate the coefficients and other essential variables to be incorporated in risk adjustment formulas [79]. Above all, promotion of optimal and appropriate diagnostic testing may prevent bias in the first place [38].

USE OF MOLECULAR TYPING FOR SURVEILLANCE

In Chapter 2 and 3 as well as the current chapter of this thesis, we stressed the implementation and standardisation of molecular typing methods for surveillance of CDI (e.g. for detecting local transmission and outbreaks and discrimin ating relapse and reinfection of CDI), and demonstrated the improving typing capacity for CDI in Europe. A sequel survey in 2017 indicated further expansion of the capacity for capillary PCR ribotyping (25/32 European countries) and the use of Multilocus Variable Tandem-repeats Analysis (MLVA) by 15 countries [16]. The advantages and disadvantages of PCR ribotyping have been discussed elsewhere [10, 54]. In summary, PCR ribotyping results can exclude the possibility of transmission when isolates are unrelated [54]. Yet, about 1:10 randomly chosen pairs of isolates has the same PCR ribotype by chance [54]. The use of capillary PCR ribotyping prevents some of the difficulties of conventional PCR ribotyping (e.g. improved discriminatory power and transfer of typing data) and was internationally standardised [11]. PCR ribotyping is useful for recognition of new emerging types, but always requires clinical alertness and knowledge. For example, PCR ribotypes evolving from 'outbreak-related' clades are not recognisable by their ribotype quotation (e.g. 826) [55] but can have the same properties and requ ire enhanced control measurements. Despite the relevance of PCR ribotyping as described in this thesis, there is only one trial performed that showed that 10% of the ribotyping results had a direct effect on infection control (and 45% for MLVA results) [80]. The use of molecular typing for discriminating false-positive from real CDI outbreaks was underlined by another study [81]. More discriminatory typing methods, such as MLVA as applied in Chapter 4 of this thesis and whole-genome sequencing can indicate transmission even when epidemiological links

are undetected [54]. In one study, use of MLVA led to a 95% similarity of outbreak detection compared to whole-genome sequencing [82]. Use of MLVA in Chapter 4 led to the interesting finding that that three fourths of the ribotype 265 strains isolated from children and adults were genetically related but did not elucidate transmission paths. This may have resulted from incomplete sampling however also illustrated the need for linked enhanced epidemiological data on previous (healthcare) exposures and validation of MLVA for other ribotypes than 027 and 078. A secondary outcome of our study was the knowledge that rapid exchange of a capillary PCR ribotyping peak patterns with other national reference laboratories for *C. difficile* provided useful information to contextualize research findings.

The added value of molecular typing data for direct patient-care remains questionable. Genotypes of *C. difficile* have been causally linked (but not always [83]) to patient mortality [84]. In chapter 5, we again demonstrated the increased mortality risk of patients with ribotype 078 but we did not find an increased mortality risk for ribotype 027/176 patients in Chapter 3. Assessing the 'virulence' of specific genotypes is troubled by confounding host factors [83] and absence of standardised control group (but a mix of other genotypes with variable virulence factors). Yet, whole-genome sequencing consistently showed that ribotype 027 was associated to higher odds of patient-patients transmission compared to other types [46, 48]. These results endorse the procedures of the Dutch national reference laboratory to directly notify hospitals after isolation of *C. difficile* ribotype 027. This type should be referred to as 'outbreak-associated' rather than 'hypervirulent'.

In conclusion, the use of molecular typing for CDI surveillance is vital but the limited resources and budget reductions in several countries (Chapter 2) force the need for well-targeted application of molecular typing in epidemiological surveillance systems, including more expensive and complex typing methodologies (e.g. whole-genome sequencing) needed for improved outbreak detection and source tracking of CDI. Additionally, the performance of these methodologies need a critical eye [30] and linkage with epidemiological data remains essential.

