National *Clostridioides difficile* Reference Laboratory Annual Report 2023

Feidhmeannacht na Seirbhíse Sláinte
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Introduction

C*lostridioides difficile* is a leading cause of healthcare associated infection with preventative measures hampered by its increasingly complex transmission patterns. Effective prevention requires a number of intervention methods, including, but not limited to, hand hygiene, contact precautions, effective environmental cleaning and antimicrobial stewardship.

The *C. difficile* National Reference Laboratory (NRL) has been in operation since the end of Q2 2022 with routine sequencing of isolate and stool samples referred to the service since July 2022. In 2023, a total of 1,033 samples were submitted to the Reference Laboratory Service with a total of 992 samples confirmed as toxin positive (96%) by way of PCR. All 992 toxin positive samples were further analysed by whole genome sequencing (WGS) and analysed for the presence of virulence markers, resistant genes and whether any relatedness amongst samples were detected against the Reference Laboratory database. Any samples that were confirmed to have a relationship, resulted in routine cluster reports issued. In addition, monthly line listings were issued to service users and Public Health Departments. Furthermore, the Reference Laboratory Service also prepared, and disrupted, ad-hoc reports to select submitting hospitals at their request, and if deemed warranted, highlighting any issues regarding clusters over a specified time frame.

Towards the end of 2023, the Reference Laboratory Service kindly received funding from AMRIC in order to upgrade one of two MiSeq sequencers to a NextSeq. Furthermore, WGS library preparations are in the process of been automated with the addition of a Tecan Fluent automated liquid robotic handler. The liquid handler was provided to PHL Dublin during the 2019 SARS-CoV pandemic and has undergone alterations in order to perform library preparations using the Illumina DNA prep protocol.

Phenotypic Analysis

During 2022, phenotypic analysis was performed on samples submitted to Reference Laboratory Service. During this time, no phenotypic resistance was detected for vancomycin and metronidazole in all *C. difficile* samples submitted to the Reference Laboratory Service. Therefore, going forward in 2023, it was decided that routine antimicrobial resistance (AMR) testing would not be preformed and instead, sentinel AMR (25% coverage) carried out which is documented in our monthly line listings. Antimicrobial susceptibility testing (AST) by minimum inhibitory concentration (MIC) was performed according to EUCAST guidelines. Interpretation of MICs was based on EUCAST breakpoints for *C. difficile*. A total of 229 isolates were subjected to phenotypic testing in 2023. All isolates were susceptible to metronidazole and vancomycin. At the time of publishing, fidaxomicin susceptibility testing was not offered by the Reference Laboratory Service due to a lack of standardised methods. However, the Reference Laboratory Service is exploring options for testing fidaxomicin going forward in 2024 which is documented in the research section below.

Whole Genome Sequencing Analysis

Whole Genome Sequencing (WGS) was performed on 992 samples that were toxin positive by way of PCR analysis. These samples were submitted by 33 hospitals nationally as part of the *C. difficile* Infection (CDI) Enhanced Surveillance Program. The hospitals included are represented by their hospital code in table 1 below. In addition, the number of samples submitted are listed in table 1, along with the predominant sequence type (ST) and the total number of STs identified within that hospital.

Table 1. Submitting hospitals and the predominant ST recovered.

Submitting Hospital Code	Number of Samples Submitted in 2023	Predominant STs	No. of STs Identified
H03	162	ST11	38
H05	5	ST44	4
H08	47	ST8	18
H10	31	ST8	15
H11	22	ST11	9
H12	1	ST6	1
H16	2	ST2	1
H17	1	ST6	1
H18	30	ST2 & ST6	18
H21	2	ST6	1
H23	25	ST2 & ST42	15
H24	12	ST8, ST37 & ST42	9
H25	14	ST54	7
H27	17	ST6	14
H28	73	ST11	20
H32	153	ST8	33
H35	24	ST11	15
H36	38	ST8	20
H40	10	ST2, ST13 & ST99	7
H42	50	ST3	19
H44	13	ST11	8
H46	30	ST11	14
H47	36	ST11	21
H50	55	ST8	25
H53	4	No Predominant ST	4
H57	3	ST11	2
H58	7	ST3	4
H60	17	ST1	9
H61	4	ST1	2
H62	19	ST11	11
H71	7	ST10	6
H80	27	ST2 & ST6	15
H83	5	ST5	4
H88	1	ST8	1

WGS analysis is performed based on core genome multi locus sequence typing (cgMLST) which compares 2,147 genes across the genome of *C. difficile* for similarities/differences. Relatedness by this method is referred to as allelic differences and this is the number represented on the branches between nodes on a phylogenetic tree. This is not to be confused with single nucleotide polymorphisms (SNPs). The Reference Laboratory Service does not perform whole genome SNP (wgSNP) analysis to date.

