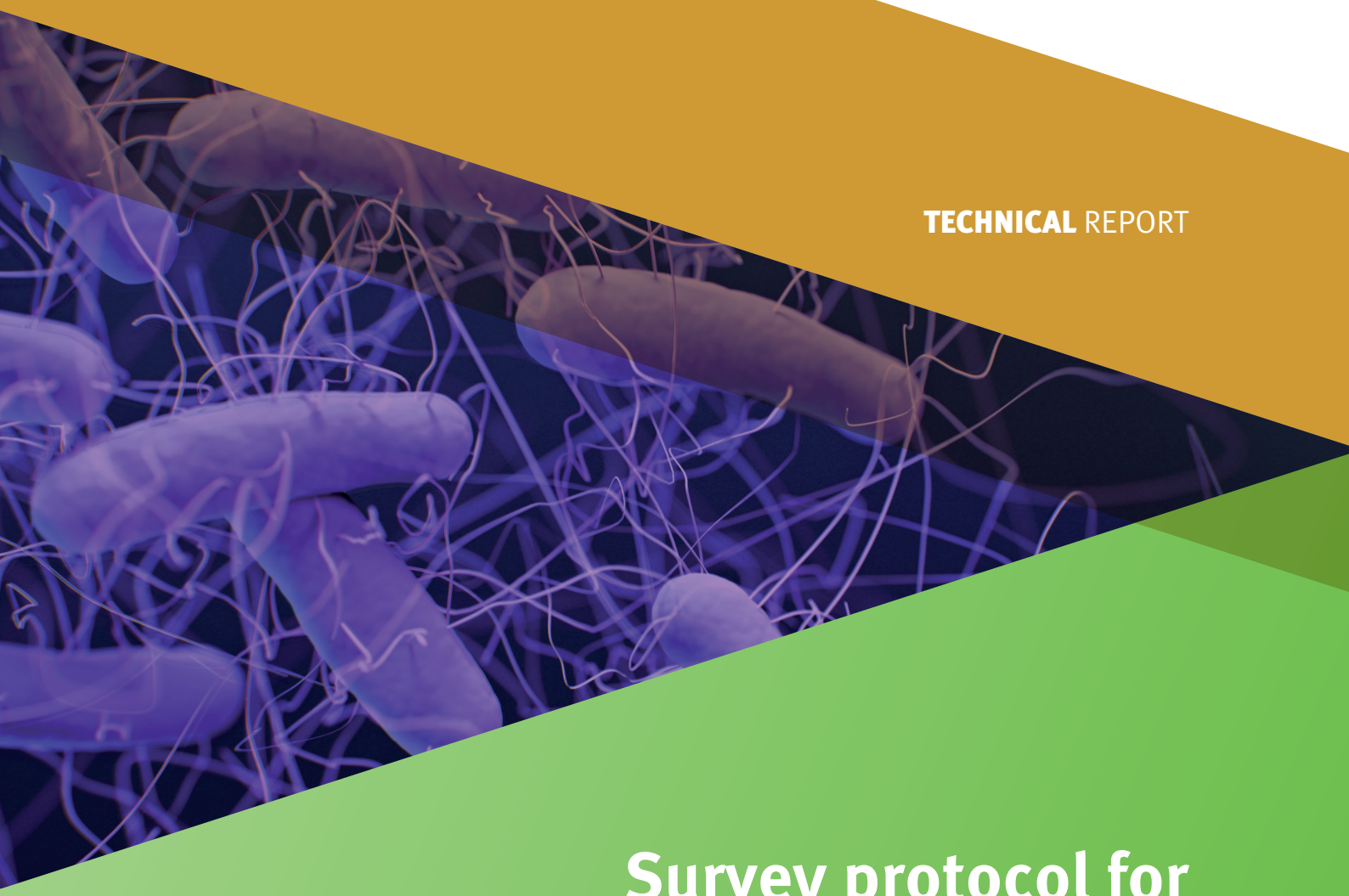


TECHNICAL REPORT

Survey protocol for whole genome sequencing of *Clostridioides difficile* isolates from tertiary acute care hospitals, EU/EEA, 2022–2023

www.ecdc.europa.eu

ECDC TECHNICAL REPORT

Survey protocol for whole genome sequencing of *Clostridioides difficile* isolates from tertiary acute care hospitals, EU/EEA, 2022–2023

This technical document of the European Centre for Disease Prevention and Control (ECDC) was coordinated and produced by Pete Kinross and Saara Kotila.

The draft document was reviewed in 2022 by the Operational Contact Points for Epidemiology, Microbiology, TESSy/IT data manager and/or Bioinformatics – Healthcare-Associated Infections *Clostridioides difficile* infection (OCPs for HAI-CDI) and/or the National Focal Points for Healthcare-Associated Infections from the following EU Member States: Czechia, Denmark, Finland, France, Hungary and the Netherlands.

Suggested citation: European Centre for Disease Prevention and Control. Study protocol for a survey of whole genome sequencing of *Clostridioides difficile* isolates from tertiary acute care hospitals, EU/EEA, 2022–2023. Stockholm: ECDC; 2024.

Stockholm, May 2024

ISBN 978-92-9498-698-6
doi: 10.2900/46926
Catalogue number TQ-05-24-212-EN-N

Contents

Figures

Tables

Abbreviations

CDI	*Clostridioides difficile* infection
cgMLST	Core genome multilocus sequence typing
EU/EEA	European Union/European Economic Area
HAI	Healthcare-associated infection
NFP	National Focal Point
NMFP	National Microbiology Focal Point
NSC	National Survey Coordinator
OCP	Operational Contact Point
RT	Ribotype
SFTP	Secure file transfer protocol
SNP	Single-nucleotide polymorphism
wgMLST	Whole-genome multilocus sequence typing
WGS	Whole genome sequencing

Executive summary

This protocol describes a survey undertaken to acquire a snapshot of the distribution of *Clostridioides difficile* strains in tertiary acute care hospitals in the European Union/European Economic Area (EU/EEA) in 2022–2023. This activity supports subsequent risk assessment by enabling the potential extrapolation of strain-specific findings from subnational, national or multi-country activities to the EU/EEA. The results could contribute to ongoing ECDC CDI surveillance activities, scientific studies or in silico analyses. ECDC will also consider the experience of this project during any upcoming update of the 'ECDC strategic framework for the integration of molecular and genomic typing into European surveillance and multi-country outbreak investigations (2019–2021)' [1].

National Focal Points (NFPs) for healthcare-associated infections (HAIs) and/or National Microbiology Focal Points (NMFPs) were invited to initiate national participation in this survey by designating National Survey Coordinators (NSCs). This protocol describes how NSCs were to recruit tertiary acute care hospitals to report *C. difficile* isolates from *C. difficile* infection (CDI) cases. The survey had funding for 990 samples/isolates (DNA or bacterial pellets), which equates to 0.5–0.9% of the estimated annual number of healthcare-associated *C. difficile* infection (HA CDI) cases in the EU/EEA.

Participating laboratories submitted DNA/pellets for *C. difficile* isolates to an ECDC laboratory contractor (Eurofins Genomics (FWC ECDC/2019/041)). The contractor was obliged to send whole genome sequencing (WGS) data back to each participating laboratory within four weeks and to store the data for three months. Participants were welcome to use the WGS data they received for their own purposes (e.g. public domain outputs). ECDC will publish the full dataset in the European Nucleotide Archive (ENA) to accompany an article co-authored with participating countries and submitted to an open access, peer-reviewed journal. Subsequently, ECDC will publish an ECDC Technical Report that contains all results from the survey and a link to the ENA database.

The preferred *C. difficile* isolates for hospitals to submit were HA CDI cases imported from another hospital (excluding recurrent cases, if feasible) or otherwise consecutively detected HA CDI or CDI cases. Tertiary acute care hospitals were the focus of this survey because they commonly receive more patient transfers than other hospital types. The number of tertiary acute care hospitals that NSCs were to recruit to this survey was proportional to the estimated number of these hospitals per country. Accordingly, the sample size calculation recommended that 50% of EU/EEA countries recruit one to five hospitals, 25% of countries recruit five to seven hospitals, and the three countries with the largest populations recruit 29–47 hospitals (Table 2). The sample size calculations and available budget for this survey implied that participating hospitals in 50% of EU/EEA countries would report one to six CDI cases per hospital, with another 40% of countries reporting six to seven cases per hospital and the remaining 10% reporting more than six cases per hospital. Countries could consider assigning one laboratory/hospital as a central coordinator for this survey. The recruited tertiary acute care hospitals may be referred to nationally as 'central' or 'regional' hospitals, such as large university teaching hospitals.

The core surveillance period for this survey was January to May 2022. Hospitals that preferred to report consecutive HA CDI or CDI cases could also report retrospectively from May 2021 onwards or prospectively up to April or May 2022. We suggested that participating hospitals store eligible samples if they exceeded the permitted maximum, as it was likely that not all countries would participate in the survey and so more samples might be permitted at a later date. The first deadline for participating laboratories to submit samples to Eurofins was 21 April 2022. By 28 April, ECDC was to inform each NSC how many more samples their hospitals could submit to Eurofins. Thereafter, if authorised by NSCs, participating laboratories could submit bacterial pellets to Eurofins up to 24 May or DNA up to 31 May 2022. In 2023, some participating countries were given another opportunity to submit samples, as more budget became available.

1 Introduction

Clostridioides difficile infection and typing in the EU/EEA

Burden of *Clostridioides difficile* infections in Europe (pre-2020)

Clostridioides difficile infections (CDI) have a strong impact on the EU/EEA population. ECDC estimates that in 2016 and 2017 there were roughly 189 526 (approximate range: 105 000–341 000) cases of healthcare-associated *C. difficile* infection (HA CDI) in acute care hospitals annually, with an estimated 7 419 (approximate range: 4 100–13 300) fatal HA CDI cases per year with CDI as a possible contributing factor [1,2]. From 2009 to 2013, HA CDI had the fourth highest burden of any single infectious disease under surveillance at the European level in terms of disability-adjusted life years (DALYs) [3]. *C. difficile* strains have different outbreak potential and varying risk of poor infection outcomes (e.g. recurrence or death) [2,4-6]. Recurrent CDI cases have a higher risk of a fatal outcome, require an approximately 3.6 times longer hospital stay and incur approximately 3.6 times higher direct and indirect healthcare costs [7]. ECDC also estimates that there were roughly 18 118 (approximate range: 500–48 200) CDI cases in long-term care facilities (LTCFs) annually in 2016 and 2017 [1].