VALIDATION AND EVALUATION OF SURVEILLANCE SYSTEMS

The validity of a surveillance system needs to be periodically evaluated in relation to its goals (e.g. outbreak detection) [85]. The most well-established framework to systematically evaluate surveillance systems was published by the CDC in 2001 [62]. The CDC framework focusses on public health surveillance systems in general and comprehends ten evaluation attributes (simplicity, sensitivity, data quality, flexibility, acceptability, positive predictive value, representativeness, timeliness, stability and usefulness) [62, 86]. Application of the framework needs prioritising and tailored use of the ten attributes for each surveillance system evaluation [62, 85]. Surveillance validation studies for CDI and HAI in general are rarely published [87, 88]. Moreover, there are no clear cutoff levels of acceptable

sensitivity and specificity for CDI surveillance [89]. In Chapter 3 of this thesis, we (partly) assessed the attributes simplicity, data quality, acceptability and usefulness of the CDC framework for Surveillance of CDI and stated that national and European Surveillance of CDI should be accompanied by validation studies. Most important evaluation attributes for CDI surveillance as highlighted earlier in this chapter are; data quality (intrinsic validity), representativeness and surveillance sensitivity and specificity. The intrinsic validity of collected surveillance data is of crucial importance for benchmarking of surveillance outcomes [89, 90]. The intrinsic validity of a surveillance data can be undermined by inter-hospital and/or international variability of the implementation and performance of a surveillance system leading to 'data quality bias' [31, 36]. ECDC has illustrated the use of point-prevalence studies for validation of HAI surveillance to evaluate data quality, as well as surveillance sensitivity and specificity [88]. Warranting representativeness of surveillance is of particular importance while designing (see Sampling of healthcare facilities for Surveillance systems) [88]. Other options include e.g. comparison of different (electronically derived) datasets and capture-recap ture methodologies [85]. Representativeness of surveillance data should ideally be verified by comparison of 'minimal' surveillance data (Chapter 3) of the complete hospital population, and enhanced surveillance data of a subset of the hospital population. In view of the increased interest in HAI incidence rates for differentiating healthcare safety between hospitals [36, 91] it is likely that reporting of hospital incidence rates of CDI will become normative in the coming years in the Netherlands and other European countries and these data can be used for validating the representativeness of (Sentinel) CDI surveillance.

IMPLICATIONS FOR INFECTION PREVENTION CONTROL

OPTIMIZING CONTROL OF CDI WITHIN HEALTHCARE-FACILITIES

CDI remains one of the most prevalent healthcare-associated infections in the United States and Europe despite the use of enhanced infection prevention control over the last decade [92, 93]. These infection prevention control measures include environmental disinfection, antibiotic stewardship and bundled interventions, usually intensified during outbreaks [94, 95]. Whereas outbreak control is achievable by using these measures, reduction of the endemic incidence of CDI remains challenging and acquires additional efforts. The CDC published an expanded roadmap for elimination of HAI including CDI in hospitals and LCTF in the United States in 2013 [96]. Notably, CDI was the only HAI that did not met the prior target (30% decrease in 2008-2013). To achieve subsequent targets, research projects to improve the knowledge of CDI transmission and a toolkit to support reduction of CDI by antibiotic stewardship were realised [96]. In fact, a recent meta-analysis showed that antibiotic stewardship can decrease CDI incidence rates with 32% [97]. Especially reduction of the use of fluoroquinolones have been linked to a decrease of CDI in England [53]. CDC currently aims for a 30% reduc-

tion of the standardised infection ratio of CDI and hospitalizations due to CDI in 2015-2020 [98]. The ECDC has not (yet) set targets for CDI reduction in Europe, but has several activities to support antibiotic stewardship [99] and infection prevention control of CDI, in addition to the initiation of European Surveillance of CDI and support of microbiological molecular typing for CDI [15]. National guidelines for infection prevention control of CDI in Europe are accessible at the website of ECDC, illustrating that most of these are out-dated as for the Netherlands. The ESCMID/ECDC infection prevention guideline for CDI [100] is currently updated and will be published in 2018. Model-based studies are useful to anticipate on the effectiveness and target population of (novel) strategies to reduce CDI with in healthcare facilities [101]. For example, one model-based study evaluated the effectiveness of vaccinations under development for CDI in a dynamic hospital setting (43% reduction) [101]. Decontamination of food was considered to have a minimal effect on transmission of CDI in another study exploiting modelling [102]. In a comprehensive model-based study, the two most effective strategies to reduce the incidence of hospital-onset CDI were daily cleaning with a sporicidal disinfectant and screening at admission, endorsing the importance of asymptomatic carriers for transmission of CDI in the hospital setting [103]. Isolating asymptomatic carriers of *C. difficile* at admission in a quasi-experimental study from Canada prevented 62% of the expected CDI [104]. Another model-based study confirmed the effectivity of screening at admission, but also illustrated this approach needs to complemented by bundled interventions [105]. Currently, a multicentre study is performed to disentangle the risk-factors of asymptomatic carriers of *C. difficile* at hospital admission and their role in hospital transmission of CDI in the Netherlands.