While not as discriminative as wgSNP analysis, cgMLST is a superior method of determining relatedness between isolates compared with standard MLST, which compares several house keeping genes, or the older method of ribotyping.

All samples sequenced by the Reference Laboratory Service have to pass a quality cut-off of ≥ 90% coverage across the core genome, otherwise repeat sequencing is performed. Once quality is met, the ST is determined. The total number of STs seen in 2023 were 71 with ST2, ST6, ST8 and ST11 as the predominant types (Fig. 1 & 2). In addition, the presence of virulence genes, as well as the presence or absence of resistant mutations were determined from the WGS data.

Mutations were assessed in the *gyrA* and *rpoC* genes, as well as two mutations in the *rpoB* gene (Fig. 3). In *gyrA*, a C to T mutation is assessed at position T82I while in *rpoC*, a G to A mutation is assessed at position D244Y. In the *rpoB* gene, a G to T mutation is assessed at position V1143F as well as a C to G mutation at position I1074K. These mutations may infer resistance to fluoroquinolones (*gyrA*) and rifampicin (*rpoB* & *rpoC*). In addition, resistance to tetracycline, erythromycin and metronizdole is also determined from the WGS data and noted in the Reference Laboratory Service database.

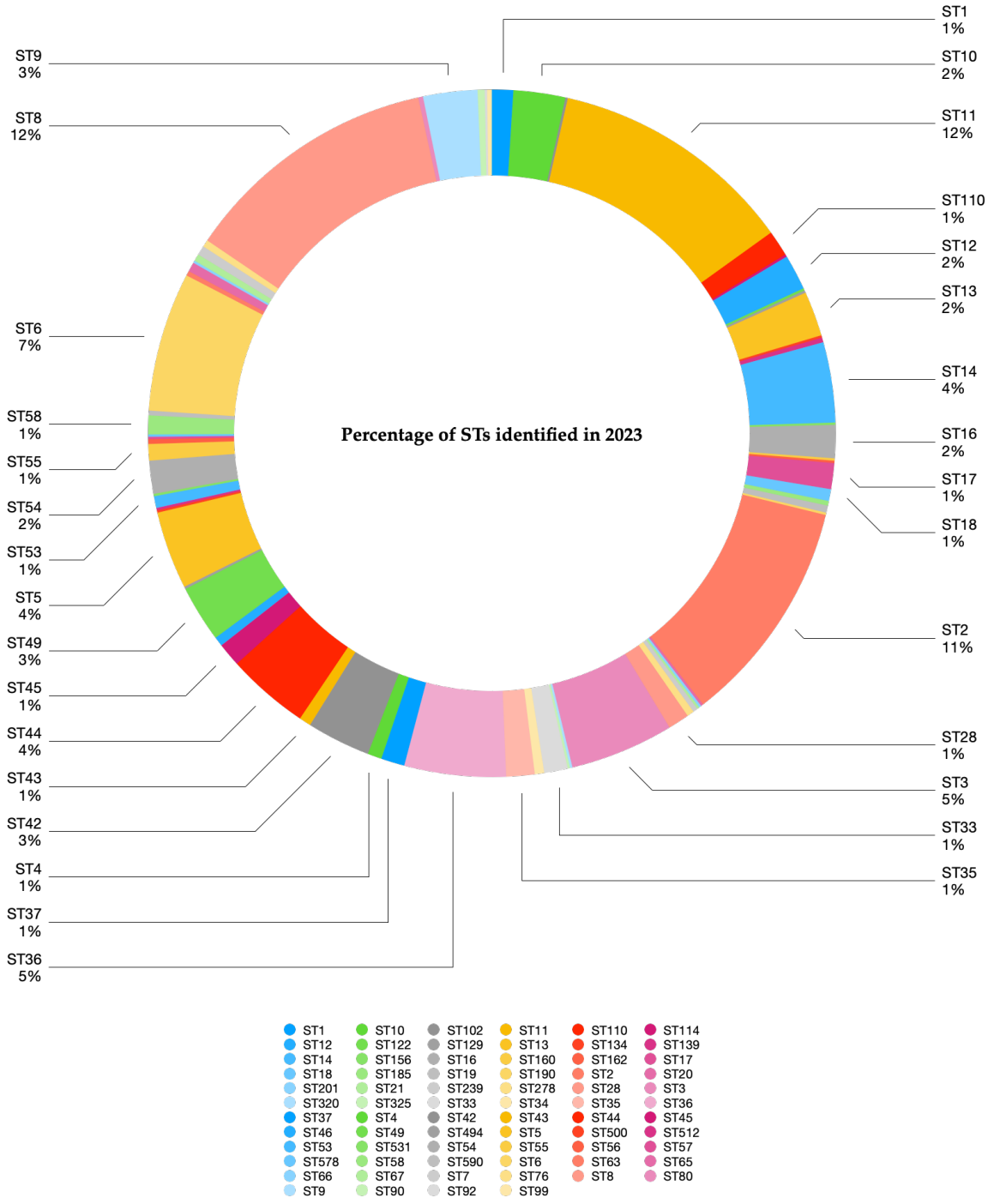


Figure 1: Percentage of STs identified from all submitting hospitals to the National Reference Laboratory in 2023. The predominant STs are ST2 (11%), ST6 (7%), ST8 (12%) and ST11 (12%).

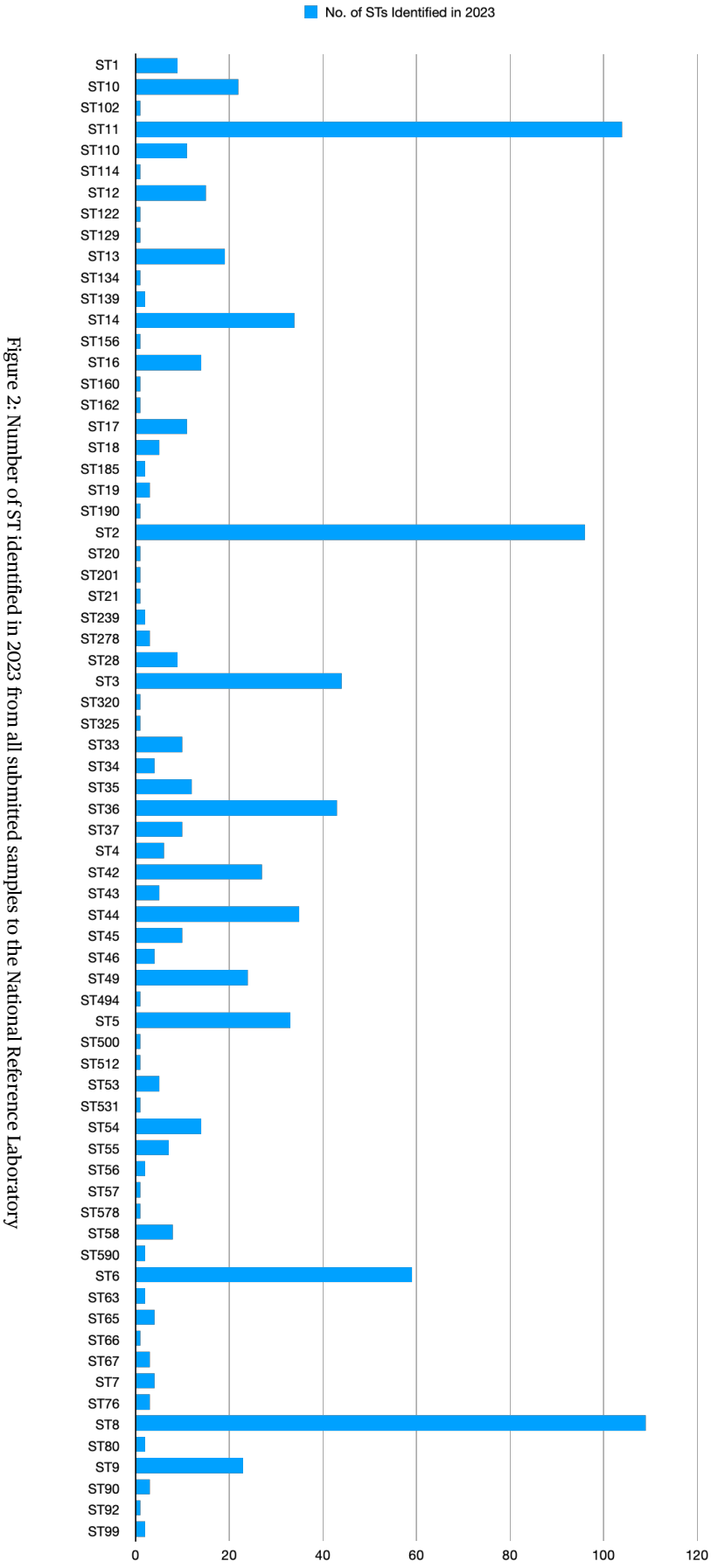
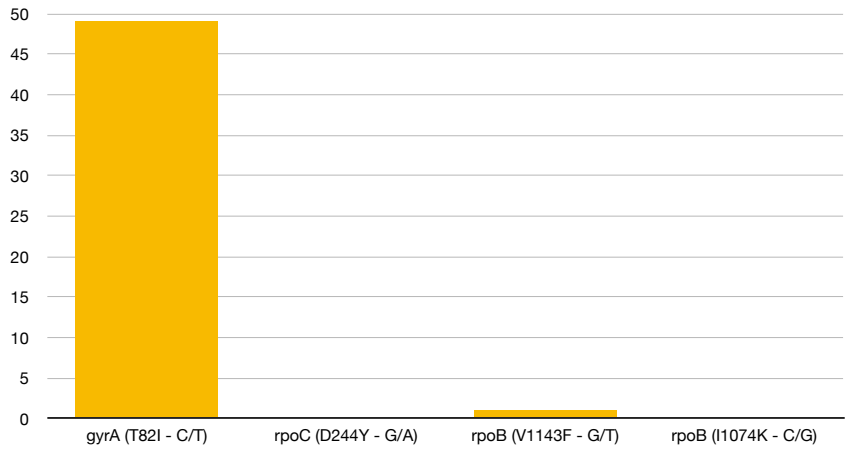