PCR ribotype distribution of *Clostridioides difficile* isolates in European healthcare

In 2008, the ECDIS study characterised 395 isolates from 73 hospitals in 26 EU/EEA countries, identifying 65 different PCR ribotypes (RTs) [8]. Since then, several multi-country studies in Europe have also incorporated whole genome sequencing (WGS) data [4,5].

Typing of *Clostridioides difficile* isolates in European healthcare

From 2010 to 2014, the ECDC ECDIS-Net project supported development of a reference *C. difficile* strain collection for more than 100 of the most common PCR RTs (referred to as the 'ECDC-Leeds-Leiden-Brazier' collection). The ECDIS-Net (2010–2014) and ECDIS-Net-2 (2016–2020) projects surveyed European countries in 2010, 2014 and 2018 to map the use of *C. difficile* subtyping methods [9,10]. The 2018 survey of 384 laboratories in 31 European countries identified that the most common routine typing method for local laboratories was PCR ribotyping, used by 85% of laboratories [10].

Clostridioides difficile whole genome sequencing data: analysis and use

WGS data have the potential to support the resolution of relapses/reinfections in recurrent CDI, the assessment of hospital infection prevention and control (IPC) performance, and the mapping of the evolution of notable strains [11].

Currently, there is no broad consensus in Europe regarding the preferred analysis scheme [11,12]. A recent study by Baktash A et al. [12] compared the discriminatory power of core genome multilocus sequence typing (cgMLST), whole genome multilocus sequence typing (wgMLST) and single nucleotide polymorphism (SNP) analysis for 100 of the previously mentioned reference RT strains. Of the 100 common RTs, 82 were distinguishable by cgMLST (SeqSphere+) at a threshold of six allelic differences. Among the remaining 18 common RTs, some RT016, RT027 and RT036 strains grouped with some RT176 strains at this threshold (i.e. all Clade 2, ST1 strains). Reducing the threshold increased the discrimination [12]. Indeed, cgMLST has been found to have a similar performance to wgMLST and SNP analysis for discrimination of RTs other than Clades 2 and 5 [12,13]. For outbreak settings, it may be preferable to have a threshold of three inter-allelic differences by cgMLST [12] or up to three single-nucleotide variants for SNP analysis [12,14].

Selected ECDC activities relevant to the survey

ECDC strategic framework for integration of genomic typing (2019–2021)

The 'ECDC strategic framework for the integration of molecular and genomic typing into European surveillance and multi-country outbreak investigations (2019–2021)' proposed multi-country *C. difficile* outbreak investigation in 2021: 'In general, Member States should receive support for the gradual use of sequence-based typing so they can participate in joint response and surveillance operations with EU/EEA Member States' [15].

ECDC integrated surveillance of *Clostridioides difficile* epidemiology and microbiology in acute care hospitals (2016–2021)

In 2016, ECDC initiated coordination of CDI surveillance using a protocol developed and piloted by the ECDIS-Net project (2010–2014) that integrates epidemiological and microbiological data (e.g. RTs) [16]. According to this protocol, from 2016 to 2021, 24/30 (80%) countries in the EU/EEA and the United Kingdom reported CDI surveillance data to ECDC for more than 3 000 hospitals for surveillance periods of 3–12 months. In 2017, 9% of all acute care hospitals in the EU/EEA participated, with 18% coverage in the participating countries (see 'ECDC Surveillance Atlas of Infectious Diseases' [17] and [18,19]).

From 2016 to 2021, 15 countries used the 'enhanced option' from the protocol (i.e. reporting RTs for more than 900 hospital surveillance periods). The dataset contains 221 PCR RT names that appear at least twice, from 8 859 CDI cases. This allows for multivariable analyses to identify RTs associated with poorer infection outcomes. These include RTs that are known to be virulent (e.g. RT027, RT001, RT078), as well as RTs with virulence suggested by smaller studies or their genotype (e.g. RT176) [20,21]. RT176 isolates share Clade 2 and ST1 with hyper-virulent RT027. ST1 strains have been increasingly prevalent among the RTs detected in the central and eastern regions of Europe [18,19,21-23].

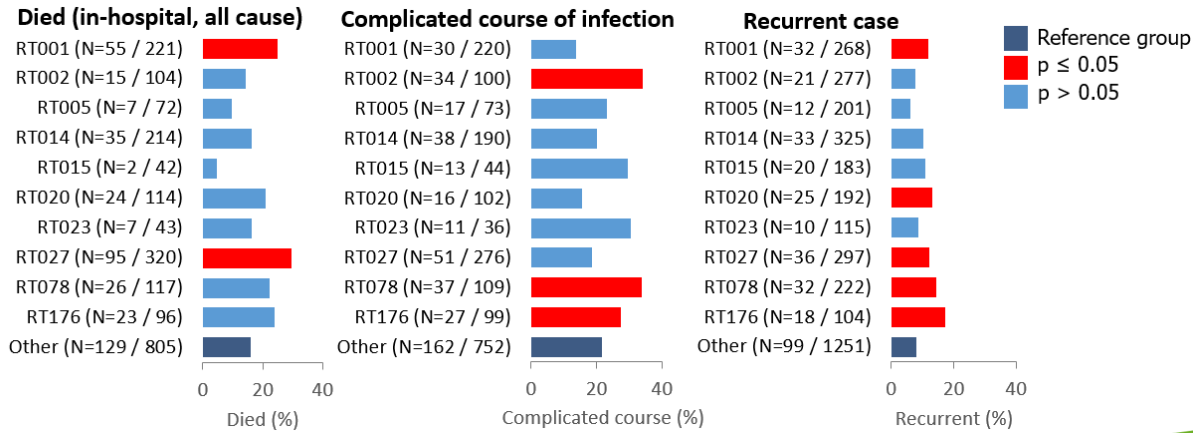
Fewer countries reported CDI data to ECDC from 2019 to 2021 compared with previous years, as the reporting schedule coincided with national and ECDC responses to the COVID-19 pandemic. Several reports document decreased CDI incidence in 2020 and 2021 in EU countries [24-26]. However, informal reports from three EU/EEA countries indicated an increased incidence, possibly linked to changes in hospital-sector antimicrobial consumption and infection control practices.

Previous ECDC survey: 'ECDC *Clostridioides difficile* infection whole genome sequencing, 2021' survey

In 2020, in a survey similar to the one reported on in this protocol, ECDC invited all OCPs for microbiology for CDI and NFPs for HAIs in all EU/EEA countries to submit clinical *C. difficile* isolates for ECDC-funded WGS. The preferred isolates were from RT176, ST1 or Clade 2, as the ECDC surveillance data had indicated that RT176 was associated with poor infection outcomes (Figure 1). The preferred surveillance period was the previous 18 months. National issues with isolate selection were discussed with ECDC and were usually solved by allowing the inclusion of historical isolates.

By June 2021, 18 countries had participated, including 14 countries that sent DNA/bacterial pellets for 148 *C. difficile* isolates. The ECDC laboratory contractor returned WGS data to each participating laboratory within four weeks. Three countries offered nationally generated sequence data (Denmark, Finland, Hungary). ECDC was not able to accept isolates from a country that offered 180 stored *C. difficile* bacterial pellets, as these had not been typed at the national level. ECDC received WGS data for 167 isolates from 15 countries, including 79 (47%) isolates identified as RT176. Initial analysis of all strains by cgMLST (≤200 allelic differences) identified three subclusters (≤6 allelic differences). Each contained strains from different countries and years, including some with different nationally assigned RTs.

Figure 1. Multi-level mixed effects logistic regression model[a] of PCR ribotype-specific outcomes of *Clostridioides difficile* infection, EU/EEA and the United Kingdom (n = 14 countries), 2016–2017



RT: ribotype.

[a] The model compares the 10 most frequently reported RTs to all other RTs, clustered by country, with McCabe score and age as modifiers.

Survey definitions

Tertiary acute care hospital

The definition of a 'tertiary acute care hospital' can vary from country to country. However, they should provide regional services, regularly taking referrals from other (primary and secondary) hospitals. They might also be referred to as a 'central', 'regional' or 'tertiary-level' hospitals, and may be university hospitals or associated with a university. They are likely to offer clinical services highly differentiated by function, with highly specialised staff and technical equipment (e.g. intensive care unit, transplantation, cardio-thoracic surgery, neurosurgery), and specialised imaging units [27,28].

Clostridioides difficile infection case

A CDI case (previously also referred to as *Clostridium difficile* infection or *C. difficile*-associated diarrhoea (CDAD)) should be aligned with the ECDC and EU case definition [27,29]. The preferred definition is:

- diarrhoeal stools or toxic megacolon;

AND

- a positive laboratory assay for *C. difficile* toxin A and/or B in stools or a toxin-producing *C. difficile* isolate detected in stool via culture or other means (e.g. a positive PCR result).

Alternatively, a national or local definition may be used.

Healthcare-associated *Clostridioides difficile* infection case

A HA CDI is defined as a case of CDI with onset of symptoms:

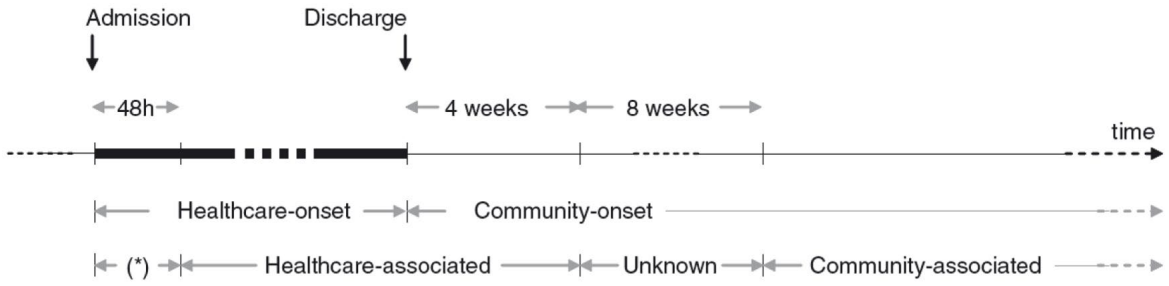
- on day three or later, following admission to a healthcare facility on day one;

OR

- within four weeks of discharge from a healthcare facility (including the current hospital or a previous stay in any other healthcare facility) EITHER in the community OR on the day of admission to a healthcare facility (day 1) or on the following day (day 2).

