Trinity College Dublin
Coláiste na Tríonóide, Baile Átha Cliath
The University of Dublin

An Roinn Talmhaíochta,
Bia agus Mara
Department of Agriculture,
Food and the Marine

An Investigation into the Epidemiology and Genetic Relatedness of pig and human *Clostridioides difficile* isolates in Ireland

Aoife Doyle

A thesis submitted to the University of Dublin in fulfilment of the requirements for the degree of Doctor of Philosophy

Department of Clinical Microbiology,
School of Medicine, Trinity College Dublin

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Declaration

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Abstract

Historically, *Clostridioides difficile* (*C. difficile*) has been associated with nosocomial diarrheal illness in older, immunocompromised patients, particularly in those on long-term antibiotics. However, cases of *C. difficile* infection are increasingly being reported among community and younger patients with no recent hospitalisation. Strains of *C. difficile* that are associated with higher virulence and more severe CDI have been found in food animals, such as pigs, which suggests the potential role for foods as a source of community-associated CDI. In addition, prophylactic antibiotics are regularly used in pig rearing, which could lead to the emergence of antimicrobial resistance. Additionally, *C. difficile* has also been widely isolated from the environment, particularly from soil and water. The objective of this study was to understand the recent shift in *C. difficile* epidemiology by exploring the impact that pigs may have on the dissemination of *C. difficile*.

This project included a longitudinal study from birth to slaughter was conducted on pig farms in Ireland with varied levels of in-feed antibiotic use. The most prevalent strains found in pig samples are associated with more severe CDI and infections in the community. This suggests the potential for *C. difficile* to be transmitted through the food chain.

Moreover, a whole genome sequencing analysis was carried out to investigate if there were any genetic relationships between *C. difficile* isolates from pigs and humans. It was clear from the results of this analysis that *C. difficile* sequence type 11 isolates from pigs and humans were closely related, and subsequently a large part of this study focused on characterising the genotype and phenotype of the isolates belonging to this sequence type. Additional sequence files for human *C. difficile* sequence type 11 isolates were obtained to elucidate if there was a relationship between pigs, different hospitals, and the environment. Resistance determinants for tetracycline were found in a high proportion of sequence type 11 *C. difficile* genomes. Tetracycline use in agriculture, coupled with selective pressure and international dissemination through the food chain, may explain the heightened prevalence of sequence type 11 in humans. The formation of a complex network of *C. difficile* isolates suggested that transmission between pigs, humans and the environment is possible. This epidemiological link

highlights the necessity of a “One Health” approach, and that the dissemination of *C. difficile* may not be confined by geographical regions or populations.

This thesis contributes valuable insights into the prevalence, characteristics, and epidemiology of *C. difficile* in animals and humans, and emphasises the potential implications for public health and food safety. The application of whole genome sequencing is superior to routine typing methodologies for exploring the epidemiology of *C. difficile*, impacting outbreak investigation, understanding phylohistory, and phylogeography of *C. difficile*.

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Presentations and Publications

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Courses

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List of Abbreviations

Abbreviation	Definition
%	Percentage
ABI	Applied Biosystems
AMOX	Amoxicillin
AMR	Antimicrobial Resistance
AMU	Antimicrobial Usage
AMURAP	Antimicrobial Use and Resistance in Animal Production (project)
ANOVA	Analysis of Variance
ARGs	Antimicrobial Resistance Genes
ASID	Australasian Society for Infectious Diseases
AST	Antimicrobial Susceptibility Testing
ATCC	American Type Culture Collection
ATP	adenosine triphosphate
AUG	Amoxicillin/clavulanic acid
BHI	Brain Heart Infusion
BHIS	Brain Heart Infusion Supplemented
bp	Base pair
BTS	Bacterial Test Standard (Bruker)
CA	Community Associated
CBA	Columbia Blood Agar
CCEY	Cefoxitin Cycloserine Egg-Yolk (agar)
CCP	Critical Control Point
CD	*Clostridioides difficile*
CDC	Centers for Disease Control and Prevention
CDI	*Clostridioides difficile* Infection
CDMN	*Clostridioides difficile* Moxalactam Norfloxacin *(agar)*
CDRN	*Clostridium difficile* ribotyping network
CDT	*Clostridioides difficile* binary toxin
CFU	Colony Forming Unit
CG	Clonal Group
CHL	Chloramphenicol
CIR	Crude Incidence Rate
CLI	Clindamycin
CLSI	Clinical & Laboratories Standards Institute
CON	Control
COVID	Coronavirus Disease
CPL	Central Pathology Laboratory
CSO	Central Statistics Office
DAFM	Department of Agriculture, Food, and the Marine
DNA	Deoxyribonucleic Acid
ECDC	European Centre for Disease Prevention and Control