Figure 2: Number of ST identified in 2023 from all submitted samples to the National Reference Laboratory



Mutation	No. Of Samples Containing Mutation
gyrA (T82I - C/T)	49
rpoC (D244Y - G/A)	0
rpoB (V1143F - G/T)	1
rpoB (I1074K - C/G)	0

Figure 3: Bar graph illustrating the number of samples containing mutations in the genes *gyrA*, *rpoB* and *rpoC*

In addition to resistance detection, WGS data was assessed to detected the following virulence genes, *cdtA*, *cdtB*, *tcdR*, *tcdA*, *tcdB*, *tcdC* and *tcdE*. The *tcdA* and *tcdB* genes are well established as the major virulence genes of *C. difficile*, responsible for the production of toxin A and B and noted as risk factors for the recurrence of CDI. In addition, the presence of the binary toxin genes (*cdtA* & *cdtB*) are also noted virulence factors previously associated with severe CDI. Furthermore, *tcdR* and *tcdC* are encoded within the pathogenicity locus which positively and negatively regulate toxin expression respectively. Finally, the *tcdE* gene codes for a protein that facilitates the release of *C. difficile* toxins to the extracellular environment. Figure 4 below illustrates the percentage of samples submitted to the Reference Laboratory Service in 2023 that contained each of these genes.