Figure 2 is a diagram to aid designation of a CDI as HA or community associated.

Figure 2. Designation of *Clostridioides difficile* infection cases as healthcare associated or community associated based on the location and time of onset of symptoms



Source: adapted from [6,27]
In practice, '48h' is interpreted as on the day of admission or on the following day. The asterisk indicates that a case may be community associated, healthcare associated or have an unknown association.

Imported healthcare-associated *Clostridioides difficile* infection case

An imported HA CDI case is defined as a HA CDI case admitted to a hospital with:

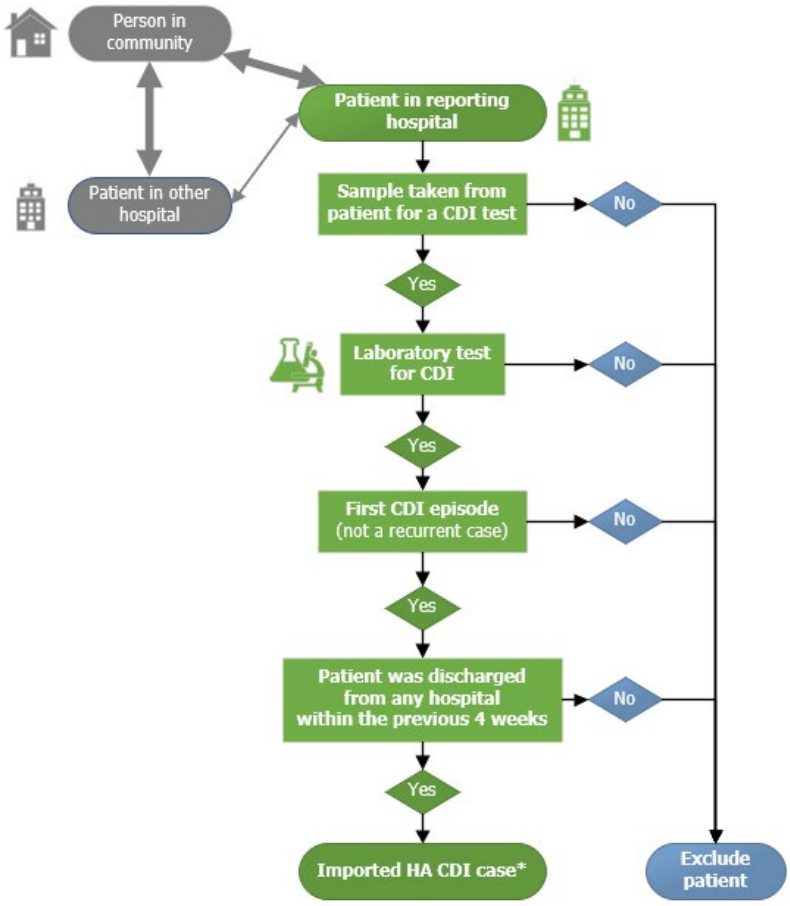
- onset of symptoms within four weeks of discharge from a previous stay any other hospital;

AND

- onset of symptoms EITHER in the community OR on the day of admission to a healthcare facility (day 1) or on the following day (day 2).

Figure 3 is a flow diagram to aid designation of an admitted patient as an imported HA CDI case. Note that this survey definition does not include two subcategories of HA CDI case origin that are specified in the ECDC CDI surveillance protocol [27]: 'long-term care facility' and 'healthcare-associated, origin of the infection not specified'.

Figure 3. Flow diagram to aid designation of an admitted patient as an imported HA CDI case[a]



CDI: Clostridioides difficile *infection; HA CDI: healthcare-associated* Clostridioides difficile *infection.*
[a] *The ECDC CDI surveillance protocol [27] does not include the definition of an imported case of a HA CDI case.*
Double-headed grey arrows indicate that patient movement may go in either direction, with patient admission/discharge (thick line) being more common than patient transfer between hospitals (thin line).

Recurrent cases

If feasible, only the first episode of an infection should be included, not recurrent cases [27]. Recurrent cases can be defined as CDI cases with a positive *C. difficile* stool specimen two to eight weeks after the last positive specimen.

Cluster/subcluster

There is currently no commonly agreed microbiological allelic/SNP distance-based cut-off for *C. difficile*. Therefore, clusters should be designated based on the available epidemiological information and the analysis objectives. In the 2021 ECDC survey focusing on RT176, visual examination of the phylogenetic tree identified three clear subclusters with a multi-country aspect. These contained strains with up to roughly six allelic differences according to single-linkage analysis of cgMLST data.

2 Survey purpose and methodology

Rationale

This survey aims to capture a snapshot of the distribution of *C. difficile* across the EU/EEA, including in countries that do not participate in ECDC-coordinated surveillance of CDI. Subsequent mapping activities will offer an indication of the distribution of the strains identified as notable. For example, strains associated with a higher outbreak potential or poorer outcomes, or that are susceptible to targeted interventions (e.g. antimicrobial stewardship of 4C antimicrobials to control fluoroquinolone-susceptible strains such as RT027 and RT176 [30], or strains with an identified reservoir). The results can support planning of surveillance, scientific studies or in silico analyses. This information will enable EU/EEA countries, including those with non-comprehensive surveillance, to focus national investigations and prevention and control activities.

Aim

The aim of this survey is to map the current genotypic distribution of *C. difficile* strains in tertiary acute care hospitals in the EU/EEA. This would support subsequent risk assessment by enabling the potential extrapolation of strain-specific findings from subnational, national or multi-country activities to the EU/EEA.

Objectives

Primary objectives

The primary objectives of this survey were to:

- acquire a snapshot of the distribution of *C. difficile* strains in tertiary acute care hospitals in the EU/EEA in 2021 and 2022;
- assess the genetic relatedness of the acquired isolates.

Secondary objectives

The secondary objectives of this survey were to:

- comment on the level of discriminatory power that may be useful for multi-country surveillance of CDI in European acute care hospitals. For example:
  - to monitor multi-country spread;
  - to support reactive interventions for (sub)national CDI prevention and control;
  - to support predictive interventions for national CDI prevention and control (e.g. antimicrobial stewardship interventions implied by subtype).
- comment on the feasibility of requesting EU/EEA countries to report isolates from imported cases of an infection from tertiary acute care hospitals, compared with the first reported isolates during a calendar month.

Intended outputs

It is proposed that the following outputs will be created in response to the survey results:

- **Scientific journal article:** The content of the article and target journal are still under discussion, but the article must be publicly accessible. Named co-authors will include the NSCs. Additionally, a co-author group (with each co-author identifiable on PubMed) will contain one to two people per country, as approved by the NFP for HAIs. All co-authors must meet ICJME criteria; drafts of the manuscript will distributed to NSCs and sent to co-authors to enable this. NSCs or NFPs for HAIs are welcome to list all people who should be in the acknowledgements by providing their details in the draft manuscript.
- **Sequence data uploaded to the European Nucleotide Archive (ENA):** The sequence data collected from the survey will be uploaded to ENA after a journal accepts the article, at the latest. Alternatively, ECDC will upload these data if three peer-reviewed journals decline acceptance of the manuscript, with permission of each participating country (i.e. NMFPs).
- **Report:** An ECDC report will be prepared in parallel to the article but will be published subsequently.
- **Nationally generated outputs:** The WGS data sent from Eurofins to participating laboratories are not under embargo. If these data are used in publications, please acknowledge ECDC (e.g. 'Whole genome sequencing was (partly) performed using funding from the European Centre for Disease Prevention and Control (ECDC)').

Sampling frame

Novel methodologies are warranted to achieve monitoring of *C. difficile* strains circulating in healthcare settings in the EU/EEA, as most countries do not have comprehensive, national, integrated CDI surveillance. In this context, the sampling frame for this survey was designed based on a methodology proposed by Donker T et al. [31] that was designed to maximise the accuracy of national estimations of the incidence of hospital-acquired CDIs when reducing the number of tested patient samples and recruited hospitals. A theoretical model of this methodology is presented in Annex 1. It is based on a mathematical modelling survey that uses known hospital transfer patterns to maximise the accuracy of estimations of the national incidence of HA CDIs in tertiary acute care hospitals, while reducing the number of tested patient samples and recruited reporting hospitals [31].

EU/EEA countries were invited to recruit tertiary acute care hospitals to submit *C. difficile* isolates, preferably from consecutively detected, imported HA CDI cases and excluding recurrent cases (Table 1). Most preferably, these should have been HA CDI cases, as not all participants would have been able to discern whether a HA CDI case was imported (Figure 3). Otherwise, although it was the least preferred option, hospitals/laboratories could report consecutively detected CDI cases of any case origin (i.e. HA CDI, community-associated CDI, unknown-associated CDI or recurrent cases; Table 1 and Figure 2).

The surveillance period permitted both retrospective and prospective reporting (Table 1). This recognised that not all hospital laboratories store *C. difficile* samples and that the incidence of imported HA CDI cases might be lower than expected because of the COVID-19 pandemic [24-26].

Table 1. Sampling frame for isolates in the survey

Order of preference	Source of isolates[a]	When detected	Surveillance period[b]
Ideal	**From imported HA CDI cases**	**Consecutively detected**	**January 2022 to May 2022**
Less preferable	From HA CDI cases	Consecutively detected	May 2021 to May 2022
Least preferable	From all CDI cases	Consecutively detected	May 2021 to May 2022

CDI: Clostridioides difficile *infection; HA CDI: healthcare-associated* Clostridioides difficile *infection.*
[a] *If feasible, isolates from recurrent cases should be excluded (i.e. only include the first CDI episode).*
[b] *In 2023, some participating countries were given another opportunity to submit samples/isolates, as additional budget became available.*

Estimated EU/EEA coverage of samples/isolates

The available budget for this survey allowed for WGS typing of 990 samples/isolates. ECDC estimated that there were roughly 189 526 (approximate range: 105 154–340 978) cases of HA CDI in acute care hospitals annually in the EU/EEA in 2016 and 2017. This implies direct coverage of about 0.5% (0.3%–0.9%) of the annual healthcare-associated *C. difficile* isolates. However, the sampling frame methodology is likely to achieve higher national coverage than standard self-reporting (Figure 6 and Annex 1 [31]).