CONTROL OF CDI BEYOND HEALTHCARE-FACILITIES; A ONE HEALTH APPROACH

As mentioned before, growing evidence supports the concept that control of CDI also requires evaluation and management of *C. difficile* reservoirs beyond healthcare-facilities. The burden of CDI in the community is considerable (Chapter 6) especially in view of the number of patients that do not visit their general practitioner with diarrhoea or submit a stool sample to search for infectious causes of diarrhoea [106]. There are no studies that tested infection prevention control strategies outside healthcare facilities. However, there has been an increased interest in studying reservoirs and transmission paths in the community setting [107]. As said, reservoirs include 'one or more epidemiologically connected populations or environments in which the pathogen can be permanently maintained and from which infection is transmitted to the target population (here: humans susceptible for CDI)' [108]. Theoretically, blocking tactics of exposure to CDI reservoirs in the community – especially for high-risk patients – could result in further control of CDI. In practice, this would be easier to achieve if contributing source populations are piglets or pigs and not asymptomatic infants and adults carrying

C. difficile as suggested in the previous paragraph. Besides, the feasibility of such a blocking tactic will depend on the detected transmission paths (e.g. meat consumption). It remains an anomaly that the community is not particularly interested in the rates of *C. difficile* contamination of food as for Salmonella species, given the same rates of these infections in the community. This is probably caused by the fact that CDI is still known as an healthcare-associated infection and is expected to change if the news on community-acquired CDI pervades in the community. Besides, healthy community residents that do use antibiotics or other drugs might consider themselves erroneously not at risk for CDI at all. The question is if that is correct. In the scientific community, *C. difficile* is now recognised as one of the pathogens needing a 'One Health' approach. By this approach, three health domains (human, animal, and environmental) are evaluated to provide an comprehensive understanding of the infection and effective strategies for infection control [109]. A proposed One Health framework cites 'directed acyclic graphs', 'risk models' and 'geographical information systems' (GIS) as important study methodologies [109]. In Chapter 6 of this thesis, we created a direct ed acyclic graph to visualise the relation between livestock exposure and CDI. Besides that, we introduced the use of geographical information systems viz. spatial scan statistics [110] in Chapter 5 and 6. Spatial clustering can trace infection sources whereas spatial and temporal connectivity between the reservoir and target population can trace (indirect) transmission paths [111]. In Chapter 6 we have elaborated on the limited application of spatial analysis in the field of *C. difficile* research and the conflicting results of published studies [60, 112, 113]. We called for further exploration of spatial settings (scale, patterns) and control data to construct spatial models for CDI. In future spatial analysis studies, discriminative molecular typing data and extensive epidemiological patient data should be integrated.

Conclusions

In Europe, including the Netherlands, a variety of laboratory diagnostics for CDI were used of which around half is suboptimal. Consequently, the burden of CDI in Europe has been underestimated for years [6]. Optimisation of CDI diagnostics improves clinical care, and sensitivity and specificity of surveillance. Epidemiological surveillance systems can endorse the use of optimal diagnostics for CDI and explore statistical strategies to remove residual bias. In the Netherlands, full adherence to European diagnostic guidelines should be strongly stimulated. After tackling the problem of underdiagnosis, future challenges relate to ascertainment of the role of free toxin detection in the faeces in CDI diagnostics and optimal testing criteria for CDI in children.