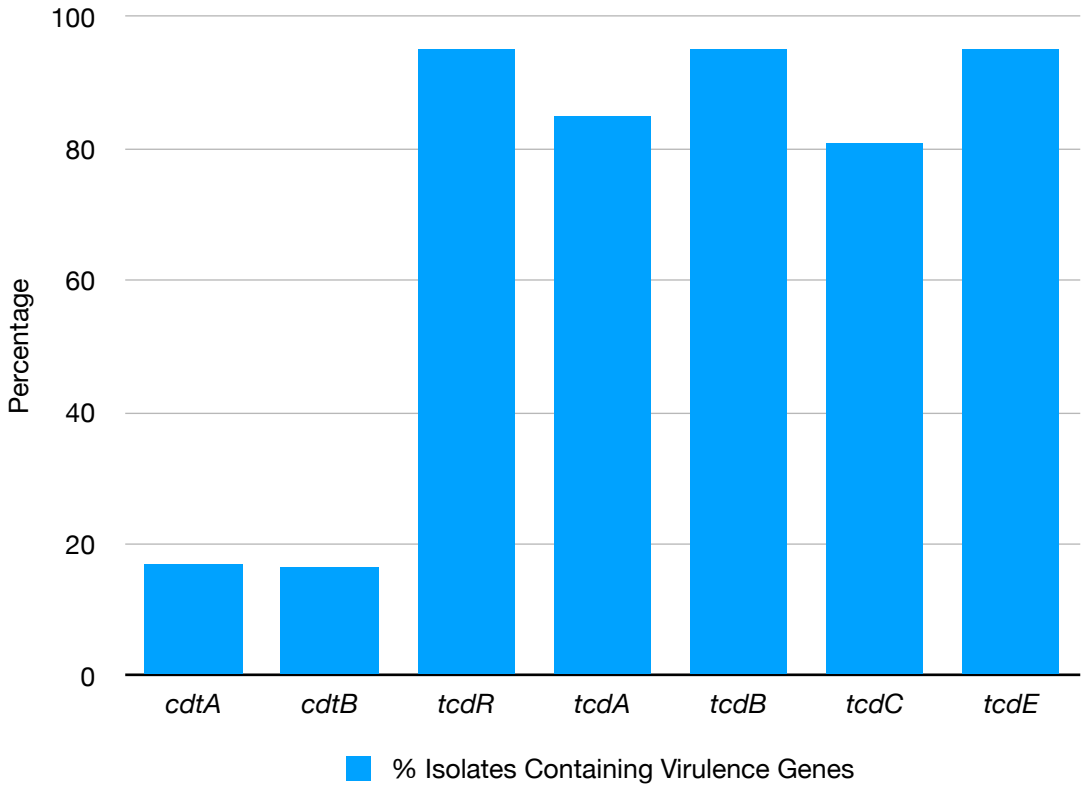


Figure 4: Bar graph illustrating the number of samples containing virulence gene factors

With the exception of the binary toxin genes *cdtA* and *cdtB*, all submitted samples contained over 80% of virulence genes associated with the production of, and facilitating release of, *C. difficile* toxins. The minority of samples that did not contain *tcdA* (15%), contained *tcdB* only. Conversely, the minority of samples that did not contained *tcdB* (5%), contained *tcdA* only.

Finally, cluster analyse was performed in order to determine whether samples were related to those stored in the Reference Laboratory Service database. Figure 5 below illustrates all samples submitted to the Reference Laboratory Service during 2023 in a minimum spanning tree (MST). Samples are colour coded based on ST. Any sample that

clustered by 2 or less allelic differences were considered closely related, or a recent transmission event, and therefore reported to our service users with a unique cluster ID code.

During 2023, the Reference Laboratory Service confirmed the presence of 143 clusters which were reported to the submitting hospitals and relevant Public Health Departments.

In addition, figure 6 illustrates an additional MST, however, the nodes are colour coded based on the submitting hospital code. Clustered samples can be seen on the same node, however, no distinct grouping pattern can be determined. While both trees (Fig. 6 & 7) appear similar in structure side by side, the comparison of both illustrates the vast differences in STs spanning across Irish hospitals.