Accurate calculation of coverage requires hospital transfer data, which are not available for most EU/EEA countries. Consequently, the estimation of EU/EEA coverage is not yet possible.

3 Survey activities

Activities of the National Survey Coordinators

ECDC requested that the NMFP or NFP for Healthcare-Associated Infections (HAIs) in EU/EEA countries designate a National Survey Coordinator (NSC) for this particular survey. Preferably, there would be one NSC per country, but NMFPs/NFPs for HAIs were able to designate more than one NSC per country. The role of an NSC was to:

- be the **main contact point** for operational communications (with ECDC and the ECDC laboratory contractor, Eurofins) during this survey.
- **recruit hospitals** to this survey and be their main contact person, as ECDC cannot[1] establish this technical/operational interaction. This might include forwarding questions to ECDC.
- **support registration of participating laboratories** with Eurofins (e.g. secure contact details of a laboratory contact point). Preferably, if feasible, propose one central laboratory to submit all samples.
- **indicate national interest in participation**, either via the email registering the participating laboratories (more details given below) or in a separate email to Typing@ecdc.europa.eu.
- **collate metadata** on isolates (Annex 4), ensure that datasets do not include any personal identifiers (i.e. in nationally added variables), submit a line list to ECDC (Annex 5) and respond to/solve data quality issues (e.g. errors) identified by ECDC. As the metadata did not request personal identifiers, NSCs were able to email these as Excel sheets to ECDC with or without password protection of the Excel sheet. Alternatively, ECDC could provide NSCs with login details for SFTP servers to share such data securely with the Centre.
- **be a named co-author and to propose co-authors for a co-author group** for approval by the NFP for HAIs/NMFPs, for a joint scientific journal article at the end of the survey (see 'Intended outputs').

Inclusion and exclusion criteria for hospitals

Any hospital that met the broad definition of a 'tertiary acute care hospital' given previously was eligible for the survey. NSCs were asked to avoid recruiting local hospitals or 'specialised acute care hospitals' unless they were specialised for CDI care. They were also asked not to include institutions other than acute care hospitals (e.g. long-term care facilities or general practitioner practices).

Number of hospitals or laboratories to recruit

The number of hospitals to recruit is indicated in Table 2 in the column labelled 'N of tertiary ACHs to recruit'. See Annex 2 for the data sources, calculations and estimations for this sample size.

Number of isolates to request from participating hospitals or laboratories

To identify the number of isolates that each recruited hospital could submit to Eurofins, NSCs were asked to divide the number of isolates indicated in the column 'Max N of isolates to submit' in Table 2 by the number of recruited hospitals (see Annex 2 for the background calculations and assumptions). For example, if Austria recruits five hospitals, each hospital can submit four isolates. Conversely, if Austria recruits four hospitals, each can submit five isolates.

[1] ECDC is only permitted to interact with EU/EEA countries through nationally nominated National Focal Points and Operational Contact Points. For more information see: www.ecdc.europa.eu/en/about-us/governance/competent-bodies

Table 2. Number of tertiary acute care hospitals to recruit to the survey and maximum number of isolates to submit to the ECDC contractor, by EU/EEA country, 2022–2023

Country	N of ACHs	Estimated N of tertiary ACHs	Recruitment of hospitals to this survey			Population in mid-2021	% EU/EEA population	Isolates to report for this survey	
			N of tertiary ACHs to recruit	% tertiary ACHs	% ACHs			Max N of isolates to submit	Estimated N of isolates per hospital
Austria	162	32.4	5	15	3	8 901 064	2	20	4
Belgium	197	39.4	5	13	3	11 522 440	2.5	26	5.2
Bulgaria	241	48.2	7	15	3	6 951 482	1.5	16	2.3
Croatia	34	6.8	1	15	3	4 058 165	0.9	9	9
Cyprus	83	16.6	3	18	4	888 005	0.2	3[b]	1.0[b]
Czechia	144	28.8	4	14	3	10 693 939	2.4	24	6
Denmark	52	10.4	2	19	4	5 822 763	1.3	13	6.5
Estonia	27	5.4	1	19	4	1 328 976	0.3	3	3
Finland	59	11.8	2	17	3	5 525 292	1.2	13	6.5
France	1 237	247.4	32	13	3	67 320 216	14.9	148	4.6
Germany	1 857	371.4	47	13	3	83 166 711	18.4	182	3.9
Greece	123	24.6	4	16	3	10 718 565	2.4	24	6
Hungary	94	18.8	3	16	3	9 769 526	2.2	22	7.3
Iceland	8	1.6	1	63	13	364 134	0.1	1	1
Ireland	60	12	2	17	3	4 964 440	1.1	11	5.5
Italy	1 134	226.8	29	13	3	59 641 488	13.2	131	4.5
Latvia	24	4.8	1	21	4	1 907 675	0.4	5	5
Lithuania	64	12.8	2	16	3	2 794 090	0.6	7	3.5
Luxembourg	12	2.4	1	42	8	626 108	0.1	2	2
Malta	4	0.8	1	100[a]	25	514 564	0.1	2	2
Netherlands	79	15.8	2	13	3	17 407 585	3.8	39	19.5
Norway	43	8.6	2	23	5	5 367 580	1.2	12	6
Poland	936	187.2	24	13	3	37 958 138	8.4	83	3.5
Portugal	225	45	6	13	3	10 295 909	2.3	23	3.8
Romania	311	62.2	8	13	3	19 328 838	4.3	43	5.4
Slovakia	107	21.4	3	14	3	5 457 873	1.2	12	4
Slovenia	21	4.2	1	24	5	2 095 861	0.5	5	5
Spain	576	115.2	15	13	3	47 332 614	10.4	104	6.9
Sweden	144	28.8	4	14	3	10 327 589	2.3	23	5.8
EU/EEA	8 058	1 611.6	218	14	3	453 051 630	100	1 006[c]	4.6

ACH: acute care hospital; Max: maximum.
[a] The percentage was rounded down from 125% of the estimated number of tertiary care hospitals in Malta to 100%.
[b] The indicated max N of isolates for Cyprus to report has been rounded up from two to three, so that there are at least as many requested isolates as requested hospitals (n = 3).
[c] Although this exceeds the budget for 990 isolates, it was foreseen that not all countries would participate. See Annex 2 for all data sources, calculations and estimations for each column in this table.

Considerations for hospital recruitment

If there were several tertiary acute care hospitals that wanted to participate, NSCs were asked to **preferentially recruit**:

- hospitals that commonly receive patient transfers from a larger number of hospitals (see Annex 2);
- hospitals that receive patient transfers relatively frequently (see Annex 2);
- hospitals that can differentiate between imported HA CDI cases and other cases (see Figure 3). Reporting imported cases provides:
  - better national coverage (see Annex 2);
  - potentially higher acceptability compared to self-reporting HA CDI cases;
  - relative anonymity of the 'source' hospital (see Annex 5).

Hospitals that were not able to participate were advised to store samples/isolates related to imported HA CDI cases. In April 2022, ECDC informed each NSC how many additional samples/isolates could be sent for ECDC-funded WGS typing, based on the utilisation of this service at that time (Table 3). In 2023, some participating countries were given another opportunity to submit samples/isolates, as additional budget became available.

Initial information for the NSC to collect from each participating laboratory

To process laboratory samples, Eurofins required each participating laboratory to register their contact details in their database. Thereafter, Eurofins sent stickers with bar codes for samples/isolates directly to these laboratory contact points via the post.

Contact information was emailed to Typing@ecdc.europa.eu and had to include (for each participating laboratory):

- Title
- First and last name
- Company/University
- Department or group (not mandatory)
- Address (street, zip/area code, city, country)
- Email address.

ECDC then forwarded this information to Eurofins.

Collating metadata on the reported isolates and submitting to ECDC

NSCs were asked to email a line list of all reported isolates to Typing@ecdc.europa.eu by 30 June 2022 (see Annex 4 for the requested metadata and Annex 5 for the data collection tool to create the line list).

Table 3. Timeline of participation and activities of ECDC, National Survey Coordinators, participating hospitals/laboratories and Eurofins, 2022[a]

Date	ECDC	National Survey Coordinatorss	Participating hospitals/laboratories	Eurofins[b]
March	• Receive contact details of participating laboratories and/or emailed intention to participate from NSCs • Send contact details of participating laboratories to Eurofins	• Recruit participating hospitals or laboratories • Ensure that ECDC is aware of each participating laboratory	• Provide contact details to NSC • Receive sample/isolate bar codes from Eurofins	• Send participating laboratories: - sample/isolate bar codes - confirmation/estimation of the format and expected maximum number of samples/isolates
Up to 22 April		• Monitor number of isolates per hospital/laboratory • Collate metadata, submitting to ECDC approximately every 4 weeks • Inform ECDC if maximum number of isolates per hospital/laboratory or country is exceeded	• Submit samples to Eurofins by first deadline (22 April 2022) • Collate metadata and submit it to NSC • If the maximum number of isolates per laboratory is exceeded, store remaining samples appropriately and inform the NSC	• Provide each participating hospital/laboratory with WGS data obtained from their submitted samples/isolates within 20 working days
22 April			**First deadline for Eurofins to receive samples/isolates**	
25 April				• Inform ECDC of the number of samples/isolates received per country by the 22 April deadline
27–28 April	• Inform NSCs how many additional samples/isolates can be submitted by the final deadlines			
Up to 29 April		• Inform participating hospitals/laboratories how many more samples/isolates they can submit to Eurofins by the final deadlines		
From 30 April		• Collate final metadata on each sample/isolate	• If informed by NSCs, submit additional samples/isolates to Eurofins by the final deadline (e.g. samples stored before the first deadline)	• Provide each participating hospital/laboratory with WGS data obtained from their submitted samples/isolates within 20 working days
24 May			**Final deadline for bacterial pellets to reach Eurofins**	
31 May			**Final deadline for DNA to reach Eurofins**	
Up to 30 June	• Receive full WGS dataset from Eurofins	• Submit updated metadata to ECDC		• Final deadline to upload all WGS data to SFTP server accessible only to ECDC and Eurofins
July to September	• Draft manuscript for scientific journal article	• Acquire list of co-authors for journal article (see 'Intended outputs')		
October to November		• Submit draft manuscript to national co-authors for comments		

NFP for HAIs: National Focal Point for Healthcare-Associated Infections; NMFP: National Microbiology Focal Point; NSC: National Survey Coordinator; WGS: whole genome sequencing.
[a] In 2023, some participating countries were given another opportunity to submit samples/isolates, as additional budget became available.
[b] Eurofins is the ECDC laboratory contractor for this survey.
Grey fields indicate that no activity was required. Green rows indicate key deadlines.