Surveillance data are used to direct and monitor the effect of local and national infection prevention control interventions, facilitate education of hospital staff, set priorities for research projects, and persuade local and national authorities to provide sufficient resources to combat CDI – as recommended by the WHO [3]. Facing the heterogeneity of European surveillance systems and a shortage of incorporation of microbiological molecular typing data [13], a standardised surveillance protocol was tested and proved to be feasible for countries with varying levels of implemented surveillance activities. Standardised European Surveillance of CDI is essential for benchmarking of CDI incidence rates (between facilities and countries) and burden estimations, and was initiated by ECDC in 2016 after completion of our study [16]. Application of capillary PCR ribotyping and more discriminatory molecular typing methodologies (e.g. whole-genome sequencing) for improved outbreak detection and source tracking of CDI should be well-targeted, given the limited resources and budget reductions in several European countries. European Surveillance of CDI would benefit from periodic evaluation and clinical validation studies, as well as incorporation of spatial data (carefully considering the sensitivity of such data).

In the Netherlands, national surveillance of CDI helps to improve local infection prevention in participating hospitals, controls the transmission of highly virulent types such as ribotype 027 and generated new hypotheses to understand and optimise the control of CDI. In this thesis, data of the Sentinel Surveillance for CDI – initiated in 2009 – was used to study (spatial) trends in the epidemiology of CDI in the Netherlands, in particular for children and the potentially zoonotic *C. difficile* ribotype 078. CDI among children did not increase, but we illustrated notable differences in the prevalence of specific ribotypes (265 and 027) compared to adults. This thesis relativized the role of pig farming to the incidence of PCR ribotype 078 in the Netherlands. There was no association between hospital incidence rates of ribotype 078 CDI and provincial pig farming density. No spatial clusters of community-onset CDI followed by hospitalisation were detected. Correspondingly, no spatial or temporal clusters were found by a community-based

study and the distribution of PCR ribotype 078 was not linked to specific geographical areas. Livestock exposure was not related to community-acquired CDI. These findings supported the hypothesis that CDI transmission in the community derives from widespread sources and not from localised environmental community point reservoirs, such as livestock farms. To identify the attribution of these reservoirs and transmission paths, 'One Health' studies incorporating highly discriminative molecular typing and modelling are needed.

To conclude, CDI should be designated as an urgent treat in European and Dutch healthcare as in the United States [114]. Future activities should focus on; i) improving of evidence-based infection prevention in hospitals, ii) evaluating the role of asymptomatic carriers in the transmission of CDI iii) provide full reporting of hospital incidence rates for CDI linked to indicators of antibiotic stewardship and patient safety, iv) application of more discriminatory molecular typing methodologies and model-based (spatial) studies for source tracking within and beyond the healthcare setting (using a 'One Health' approach).

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Dutch summary

Clostridium difficile infectie (CDI) is sinds het veelvuldig gebruik van antibiotica in de gezondheidszorg uitgegroeid tot de meest voorkomende zorg-gerelateerde infectie in Noord-Amerika en Europa. Bij patiënten met een verstoorde darmflora (hoofdzakelijk door antibiotica) kan de bacterie hevige diarree en soms een levensbedreigende darmontsteking veroorzaken door de vorming van 'toxines' (gifstoffen). In Nederlandse ziekenhuizen treden 3 CDI gevallen per 10.000 verpleegdagen op. Bij mensen met gezonde darmflora en afweer geeft de darmbacterie geen klachten. Circa 14% van de patiënten met een CDI sterft binnen 30 dagen na het stellen van de diagnose, waarvan 4% (deels) door de directe gevolgen van de darminfectie **(HOOFDSTUK 4 EN 5)**. *C. difficile* is een bekende oorzaak van diarree-uitbraken in ziekenhuizen en verpleeghuizen, maar ook buiten zorginstellingen is *C. difficile* alom in onze leefomgeving aanwezig. Uit recente studies blijkt dat meer dan de helft van de ziekenhuispatiënten met CDI de bacterie waarschijnlijk elders heeft opgelopen, maar het brononderzoek is zeer complex.