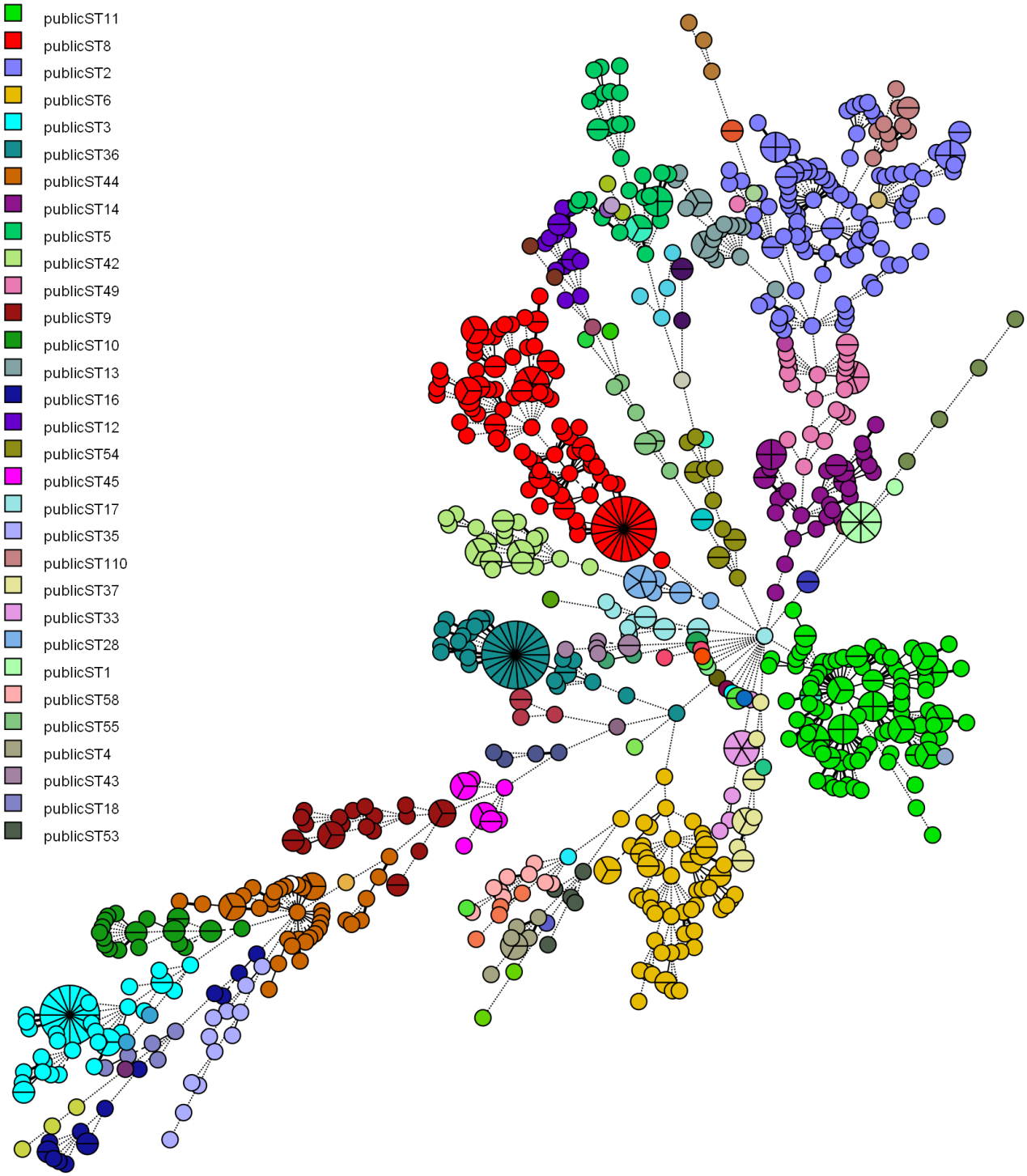


Figure 5: Minimum spanning tree (MST) of 2023 Isolates submitted to the Reference Laboratory. The tree is colour coded by sequence type (ST) and illustrates how each ST is grouped together. The legend illustrates only a selection of STs, of which are the most prevalent. This includes ST2, ST3, ST6, ST8, ST11 and ST36.