Activities for participating hospitals/laboratories

Registering interest in participation in this survey

To participate in the survey, hospitals/laboratories required the approval of an NSC. NSCs were responsible for providing ECDC with the contact details of participating laboratories. ECDC submitted these to the ECDC laboratory contractor, Eurofins. Eurofins sent bar code stickers for laboratory samples/isolates to participating laboratories and paid the shipment costs.

Total number of *Clostridioides difficile* samples/isolates to prepare

NSCs informed each laboratory of the maximum number of samples/isolates to collect. The number of samples/isolates per country depended on the population size and the number of tertiary acute care hospitals in the country (see 'Sampling frame' and Annex 2). The available budget suggested that participating hospitals in 50% of EU/EEA countries could submit isolates from one to six CDI cases per hospital, with 40% of countries able to submit six to seven cases per hospital.

Surveillance period and inclusion/exclusion criteria for *Clostridioides difficile* infection cases

It was preferable that hospitals/laboratories reported isolates from consecutive imported HA CDI cases sampled between January and May 2022 (Table 1 and Figure 3). Table 1 lists additional options (e.g. consecutive HA CDI or CDI cases sampled between May 2021 and April/May 2022; see '1.3 Survey definitions'). If feasible, recurrent cases were excluded (i.e. only samples/isolates from the first episode of an infection were submitted [27]); the metadata (Annex 4) indicates whether a case was recurrent. Further guidance is available in section '1.3 Survey definitions' and the flow diagram in Figure 3.

Collecting metadata for each submitted sample/isolate

Each participating hospital/laboratory had to provide at least the three mandatory variables and preferably the four recommended variables and, if possible, any number of the optional variables. Table 4 summarises the requested variables by type, indicating the requirement for each. See Annex 4 for descriptions of all the metadata variables and the values that could be reported using the data collection tool (Annex 5). These tables were also available as one Excel sheet that was emailed to NSCs.

Submitting microbiological samples to the ECDC laboratory contractor

The methodological instructions for submitting DNA and bacterial pellets to Eurofins are in section '4. Microbiological preparation of samples/isolates'.

Submitting whole genome sequencing data to ECDC

ECDC provided NSCs with an STFP server to transfer WGS data to ECDC (see section '4. Microbiological preparation of samples/isolates').

Table 4. Data to collect for each reported sample/isolate*

Variable type	Number of variables	Requirement	Description*
ID	2	Mandatory	Reporting country
			Isolate identifier
Isolate selection	1	Mandatory	How the isolate was selected for this survey
	2	Recommended	Part of a known outbreak
			Recurrent CDI
Date	2	Recommended	Date used for statistics
			Type of date used for statistics
Hospital description	1	Recommended	Hospital ID
	3	Optional	Hospital type
			Region where the reporting hospital is located
			Address of the reporting hospital or laboratory
Subtype information	4	Optional	PCR ribotype of *C. difficile* isolate
			Reason for typing
			Sequence type
			Genetic clade
Antimicrobial susceptibility	6	Optional	Final interpretation of metronidazole susceptibility testing results
			MIC method for metronidazole susceptibility testing
			Final interpretation of vancomycin susceptibility testing results
			MIC method for vancomycin susceptibility testing
			Final interpretation of fidaxomicin susceptibility testing results
			MIC method for fidaxomicin susceptibility testing
CDI outcome	2	Optional	Fatal CDI outcome
			Severe CDI outcome

CDI: Clostridioides difficile *infection.*
** See Annex 4 for full details on the metadata for each variable.*

4 Microbiological preparation of samples/isolates

Overview

- Samples/isolates could be submitted to the survey as:
  - Sequences OR
  - Extracted DNA from countries with no WGS capacity OR
  - Bacterial pellets/cultures from countries with no WGS or DNA extraction capacity.
- ECDC covered the costs related to WGS or DNA extraction.
- Samples/isolates submitted without prior written approval from ECDC could not be processed.
- The ECDC laboratory contractor for this project was Eurofins Genomics. The contractor has two sites in Germany (Konstanz and Ebersberg) and had already supported the ECDC-led PCR RT 176 WGS pilot project.
- The submitting laboratory covered the costs for sample preparation and packaging. Eurofins funded the shipment from the sending laboratory to Eurofins.
- Eurofins shared the resulting sequences with the sending laboratory and ECDC through an SFTP site:
  - Isolates and DNA sent to the contractor are owned by the sending laboratory and the contractor will destroy them after an agreed-upon storage period.
  - The resulting WGS data are owned by the sending laboratory and ECDC, and will be used for the purposes of the survey and EU-level surveillance. They will be subsequently uploaded to public sequence repositories with minimal metadata (pseudonymised identifier, species, host, country, year).
- Nationally generated WGS data will also need to be uploaded to public repositories when included in a published article.

Submitting nationally generated whole genome sequencing data to ECDC

Uploading data

An ECDC SFTP site could be arranged by ECDC for each country or national tools could be used for the data transmission.

Data handling

Any sequences referenced in scientific articles would need to be uploaded to public repositories with minimal metadata (i.e. pseudonymised identifier, species, host, country, year), as per common scientific publishing practices. The European Surveillance System (TESSy) policy on data submission and access and use of data within TESSy [1] states (in 4. Data handling):

> 'The data originate from Member States, who submit it in compliance with the EU regulations including EC decisions (notably Decision 1082/2013/EU), and other data providers who submitted in accordance with this policy. ECDC is the data controller, holds the data in its trusteeship, and can assist Member States in data uploading and reporting. ECDC is also entrusted with the technical implementation of the publication of the data and the granting of data access in accordance with this policy.'

Preparation of DNA and bacterial pellets/cultures

See Annex 3 for a description of how laboratories were asked to prepare DNA and bacterial pellets/cultures. More details were sent to countries after confirming their interest to in submitting either sample type.

5 Planned analyses

The analyses planned for after the survey include, but may not be limited to, the following.

Description of dataset

The following summary statistics describing survey participation, by country and overall, will be analysed:

- the number of participating countries and hospitals, including a comparison with the eligible/estimated sample size;
- the rate at which participating hospitals reported imported CDI cases compared with the expected rate based on ECDC TESSy data for 2016 and 2017;
- the number of samples/isolates received, also by data source (i.e. DNA, bacterial pellet, data).

The following summary statistics of the metadata will also be analysed:

- the data completeness of each variable, by country and overall (for dates, this will include the granularity of the data, i.e. the proportion of isolates with a reported year, quarter, month or day);
- a time series plot for samples/isolates with known dates, highlighting date ranges for national participation and a description of reported date type;
- a map of Europe indicating the geographical location of participating hospitals/laboratories (from those that provided location data);
- the available subtype, antimicrobial susceptibility and CDI outcome data.

Whole genome sequencing analysis

Whole genome sequencing of samples/isolates was conducted by Eurofins and the results were analysed by ECDC. The minimum WGS pipelines available at ECDC are cgMLST (incl. 1999 loci) and wgMLST (incl. 8745 loci) schemes available in BioNumerics 7.6.3, with the following parameters:

- including default settings for trimming and allele calling, assembly SPAdes 3.7.1, post-assembly optimisation by mapping reads back onto the assembly and keeping the consensus;
- allele calling from assembly only;
- quality control that includes at least the proportion of detected core loci per isolate and assembly length, based on analyses of other pathogens.

Optimally, sequence data would be analysed using national WGS pipelines when available in a public repository to evaluate correspondence and aligned conclusions.

Visualisation of sequence data

cg/wgMLST-based single-linkage trees (showing ≤200 allelic differences) will be used to interpret the sequence data and minimum spanning trees will be used to zoom into interesting clusters. Graphical methods (e.g. colour coding) will indicate subcategories of mandatory and recommended variables (e.g. country, date) within the trees. Separate visualisations of the same trees will indicate subcategories of the optional variables, in particular PCR RT.

The data completeness of the optional variables is expected to be relatively low (i.e. only some countries will report data for antimicrobial susceptibility and CDI outcome data). Still, plotting even sparse data on phylogenetic trees may indicate international subclusters that are worthy of additional investigation.

MicroReact software may be used to generate more interactive visualisations.

Summary statistics of the detected clusters

The following summary statistics of the detected clusters will be analysed:

- ad hoc microbiological cluster identification with no strict (multi-country) cluster definition (hopefully aided by epidemiological data);
- test description of the analysis, with quantification as appropriate (e.g. number of countries per (sub)cluster);
- descriptions of the equivalence between PCR RT and identified subclusters;
- metadata from any particularly notable subclusters, including time series plots.

Processing personal data

The survey did not request or collect personal identifiers. No data were collected that could identify CDI cases (e.g. national ID number, age, sex, date of admission, hospital ward). Additionally, the metadata did not include any identifier of the source hospital for imported cases. Still, NSCs had to ensure that any submitted data did not contain any identifiers so that no link to a person could be established.

As described in the 'Intended outputs' section of this report, ECDC will store data from this survey according to ECDC Data Protection standards[2] and will publish the results in aggregated form. However, if ECDC receives a request for public access to the individual replies, the Centre will disclose them (without personal data) unless the participant explicitly requested and justified keeping the information confidential when they submitted the data.