Sinds 2005 zijn een groot aantal Nederlandse zorginstellingen getroffen door CDI uitbraken van een meer antibioticaresistent *C. difficile* subtype dat zich vanuit Canada en de Verenigde Staten naar Europa heeft verspreid. Patiënten met CDI bleken ernstiger ziek te worden en vaker aan de infectie te overlijden dan voorheen bekend. Middels moleculaire typering werd dit subtype aangeduid als 'PCR ribotype 027'. In Nederland werd, net als in andere Europese landen, een nationaal referentielaboratorium opgezet ter ondersteuning van de infectiebestrijding middels moleculaire typering en surveillance onderzoek. In de loop van 2006 werd er een grote afname van PCR ribotype 027 waargenomen, alhoewel uitbraken bleven voorkomen. In 2009 voerde het referentielaboratorium een doorlopend nationaal surveillanceprogramma in een deel van de Nederlandse ziekenhuizen in ('Sentinel surveillance'). Hiermee werd beoogd het voorkomen van CDI in Nederland beter te monitoren en uitbraken sneller te kunnen opmerken en bestrijden.

Sinds de verspreiding van PCR ribotype 027 in Europa roept het 'European Centre for Disease Prevention and Control' (ECDC) op tot nationale surveillance in alle Europese landen. In 2011 bleken slechts 14 van de 31 Europese landen (45%) surveillance toe te passen en met beperkt gebruik van moleculaire typering. Berekeningen van de incidentie en ziektelast van CDI in Europa waren niet goed mogelijk door het gebruik van uiteenlopende en vaak suboptimale laboratoriumdiagnostiek-, typerings- en surveillancemethoden. Signalen van internationale uitbraken of verspreiding van subtypes met een toegenomen ziektelast waren hierdoor lastig te herkennen. In 2010 initieerde ECDC een vierjarig project genaamd 'European CDI Surveillance network (ECDIS-Net)' om de diagnostiek en

surveillance op Europese schaal te verbeteren. Het eerste deel van dit proefschrift omvat twee studies van ECDIS-Net om de (mogelijkheden tot) standaardisatie van laboratoriumdiagnostiek, typerings- en surveillancemethoden voor *C. difficile* te onderzoeken.

De laboratoriumdiagnostiek van CDI dient te bestaan uit het uitvoeren van meerdere testen in een 'algoritme'. Door één enkele test te gebruiken of een incorrecte combinatie van testen kan CDI worden gemist of juist onterecht worden vastgesteld. **HOOFDSTUK 2** van dit proefschrift beschrijft dat slechts 46% van de ECDIS-Net laboratoria in 2014 een optimaal algoritme gebruikte om CDI te diagnosticeren. In 2011 was dit percentage 19%. De studie gaf meer inzicht in de barrières voor het implementeren van optimale laboratoriumdiagnostiek in Europese laboratoria, welke werd gebruikt bij de totstandkoming van een nieuwe Europese richtlijn voor de diagnostiek van CDI in 2016. Surveillanceprogramma's kunnen het gebruik van optimale laboratoriumdiagnostiek stimuleren en statistische methoden toepassen om vertekening (bias) door diagnostische variabiliteit zo goed mogelijk te corrigeren.

Met moleculaire typering kunnen thans ongeveer 600 verschillende PCR ribotypen van *C. difficile* onderscheiden worden. Door het genetisch materiaal van *C. difficile* bij verschillende ziektegevallen te vergelijken, krijgt men inzicht in de verspreiding van de bacterie binnen een zorginstelling, tussen zorginstellingen of op grotere schaal. **HOOFDSTUK 2** laat zien dat de Europese capaciteit voor PCR ribotypering tussen 2011 en 2014 toenam van 65 tot 72%. De capaciteit voor een internationaal gestandaardiseerde vorm van PCR ribotypering met een verbeterd onderscheidend vermogen ('capillaire' PCR ribotypering) steeg van 23 tot 50%. Optimale toepassing van bestaande en verbeterde moleculaire typeringsmethoden is belangrijk, maar niet vanzelfsprekend door de beperkte middelen en bezuinigingen in verschillende Europese landen. De ontwikkeling en toepassing van nieuwe typeringsmethoden, zoals bijvoorbeeld 'whole-genome sequencing' met gebruik van nagenoeg het complete bacteriële genoom, vindt slechts in enkele laboratoria plaats.