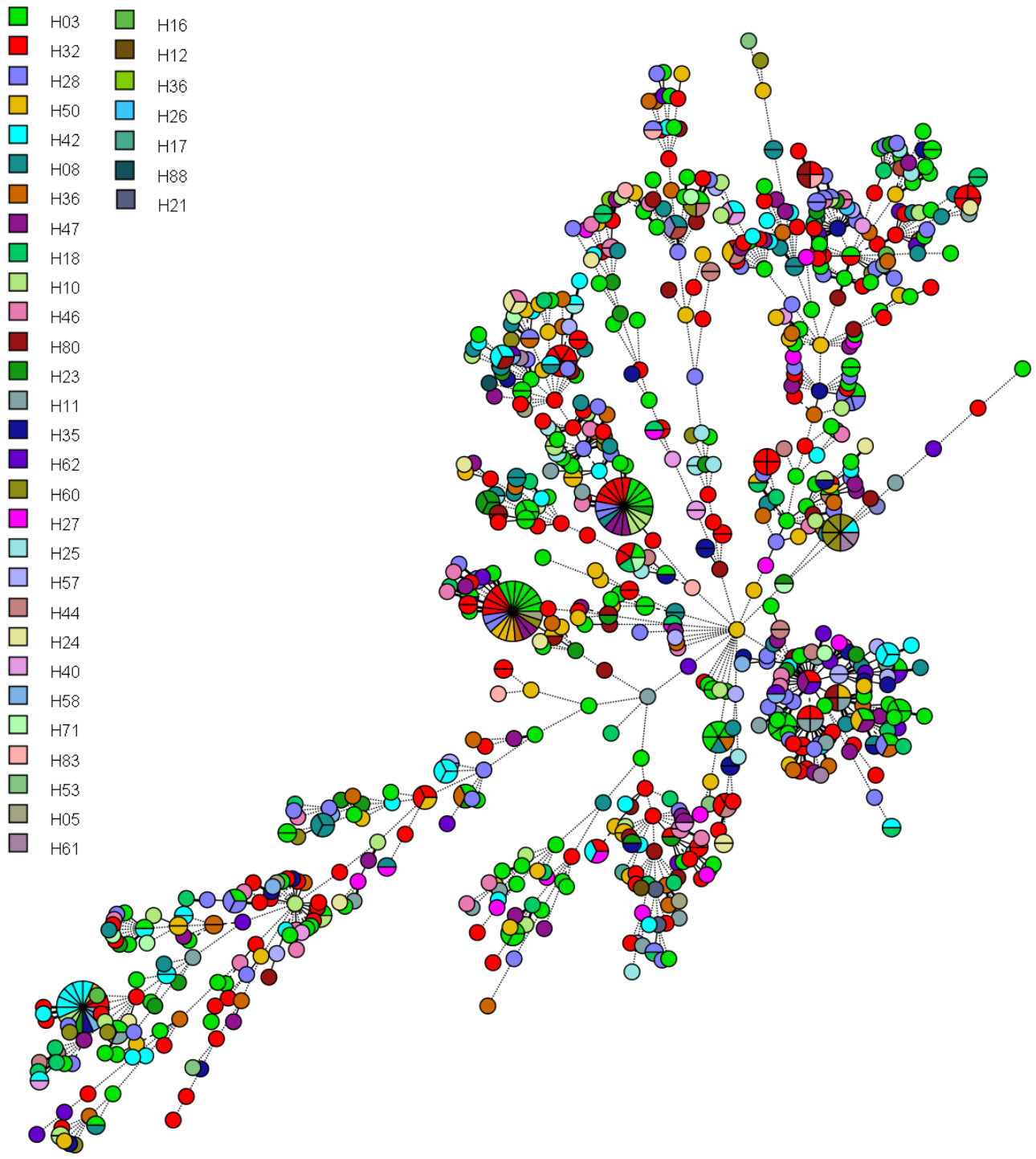


Figure 6: Minimum spanning tree (MST) of 2023 Isolates submitted to the Reference Laboratory. The tree is colour coded by hospital code. Some isolates cluster which can be seen from those that are on the same nodes.However, in generally, there is no distinct pattern.

Research Projects

Development of Fidaxomicin Susceptibility Testing

Dr. Tee Keat Teoh M.B. B.Ch. B.A.O. M.D. FRCPath. UK, FFac. Path. FRCPI
Dr. Peter Flanagan Ph.D. M.Sc. B.Sc.

In 2024, the Reference Service will begin a study to determine fidaxomicin susceptibility concentrations against *C.difficile* isolates. This will be performed by way of disc diffusion assay were multiple concentrations of fidaxomicin will be impregnated into 6 mm blank susceptibility discs and placed on fully confluent agar. To date, the Reference Laboratory Service has one control fidaxomicin resistant isolate and one clinical isolate that demonstrated intermediate fidaxomicin resistance based off of WGS analysis. This particular isolate has a GTT to GAT nucleotide substitution resulting in a V1143D mutation and is the only known isolate to date in our database that has a mutation that may infer decreased susceptibility to fidaxomicin. At the time of publication, the Reference Laboratory Service has begun initial testing with the control isolate, clinically resistant isolate (determined by WGS) and a selection of clinal isolates deemed susceptible based on WGS. A 20 μg concentration has been used in an initial pilot study with the resistant isolates demonstrating no zone of inhibition and the susceptible isolates showing X mm zones of inhibition. The Reference Laboratory Service will provide an update to this on going work in a future report.