[2] For more information, see https://www.ecdc.europa.eu/en/publications-data/european-surveillance-system-tessy

References

1. Suetens C, Latour K, Kärki T, Ricchizzi E, Kinross P, Moro ML, et al. Prevalence of healthcare-associated infections, estimated incidence and composite antimicrobial resistance index in acute care hospitals and long-term care facilities: results from two European point prevalence surveys, 2016 to 2017. Euro Surveill. 2018;23(46):800516. Available at: https://www.eurosurveillance.org/content/10.2807/1560-7917.ES.2018.23.46.1800516
2. Kinross P. The ECDC CDI surveillance protocol and European CDI surveillance data (S0692). Presented at: European Congress of Clinical Microbiology & Infectious Diseases; 15 April 2019; Amsterdam, the Netherlands. Available at: https://2019.eccmid.org/scientific_programme/preliminary_programme/scientific_symposia
3. Cassini A, Plachouras D, Eckmanns T, Abu Sin M, Blank HP, Ducomble T, et al. Burden of Six Healthcare-Associated Infections on European Population Health: Estimating Incidence-Based Disability-Adjusted Life Years through a Population Prevalence-Based Modelling Study. PLoS Med. 2016 Oct;13(10):e1002150. Available at: https://www.ncbi.nlm.nih.gov/pubmed/27755545
4. Combatting Bacterial Resistance in Europe – Clostridioides Difficile Infection (COMBACTE-CDI). COMBACTE Abstracts ECCMID 2020 Available. Paris: ESCMID [Accessed: 8 Mar 2022]. Available at: https://www.combacte.com/news/combacte-abstracts-eccmid-2020-available
5. Davies KA, Ashwin H, Longshaw CM, Burns DA, Davis GL, Wilcox MH, et al. Diversity of *Clostridium difficile* PCR ribotypes in Europe: results from the European, multicentre, prospective, biannual, point-prevalence study of *Clostridium difficile* infection in hospitalised patients with diarrhoea (EUCLID), 2012 and 2013. Euro Surveill. 2016;21(29):30294. Available at: https://www.eurosurveillance.org/content/10.2807/1560-7917.ES.2016.21.29.30294
6. Kuijper EJ, Coignard B, Tüll P, ESCMID Study Group for *Clostridium difficile*, EU Member States, European Centre for Disease Prevention and Control (ECDC). Emergence of *Clostridium difficile*-associated disease in North America and Europe. Clinical Microbiology and Infection. 2006;12 Suppl 6:2-18. Available at: http://www.ncbi.nlm.nih.gov/pubmed/16965399
7. Wingen-Heimann S. A pan-European cost-of-illness analysis of patients with *Clostridioides difficile* infection: results of COMBACTE-CDI. (2962). Presented at European Congress of Clinical Microbiology & Infectious Diseases; 9 July 2021; Vienna, Austria.
8. Bauer MP, Notermans DW, van Benthem BH, Brazier JS, Wilcox MH, Rupnik M, et al. *Clostridium difficile* infection in Europe: a hospital-based survey. Lancet. 2011;377(9759):63-73.
9. van Dorp SM, Notermans DW, Alblas J, Gastmeier P, Mentula S, Nagy E, et al. Survey of diagnostic and typing capacity for *Clostridium difficile* infection in Europe, 2011 and 2014. Euro Surveill. 2016;21(29):30292. Available at: https://www.eurosurveillance.org/content/10.2807/1560-7917.ES.2016.21.29.30292
10. Rada S, Kanitz E, Schmid D, Suetens C, Kuijper EJ, Kinross P. Cross-sectional survey of *Clostridioides difficile* infection diagnostic and typing capacity in 31 European countries in 2018. Presented at International *Clostridium difficile* Symposium (ICDS); 14 October 2020; Bled, Slovenia. Available at: https://www.icds.si/posters-2020
11. Janezic S, Rupnik M. Development and Implementation of Whole Genome Sequencing-Based Typing Schemes for *Clostridioides difficile*. Front Public Health. 2019;7:309. Available at: https://www.ncbi.nlm.nih.gov/pubmed/31709221
12. Baktash A, Corver J, Harmanus C, Smits WK, Fawley W, Wilcox MH, et al. Comparison of Whole-Genome Sequence-Based Methods and PCR Ribotyping for Subtyping of *Clostridioides difficile*. Journal of Clinical Microbiology. 2022;60(2):e0173721.
13. Bletz S, Janezic S, Harmsen D, Rupnik M, Mellmann A. Defining and Evaluating a Core Genome Multilocus Sequence Typing Scheme for Genome-Wide Typing of *Clostridium difficile*. Journal of Clinical Microbiology. 2018;56(6).
14. Eyre DW, Cule ML, Wilson DJ, Griffiths D, Vaughan A, O'Connor L, et al. Diverse sources of *C. difficile* infection identified on whole-genome sequencing. The New England Journal of Medicine. 2013;369(13):1195-205.
15. European Centre for Disease Prevention and Control (ECDC). ECDC strategic framework for the integration of molecular and genomic typing into European surveillance and multi-country outbreak investigations (2019–2021). Stockholm: ECDC; 2019. Available at: https://www.ecdc.europa.eu/en/publications-data/ecdc-strategic-framework-integration-molecular-and-genomic-typing-european
16. van Dorp SM, Kinross P, Gastmeier P, Behnke M, Kola A, Delmee M, et al. Standardised surveillance of *Clostridium difficile* infection in European acute care hospitals: a pilot study, 2013. Euro Surveill. 2016;21(29):30293. Available at: https://www.eurosurveillance.org/content/10.2807/1560-7917.ES.2016.21.29.30293
17. European Centre for Disease Prevention and Control (ECDC). Surveillance Atlas of Infectious Diseases - *Clostridioides difficile* infection data 2019. Stockholm: ECDC; 2022. Available at: https://atlas.ecdc.europa.eu
18. European Centre for Disease Prevention and Control (ECDC). *Clostridium difficile* infections. In: ECDC. Annual epidemiological report for 2016. Stockholm; ECDC: 2018. Available at: https://www.ecdc.europa.eu/en/publications-data/healthcare-associated-infections-clostridium-difficile-infections-annual
19. European Centre for Disease Prevention and Control (ECDC). *Clostridium difficile* infections. In: ECDC. Annual epidemiological report for 2017 (in press). Stockholm: ECDC.
20. Dresler J, Krutova M, Fucikova A, Klimentova J, Hruzova V, Duracova M, et al. Analysis of proteomes released from in vitro cultured eight *Clostridium difficile* PCR ribotypes revealed specific expression in PCR ribotypes 027 and 176 confirming their genetic relatedness and clinical importance at the proteomic level. Gut Pathogens. 2017;9(1):45. Available at: https://doi.org/10.1186/s13099-017-0194-9
21. Obuch-Woszczatynski P, Lachowicz D, Schneider A, Mol A, Pawlowska J, Ozdzenska-Milke E, et al. Occurrence of *Clostridium difficile* PCR-ribotype 027 and it's closely related PCR-ribotype 176 in hospitals in Poland in 2008-2010. Anaerobe. 2014;28:13-7. Available at: https://www.ncbi.nlm.nih.gov/pubmed/24799338
22. Novakova E, Stefkovicova M, Kopilec MG, Novak M, Kotlebova N, Kuijper E, et al. The emergence of *Clostridium difficile* ribotypes 027 and 176 with a predominance of the *Clostridium difficile* ribotype 001 recognized in Slovakia following the European standardized *Clostridium difficile* infection surveillance of 2016. Int J Infect Dis. 2020;90:111-5.

23. Pituch H, Obuch-Woszczatynski P, Lachowicz D, Wultanska D, Karpinski P, Mlynarczyk G, et al. Hospital-based *Clostridium difficile* infection surveillance reveals high proportions of PCR ribotypes 027 and 176 in different areas of Poland, 2011 to 2013. Euro Surveill. 2015;20(38):30025. Available at: https://www.eurosurveillance.org/content/10.2807/1560-7917.ES.2015.20.38.30025
24. Granata G, Petrosillo N, Al Moghazi S, Caraffa E, Puro V, Tillotson G, et al. The burden of *Clostridioides difficile* infection in COVID-19 patients: A systematic review and meta-analysis. Anaerobe. 2021:102484. Available at: https://pubmed.ncbi.nlm.nih.gov/34843959
25. Hazel K, Skally M, Glynn E, Foley M, Burns K, O'Toole A, et al. The other 'C': Hospital-acquired *Clostridioides difficile* infection during the coronavirus disease 2019 (COVID-19) pandemic. Infection Control & Hospital Epidemiology. 2021:1-2. Available at: https://www.ncbi.nlm.nih.gov/pmc/articles/PMC7870903/
26. Ponce-Alonso M, Sáez de la Fuente J, Rincón-Carlavilla A, Moreno-Nunez P, Martínez-García L, Escudero-Sánchez R, et al. Impact of the coronavirus disease 2019 (COVID-19) pandemic on nosocomial *Clostridioides difficile* infection. Infection Control & Hospital Epidemiology. 2021;42(4):406-10. Available at: https://pubmed.ncbi.nlm.nih.gov/32895065
27. European Centre for Disease Prevention and Control (ECDC). European Surveillance of *Clostridioides difficile* infections. Surveillance protocol version 2.4. Stockholm: ECDC; 2019. Available at: https://www.ecdc.europa.eu/en/publications-data/european-surveillance-clostridium-difficile-infections-surveillance-protocol-2
28. European Centre for Disease Prevention and Control (ECDC). Healthcare-associated Infections Surveillance Network (HAI-Net). Stockholm: ECDC [Accessed: 8 Mar 2022]. Available at: https://www.ecdc.europa.eu/en/about-us/partnerships-and-networks/disease-and-laboratory-networks/hai-net
29. European Commission (EC). Commission Implementing Decision (EU) 2018/945 of 22 June 2018 on the communicable diseases and related special health issues to be covered by epidemiological surveillance as well as relevant case definitions. Brussels: EC; 2018. Available from: https://eur-lex.europa.eu/legal-content/EN/TXT/PDF/?uri=CELEX:32018D0945
30. Lawes T, Lopez-Lozano JM, Nebot CA, Macartney G, Subbarao-Sharma R, Wares KD, et al. Effect of a national 4C antibiotic stewardship intervention on the clinical and molecular epidemiology of *Clostridium difficile* infections in a region of Scotland: a non-linear time-series analysis. The Lancet Infectious Diseases. 2017;17(2):194-206. Available at: https://pubmed.ncbi.nlm.nih.gov/27825595
31. Donker T, Smieszek T, Henderson KL, Walker TM, Hope R, Johnson AP, et al. Using hospital network-based surveillance for antimicrobial resistance as a more robust alternative to self-reporting. PloS one. 2019;14(7):e0219994.
32. European Centre for Disease Prevention and Control (ECDC). Point prevalence survey of healthcare-associated infections and antimicrobial use in European acute care hospitals, 2016–2017. Stockholm: ECDC; 2023. Available at: https://www.ecdc.europa.eu/en/publications-data/point-prevalence-survey-healthcare-associated-infections-and-antimicrobial-use-5
33. Eurostat. Population & demography - overview. Brussels: European Commission; 2022. Available at: https://ec.europa.eu/eurostat/web/population-demography
34. Boekhoud IM, Hornung BVH, Sevilla E, Harmanus C, Bos-Sanders IMJG, Terveer EM, et al. Plasmid-mediated metronidazole resistance in *Clostridioides difficile*. Nature Communications. 2020;11(1):598. Available at: https://doi.org/10.1038/s41467-020-14382-1