ECDIS-Net leidde tot de realisatie van een gestandaardiseerd Europees surveillanceprotocol met ondersteuning van moleculaire typering, om de infectiebestrijding van CDI op grotere schaal te kunnen verbeteren. In **HOOFDSTUK 3** van dit proefschrift werd dit surveillanceprotocol getest in 14 Europese landen in 2013. Implementatie van het protocol was haalbaar in alle deelnemende landen met een acceptabele en bruikbare hoeveelheid data. Opvallend was het hoge aantal patiënten met symptomen van CDI bij ziekenhuisopname (49%) en de hoge prevalentie van PCR ribotype 027 (30%). Ook een andere studie toonde aan dat er nog steeds grote uitbraken van PCR ribotype 027 plaats vinden, met name in Oost-Europa. Na afronding van deze studie heeft het ECDC het Europese surveillanceprogramma van CDI verder geoptimaliseerd en toegankelijk ge-

maakt voor alle Europese landen. In 2017 namen ziekenhuizen in minstens 21 landen deel. Verdere aandacht is nodig voor verbetering van gebruikte surveillance definities, het verkrijgen van een representatieve populatie en het valideren van verkregen data.

In het tweede deel van dit proefschrift werden gegevens van het Sentinel surveillanceprogramma in Nederlandse ziekenhuizen en een patiënt-controle onderzoek in de huisartsenpraktijk gebruikt om veranderingen in de epidemiologie van CDI in Nederland te bestuderen. Hierbij lag de nadruk op recente ontwikkelingen, zoals de prevalentie van CDI bij kinderen, de verspreiding van *C. difficile* vanuit de vee/varkenshouderij of andere mogelijke bronnen van CDI buiten het ziekenhuis.

De afgelopen jaren werd er een toename van CDI bij kinderen in de Verenigde Staten gerapporteerd. Het Sentinel surveillanceprogramma registreert kinderen vanaf 2 jaar met CDI, omdat de betekenis van *C. difficile* bij kinderen jonger dan 2 jaar onduidelijk is. **HOOFDSTUK 4** van dit proefschrift beschrijft de analyse van klinische en microbiologische surveillancegegevens van kinderen met CDI in Nederlandse ziekenhuizen. Er bleek geen toename van het aantal CDI meldingen bij kinderen (3% van alle CDI) in 26 Nederlandse ziekenhuizen in 2009-2015. Ongeveer een derde van de kinderen had een ernstige infectie, maar slechts 3% had complicaties en geen van de kinderen stierf aan de gevolgen van CDI. Kinderen waren relatief vaak besmet met PCR ribotype 265 vergeleken met volwassenen (15 versus 1%). Een specifieke moleculaire analyse middels Multiple-locus variabele-number tandem-repeat analyse (MLVA) toonde aan dat driekwart van de ribotype 265-stammen van kinderen genetisch gerelateerd waren aan die van volwassenen. Ribotype 265 bleek bij navraag zeer zeldzaam of afwezig in verschillende landen ter wereld behalve in het naburige België (ook bij jonge kinderen). Het bleef onduidelijk hoe dit type zich kan hebben verspreid. Het uitbraak-geassocieerde PCR ribotype 027 werd niet gevonden bij kinderen. Deze verschillen behoeven verdere opheldering.

Na bestrijding van PCR ribotype 027 in Nederland ontstond er een hoge ziektelast van *C. difficile* PCR ribotype 078 (13% van alle CDI), wat mogelijk van dier op mens overdraagbaar is. Uit eerdere studies bleek dat een kwart van de geteste Nederlandse varkensboeren positief testte op ribotype 078 en dat varkens en hun omgeving vaak verontreinigd zijn met dit type. Er werden genetisch identieke bacteriën in varkens en mensen aangetroffen middels whole-genome sequencing. In **HOOFDSTUK 5** van dit proefschrift onderzochten we of een verhoogde dichtheid aan varkens leidt tot een hogere incidentie van ribotype 078 CDI in de daar gelegen ziekenhuizen. Er bleek echter geen verband tussen de ziekenhuisincidentie van ribotype 078 en de provinciale varkensdichtheid. Tevens zochten we naar geografische clustering van CDI ziektegevallen die opgenomen werden in het ziekenhuis maar voor opname klachten hadden gekregen. Er werden geen

geografische clusters gevonden. Patiënten met PCR ribotype 078 hadden wel een verhoogd sterfterisico in vergelijking met andere CDI patiënten (relatief risico 1.35) zoals eerder vastgesteld. Om verspreiding van CDI verder te onderzoeken zal dus nog breder moeten worden gekeken. Momenteel worden infecties in toenemende mate onderzocht in de context van een ecologisch systeem tussen mens, dier en omgeving; het zogenaamde 'One Health'-concept.