Investigation of Genotypic Sequences in order to Correlate with Phenotypic AMR EUPHEM Fellowship

Dr. Eleanore McNamara M.B. FRCPath UK FFPath FRCPI
Dr. Anne Carroll PhD. Dip.Mgmt. DipSTLiH. B.Sc. FACSLM
Dr. Lieke Brouwer M.D. Ph.D.

Currently, the Reference Laboratory determine antimicrobial resistance in *C. difficile* isolates to a limited panel of antibiotics. Screening for a wider panel would allow better monitoring of the changes in antimicrobial resistance in *C. difficile* within Ireland over

time. However, phenotypic testing is time consuming and labour intensive, and screening for a wider panel of antibiotics on a routine basis cannot be performed.

Therefore, the Reference Laboratory Service undertook genomic sequencing to determine, to what extent, genotypic determinants of AMR correlate with phenotypic resistance. This study involved looking at 99 *C. difficile* isolates taken from 2022, and testing them phenotypically for resistance to eight antibiotics (vancomycin, rifampicin, metronidazole, cefoxitin, clindamycin, moxifloxacin, tetracycline and imipenem). All isolates were subjected to WGS and the genomes screened for a panel of acquired genes and point mutations that was constructed specifically for this purpose. The results of this project are currently pending publication.

It is hoped that through this project, we can identify antibiotics for which the genotypic markers can, to a large extent, predict phenotypic resistance in *C. difficile* isolates. If this is the case, we will be able to monitor more closely the patterns and changes of antimicrobial resistance in the *C. difficile* isolates circulating in Ireland, without the need for labour intensive phenotypic testing.

Publications

Genotypic and Phenotypic Antimicrobial Resistance of *Clostridioides difficile* in The Irish Population, 2022 - L. Brouwer, A. Carroll & E. McNamara *Under review with the Journal of Anaerobe*

Concluding Remarks

Now in its 2nd year, and with the purchase of the NextSeq and automation of libraries using the Tecan Fluent liquid handling system, the Reference Laboratory Service aims to stream line processes in 2024 at a cost saving to the laboratory. Furthermore, the automated liquid handler system will allow re-deployment of senior medical scientists in order to up skill their knowledge of bioinformatic analysis using command line tools. It is hoped that the National Reference Service will begin to run command line whole genome single nucleotide polymorphism (wgSNP) analysis alongside core genome multi-locus (cgMLST) analysis. Currently, the Molecular Epidemiologist and Senior Scientist are trained in command line analysis. Both senior Medical Scientists employed by the National Reference Laboratory Service have no training. Up skilling of the Senior Medical Scientists will greatly increase the analysis and research capabilities, as well as future proof the National Reference Service going forward.

With the liquid handler system in place, all sample extractions will be performed on Fridays with libraries, including VTEC and Campylobacter, prepared on Mondays for sequencing to commence that evening or the following morning. However, the Service does appreciate that from time to time, urgent outbreak scenarios my arise. In this instance, the Reference Service will retain a small number MiSeq Micro kits on site for ad-hoc sequence for increased turn around times in the event of these priority/outbreak cases. However, this service will only be provided where warranted.

In order to engage with our service users, the *C. difficile* National Reference Laboratory will aim to host a morning webinar in order to determine feedback on the service. In addition, we will aim to teach our users of the work flow and explain how the service will advance over the coming year. It is planned that cluster reporting will decrease and instead, line listing reports issued more frequently. This will also be discussed and users given a demonstration on how to filter the line listing.

As the Reference Laboratory Service is still in its infancy, we do envision changes may still occur in order to streamline processes and as we develop new process. For example, the possibility of fidaxomicin testing. We do encourage our service users to not hesitate to contact use using the new NRL email address cdiff.nrl@hse.ie should they have any queries going forward.

Lastly, we look forward to continuing working with all our service users to continue to progress the service for the benefit of all stakeholders across the country. We plan to collaborate closely with the HPSC, AMRIC, Departments of Public Health and hospital-based Microbiologists and Infection Prevention and Control Teams to ensure that the work done ultimately benefits the general public and patients.