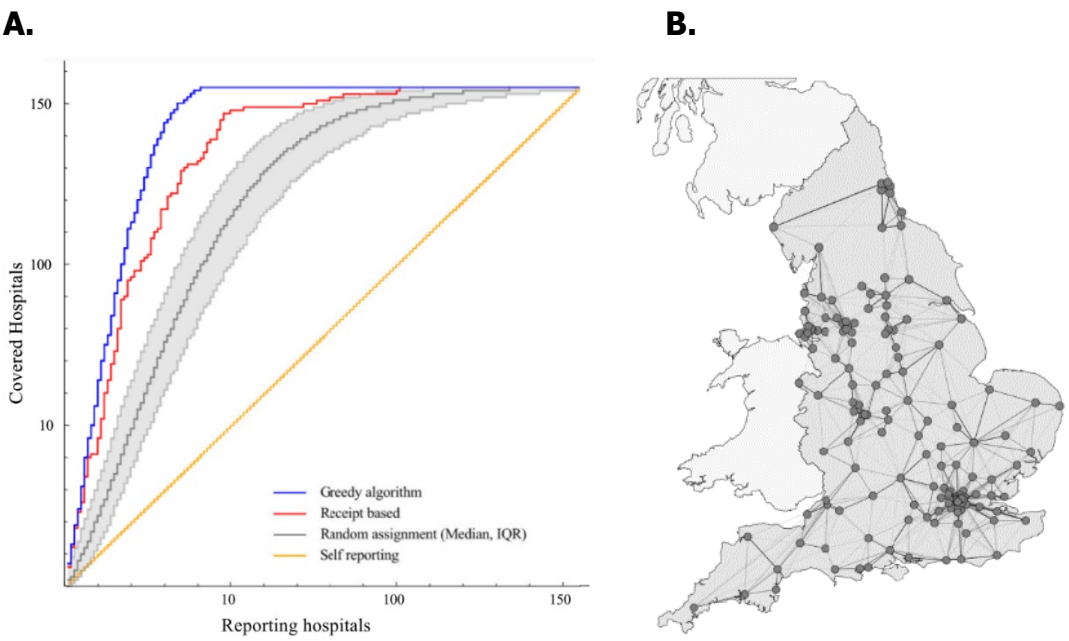
Annex 1. Theoretical model for the sampling frame used in this survey

As comprehensive, national, integrated surveillance of HA CDI is not feasible in many EU/EEA countries, alternative methodologies are warranted to achieve monitoring of nationally circulating *C. difficile* strains. In this context, the sampling frame for this survey was designed based on a methodology proposed by Donker T et al. [31] that was designed to maximise the accuracy of national estimations of the incidence of hospital-acquired CDIs when reducing the number of tested patient samples and recruited hospitals. This methodology utilised a mathematical model that incorporated known patient transfer patterns in England.

As a theoretical exercise to demonstrate how this methodology could be used for this survey, Figure 1A presents four sampling strategies to acquire coverage of all 155 acute care hospitals in England. The yellow line shows a strategy in which hospitals only self-report their own (i.e. non-imported) cases. The three other lines present sampling schemes in which hospitals only report imported cases. Each has a different strategy to sequentially add hospitals to a reporting set. The red line shows a 'receipt-based' strategy in which hospitals are added sequentially, based on their total number of received patient transfers (most to least). The blue line presents a 'greedy algorithm', in which the addition of hospitals is based on the total number of hospitals that supply them with patient transfers (most to least). If addition of a hospital by this strategy did not increase national coverage (i.e. connections to other hospitals), then the next hospital was added based on the strategy presented in the red line.

In both the 'greedy algorithm' and the receipt-based strategy, the first added hospitals were mostly tertiary acute care hospitals, while specialist hospitals were mostly added last. According to the 'greedy algorithm', more than 1 000 patient samples obtained from 41 of the 155 pre-chosen acute care hospitals would accurately estimate national incidence, while obtaining samples from 13 of the 155 hospitals would estimate the incidence of 50% of the national hospitals. The grey line and shaded area present the results from 100 iterations of random sequential allocation of hospitals to the reporting set, with national coverage calculated based on the total number of hospital connections that supply patient transfers.

Figure 1A. Theoretical model for a sampling frame for the incidence of HAIs from a hospital network in England, as used in this survey



Source: Adapted from [31]
Part (A) presents the number of covered hospitals, as a function of the number of reporting hospitals, by type of sampling scheme. Part (B) gives the distribution of hospitals according to the 'greedy algorithm' and the minimal set of reporting hospitals needed to report on all hospitals, as found using the greedy algorithm. Dark grey dots show the reporting set and lines show the links over which patients previously discharged from other hospitals are included.

Annex 2. Calculation of the numbers of hospitals and samples/isolates

Estimated frequency of importation of HA CDI cases into tertiary acute care hospitals in the EU/EEA

The ECDC CDI surveillance data (reported to TESSy) contain data for 2016–2020. These data were reported by 24 EU/EEA countries, of which 18 had hospital surveillance periods ranging from 3–12 months in any year. Of these, 13 (72%) countries ever reported any cases that met the survey definition of an imported HA CDI case (i.e. HA CDI from the current hospital (HA-CURR) or HA CDI from another hospital (HA-OHOSP), see '1.3 Survey definitions' [27]). The most complete data are available for 2016 and 2017, with eight countries reporting imported HA CDI cases from 51 (42%) of their 122 participating tertiary acute care hospitals. In these 51 hospitals, 313/5 285 (6%) CDI cases were imported HA CDI. The median frequency of importation of a HA CDI case to these tertiary acute care hospitals was 2.3 cases per month.

Estimation of sample size, assuming all participating countries report only imported HA CDI cases

A sample size calculation was made, according to different participation scenarios, in order to match the maximum number of isolates permitted by the budget (990 isolates). Each scenario incorporated the same estimated median frequency of HA CDI importation (2.3 cases per month) but varied in terms of the number of participating countries, the average number of participating hospitals per country and the duration of the surveillance periods. There were plausible scenarios that assumed recruitment of seven tertiary acute care hospitals per country. This implied that each country could recruit (on average) 1 in every 7.5 of these major, probably university-based hospitals (Table 2).

Every scenario assumed that countries would only report imported HA CDI cases. However, countries were permitted to use other sampling frames (Table 1). Therefore, every EU/EEA country was permitted to submit the same number of isolates for ECDC-funded typing, adjusted by population size (Table 2). In the timeline (Table 4), the inclusion of a first deadline to submit cases and metadata in April 2022 permitted ECDC to allocate any unused typing budget to the participating countries for further typing. The allocation gave preference to countries that have infrequent participation in international studies and surveillance activities and/or to countries with less national capacity for WGS of *C. difficile* and/or to countries that could report imported HA CDI cases excluding recurrent cases from tertiary acute care hospitals. Similarly, in 2023, when additional budget was available for this activity, these same criteria were applied.

Calculations used to generate the number of hospitals to recruit

The following calculations were used to generate the number of hospitals to recruit (Table 2):

- '**Number of acute care hospitals**' was acquired from the denominator data provided by EU/EEA countries for the 'ECDC point prevalence survey (PPS) of European acute care hospitals, 2016–2017' [32].
- '**Estimated number of tertiary acute care hospitals**' was based on data from that PPS, in which 20.0% of all participating acute care hospitals were tertiary hospitals (likely to be an overestimation, because tertiary hospitals were more likely to participate in the PPS).
- '**Number of tertiary acute care hospitals to recruit**' was based on a calculation to recruit an average of seven acute care hospitals per country, adjusted for each country by the estimated number of tertiary care hospitals in that country. If only half the EU/EEA countries participated, each recruiting seven hospitals, and they only reported isolates from imported HA CDI cases (estimated at 2.3 isolates per month) for a four-month surveillance period, there would be 969 isolates (i.e. close to the 990 maximum).
- '**% tertiary acute care hospitals**' and '**% acute care hospitals**' were calculated by dividing the 'Number of tertiary hospitals to recruit' by the 'Number of acute care hospitals' and 'Estimated number of tertiary acute care hospitals', respectively.
- '**Maximum number of isolates to submit**' is calculated by multiplying the maximum number of isolates for typing (n = 990) by the '% EU/EEA population', according to mid-2021 population estimates from Eurostat (published on 4 October 2020), rounded up to the nearest integer [33].

Annex 3. Technical specifications for laboratory samples for whole genome sequencing

Samples sent without prior approval from ECDC could not be processed (i.e. only samples sent by laboratories that were recruited by nationally designated NSCs and only the number of isolates indicated by Eurofins/ECDC).

DNA samples

DNA samples were purified double-stranded high molecular weight genomic DNA, with the following characteristics:

- Recommended ≥200–500 ng
- Up to 100 µl total volume
- Recommended concentration ≥2 ng/µl
- An OD 260/280 ≥1.8 and an OD 260/230 ≥1.9
- Preferably dissolved in RNAse-, DNAse- and protease-free Tris-HCl buffer (pH 8.0–8.5).