HOOFDSTUK 6 van dit proefschrift beschrijft onderzoek naar geografische clustering en omgevingsrisicofactoren (met name blootstelling aan vee) van CDI buiten het ziekenhuis. Hierbij hebben we gebruikgemaakt van data van een eerder afgerond patiënt-controle onderzoek in de huisartsenpraktijk. De hypothese was dat clustering van CDI buiten het ziekenhuis zou kunnen wijzen op nog niet eerder gedetecteerde omgevingsbronnen van *C. difficile*, zoals veehouderijen. Er werden echter geen geografische clusters gevonden en blootstelling aan vee verhoogde de kans op CDI niet (Odds Ratio 0.99). Mogelijk wordt vindt verspreiding van CDI dus plaats via wijdverspreide bronnen (zoals dragers van *C. difficile* zonder klachten) en niet door plaatselijke omgevingsbronnen. Momenteel loopt een nieuw onderzoek naar de risicofactoren en besmettelijkheid van dragers van *C. difficile* bij ziekenhuisopname.

CDI blijft vooralsnog de meest voorkomende zorg-gerelateerde infectie in Europa met een aanzienlijke sterfte. Hoewel de incidentie van CDI in Nederland al jaren stabiel is, blijven uitbraken voorkomen. Er zijn aanvullende maatregelen nodig om CDI verder te bestrijden, waarbij de belangrijkste aanbevelingen vanuit dit proefschrift zijn: i) verbetering van op onderzoek berustende infectiepreventie in ziekenhuizen, ii) het realiseren van optimale laboratoriumdiagnostiek voor CDI in alle ziekenhuizen, iii) meer aandacht voor de rol van dragers bij de verspreiding van *C. difficile*, iii) continue monitoring van de ziekenhuisincidentie van CDI in alle ziekenhuizen als indicator voor patiëntveiligheid, iv) toepassing van hoog-onderscheidende moleculair typeringsmethoden in een 'One Health' context om het brononderzoek van CDI te laten slagen.

Concluderend biedt dit proefschrift verschillende opties voor verbetering van surveillance en infectiebestrijding van CDI.

List of publications

van Dorp SM, Hensgens MPM, Dekkers OM, Demeulemeester A, Buiting A, Bloembergen P et al. Spatial clustering and livestock exposure as risk factor for community-acquired *Clostridium difficile* infection. *Clin Microbiol Infect. 2018; Epub Aug 1.*

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About the author

Sofie van Dorp was born in Overveen, the Netherlands on August 9, 1985. She completed her secondary school (gymnasium) education at the Kennemer Lyceum in Overveen in 2003. She studied Medicine at the Free University of Amsterdam from 2003-2010. In 2008, she did a 3-month research internship at the Anahuac University in Mexico City analysing data from a Social and Health Assessment survey among adolescents in Mexico City. She completed her training as a medical doctor with internships at the department of internal medicine and neurology in Paramaribo, and a public health clinic located in the Sipaliwini district of Surinam. Afterwards, she started working at the Rijnland Hospital in Leiderdorp at the department of internal medicine and followed a 4-month training at the IC unit. In 2012, she started her first research project on CDI as part of ECDIS-Net under the supervision of prof. E.J. Kuijper, medical microbiologist. During her PhD-research period, she expanded her work in ECDIS-Net and coordinated activities of the national reference laboratory for *C. difficile* in the Netherlands in cooperation with the National Institute for Public Health and the Environment (RIVM). To improve her research competencies, she followed several trainings at the department of Clinical Epidemiology at the LUMC. She enjoyed international collaboration with *C. difficile* researchers from all over Europe and other parts of the world, and presented her work at national and international conferences. Furthermore, she supported research projects on *C. difficile* and highly resistant microorganisms in nursing homes. In 2017, she worked at the department of clinical geriatrics at the Slotervaart Hospital in Amsterdam and started her training as a clinical geriatrician. She is married to Sebastiaan van Denderen and mother of two sons.

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