Samples submitted as pellets

Packaging of samples submitted as pellets had to comply with international shipment regulations for biohazardous material. The instructions were as follows:

- Ship 2 × 1 ml overnight culture at least with an optical density of 1 at 600 nm, expecting in each culture on average 8×10^8 cells;

  OR

- A bacterial pellet from equivalent cultures should be provided in 2 ml screw cap tubes;

  OR

- Plated colonies on agar plates (at least 10 colonies with a diameter 0.8 mm).

Annex 4. Metadata for reported samples/isolates

NSCs obtained at least the three mandatory metadata variables (reporting country, isolate identifier, how the isolate was selected for this survey) from participating hospitals for each sample/isolate sent for typing, as well as recommended and/or optional variables, as possible. Reporting hospitals could use national definitions, though preferred definitions were given. Table 1A and 2A (Annex 5) were also available as one Excel sheet that was emailed to NSCs.

Table 1A. Metadata requested for each isolate

Variable name	Full name	Required	Description	Permitted values
ID				
ReportingCountry	Reporting country	Mandatory	The country reporting the record (two-letter country code).	AT, BE, BG, CY, etc.
IsolateId	Isolate identifier	Mandatory	Unique identifier for each isolate within the data source/lab system related to the case. If the laboratory is participating in ECDC CDI surveillance, it can consider storing the relationship between IsolateId and PatientId separately in case these codes are linked later.	NUM
Isolate selection				
HowSelected	How the isolate was selected for this survey	Mandatory	Methodology used to select isolates for this survey, i.e. only imported HA CDI cases (preferred), consecutively detected HA CDI cases (less preferable) or all consecutively detected CDI cases (least preferable).	I = imported HA CDI cases; H = consecutively detected HA CDI cases; C = all consecutively detected CDI cases.
Outbreak	Part of known outbreak	Recommended	Is this isolate part of a known outbreak?	Y/N/Unk
RecurrentCDI	Recurrent CDI	Recommended	Choose 'Yes' if the patient had an episode of CDI (return of diarrhoeal stools with a positive laboratory test after the end of treatment) for more than two weeks and less than eight weeks following the onset of a previous episode.	Y/N/Unk
Date				
DateUsedForStatistics	Date used for statistics	Recommended	Any date associated with the isolate is accepted. Specify which date type is used for this variable in the free text variable TypeOfDate. If multiple dates are available for an isolate, the order of preference for date to report is (most to least preferable): date of onset, date sample taken, date laboratory received the isolate, date laboratory typed, date of laboratory report.	dd/mm/yyyy; mm/yyyy; Q/yyyy
TypeOfDate	Type of date used for date for statistics	Recommended	Specify what type of date was reported in the variable 'DateUsedFor Statistics' (e.g. 'date lab received sample').	Free text
Hospital description				
HospitalId	Hospital ID	Recommended	Unique identifier for each hospital that reports any isolates in this survey (not the source hospital for an imported case). EU/EEA country selected and generated. It is recommended to keep the Hospital ID the same across all ARHAI surveillance protocols (PPS, ICU, ESAC-Net, EARS-Net) from one year to another.	Free text
HospitalDescription	Hospital type	Recommended	The hospital type, according to any national designation scheme (e.g. 'University teaching hospital') or the ECDC scheme (e.g. 'Tertiary hospital').	Free text
HospitalLocation	Region where the reporting hospital is located	Optional	Geographical area (e.g. NUTS 1 or NUTS 2 code) where the hospital that reported this imported CDI case (not the source hospital of an imported case) is located.	NUTS 2 or NUTS 1, or free text
LaboratoryLocation	Region where the reporting laboratory is located	Optional	Recommended if 'HospitalLocation' is unavailable. Geographical area (e.g. NUTS 1 or NUTS 2 code) of the typing laboratory.	NUTS 2 or NUTS 1, or free text
Address	Address of the reporting hospital or laboratory	Optional	Recommended only if the NUTS 1 or NUTS 2 code is unknown for the reporting hospital and laboratory; otherwise, this variable is not required. Preferably provide the address of the hospital that reported this case, or otherwise the reporting laboratory. This address will be used by ECDC to identify the NUTS 1 or NUTS 2 code	Free text
ParticipSurvNetCDI	Participation in ECDC CDI surveillance network	Optional	Participation of the reporting hospital in ECDC surveillance network of *Clostridioides difficile* infections during the previous year.	Y/N/Unk

Variable name	Full name	Required	Description	Permitted values
Subtype information				
PCRRibotype	PCR ribotype of *C. difficile* isolate	Optional	PCR ribotype of *C. difficile* isolate, if known.	Free text
RibotypeMethod	Reason for typing	Optional	Method used to acquire PCR ribotype from *C. difficile* isolate.	RibotypeMethodHAICDI: CE-PCR = Capillary-based PCR; G-PCR = Standard, gel-based PCR; O = Other; WGS = Whole genome sequencing
SequencyType	Sequency type (ST)	Optional	Sequence type of the *C. difficile* isolate, if known.	Free text
GeneticClade	Genetic Clade	Optional	Clade of the *C. difficile* isolate, if known.	Free text
Antimicrobial susceptibility				
METSIR	Final interpretation of metronidazole susceptibility testing results	Optional	Final interpretation of the results of all different susceptibility tests performed for metronidazole. Please use, in order of preference (most to least preferable) EUCAST clinical breakpoints, EUCAST ECOFF, CLSI or national breakpoints.	SIRHAIPPS: S = Susceptible, standard dosing regimen; R = Resistant; UNK = Unknown; NA = Not applicable; I = Susceptible, increased exposure; IR = Intermediate or Resistant (non-susceptible, old classification).
METMICMethod	MIC method for metronidazole susceptibility testing	Optional	Method used to acquire MIC result for metronidazole. The agar type should be reported, due to haem-inducible resistance [34].	Free text
VANSIR	Final interpretation of vancomycin susceptibility testing results	Optional	Final interpretation of the results of all different susceptibility tests performed for vancomycin. Please use, in order of preference (most to least preferable) EUCAST clinical breakpoints, EUCAST ECOFF, CLSI or national breakpoints.	SIRHAIPPS: S = Susceptible, standard dosing regimen; R = Resistant; UNK = Unknown; NA = Not applicable; I = Susceptible, increased exposure; IR = Intermediate or Resistant (non-susceptible, old classification).
VANMICMethod	MIC method for vancomycin susceptibility testing	Optional	Method used to acquire MIC result for vancomycin.	Free text
FDXSIR	Final interpretation of fidaxomicin susceptibility testing results	Optional	Final interpretation of the results of all different susceptibility tests performed for fidaxomicin, according to local/national breakpoints, if applicable. To date, EUCAST has not set the fidaxomicin breakpoint or ECOFF for *C. difficile*, due to the variability between published studies (see EUCAST clinical breakpoints – bacteria (v 12.0)).	SIRHAIPPS: S = Susceptible, standard dosing regimen; R = Resistant; UNK = Unknown; NA = Not applicable; I = Susceptible, increased exposure; IR = Intermediate or Resistant (non-susceptible, old classification).
FDXMICMethod	MIC method for fidaxomicin susceptibility testing	Optional	Method used to acquire the MIC/SIR result for fidaxomicin (e.g. agar dilution).	Free text
CDI outcome				
FatalOutcome	Fatal CDI outcome	Optional	CDI case had national definition of a CDI-associated fatal outcome (e.g. in-hospital death <30 days of admission).	Y/N/Unk
SevereOutcome	Severe CDI outcome	Optional	CDI case had a severe infection according to the national definition. Preferably use the ECDC/ESCMID definition of a 'complicated course of infection'. For example, admission to a healthcare facility for treatment of a community-onset CDI or CDI that resulted in, for example, ICU admission, toxic megacolon, surgery or death <30 days if CDI is primary/contributory cause (see '1.3 Survey definitions' or [27]).	Y/N/Unk

CDI: Clostridioides difficile *infection; CLSI: Clinical and Laboratory Standards Institute; EARS-Net: European surveillance of antimicrobial resistance network; ECOFF: epidemiological cut-off value; ESAC-Net: European surveillance of antimicrobial consumption network; ESCMID: European Society for Clinical Microbiology and Infectious Diseases; EUCAST: European Committee of Antimicrobial Susceptibility Testing; HA CDI: healthcare-associated* Clostridioides difficile *infection; ICU: HAI incidence in intensive care units; NUM: number; PPS: European point prevalence survey of healthcare-associated infections and antimicrobial use in European acute care hospitals; Unk: Unknown.*

Annex 5. Data collection tool to report line list metadata for sent samples/isolates

Participating hospitals were asked to add at least the mandatory (M) variables to this data collection tool, which could be transferred to Excel. The NSCs collated the line lists and submitted them to ECDC via email or via a dedicated SFTP server for their country. Details regarding the desired metadata (Table 1A; Annex 4) and Table 2A were also available as one Excel sheet that was emailed to NSCs.

Table 2A. Line list to report metadata for each reported sample/isolate

ID		Isolate selection			Date		Hospital description				Subtype information					Antimicrobial susceptibility						CDI outcome	
ReportingCountry	IsolateId	HowSelected	Outbreak	RecurrentCDI	DateUsedForStatistics	TypeOfDate	HospitalId	HospitalLocation	LaboratoryLocation	Address	ParticipSurvNetCDI	PCRRibotype	RibotypeMethod	SequencyType	GeneticClade	METSIR	MTRMICMethod	VANSIR	VANMICMethod	FDXSIR	FDXMICMethod	FatalOutcome	SevereOutcome
M	M	M	R	R	R	R	R	O	O	O	O	O	O	O	O	O	O	O	O	O	O	O	O

CDI: Clostridioides difficile *infection; M: mandatory; O: optional; R: recommended.*

European Centre for Disease Prevention and Control (ECDC)

Gustav III:s Boulevard 40
16973 Solna, Sweden

Tel. +46 858601000
ECDC.info@ecdc.europa.eu

www.ecdc.europa.eu

Follow ECDC on social media
Twitter: @ECDC_EU
Facebook: www.facebook.com/ECDC.EU
Linkedin: www.linkedin.com/company/ecdc/

Publications Office
of the European Union