EIA	Enzyme Immunoassay
EPL	Enteric Pathogens Laboratory
ERY	Erythromycin
ESCMID	European Society of Clinical Microbiology and Infectious Diseases
ESGCD	European Society of Clinical Microbiology and Infectious Diseases Study Group for *Clostridium difficile*
EU	European Union
EUCAST	European Committee on Antimicrobial Susceptibility Testing
FAA	Fastidious Anaerobic Agar
FAM	6-Carboxyfluorescein
FASTQ	Text-based sequencing data file format
FASTQC	Quality control analysis tool for FASTQ files
FMT	Faecal Microbiota Transplant
FOX	Cefoxitin
GDH	Glutamate Dehydrogenase
GDPR	General Data Protection Regulation
GERD	Gastroesophageal reflux disease
GI	Gastrointestinal
GP	General Practice
HA	Healthcare Associated
HACCP	Hazard Analysis & Critical Control Point
HAU	High Antibiotic Use
HCCA	a-Cyano-4-hydroxycinnamic acid
HPSC	Health Protection Surveillance Centre
HSE	Health Services Executive
IBS	Irritable Bowel Syndrome
ICU	Intensive Care Unit
ID	Infectious Disease
IDSA	Infectious Diseases Society of America
IDT	Integrated DNA Technologies
IMI	Imipenem
ISR	Intergenic Spacer Region
IVD	In vitro Diagnostics
LAU	Low Antibiotic Use
MALDI	Matrix-Assisted Laser Desorption/Ionization-Time Of Flight
MAU	Moderate Antibiotic Use
MBT	MALDI Biotyper
MDR	Multi Drug Resistant
MHB	Mueller Hinton Broth
MIC	Minimum Inhibitory Concentration
MLS_B	Macrolide-Lincosamide-Streptogramin B
MLST	Multilocus Sequence Typing
MRD	Maximum Recovery Diluent

MRSA	Methicillin Resistant *Staphylococcus aureus*
MS	Mass Spectrometry
MSP	Matrix-Assisted Laser Desorption/Ionization-Time of Flight steel plate
MST	Minimum Spanning Tree
MXF	Moxifloxacin
NAAT	Nucleic Acid Amplification Test
NAP	North American Pulsed-field gel electrophoresis type
NCBI	National Center for Biotechnology Information
NCEC	National Clinical Effectiveness Committee
NCTC	National Collection of Type Cultures
NEB	New England Biolabs
NJ	Neighbour Joining
NRL	National Reference Lab
OPT-80	Fidaxomicin
PCR	Polymerase Chain Reaction
PEN	Penicillin
PFGE	Pulsed Field Gel Electrophoresis
PH	Pig (High Antibiotic Use)
PIP	Piperacillin
PL	Pig (Low Antibiotic Use)
PM	Pig (Moderate Antibiotic Use)
PMC	Pseudomembranous colitis
POS	Positive
PPE	Personal Protective Equipment
PPI	Proton Pump Inhibitor
PRJNA	BioProject accession number
QRDR	Quinolone Resistance-determining Regions
RNA	Ribonucleic Acid
rRNA	ribosomal Ribonucleic Acid
RT	Ribotype
SDW	Sterile Distilled Water
SEM	Standard Error of the Mean
SJH	St James's Hospital
SKESA	Strategic k-mer Extension for Scrupulous Assemblies
SLS	Scientific Laboratory Supplies
SNP	Single Nucleotide Polymorphism
SNV	Single Nucleotide Variant
SPD	Sir Patrick Dun Laboratory
ST	Sequence Type
TBE	Tris-borate Ethylenediaminetetraacetic acid (EDTA)
TCD	Trinity College Dublin
TES	2-Tris(hydroxymethyl)-methyl-2-amino 1-ethanesulfonic acid (buffer)
TET	Tetracycline

™	Trademark
UCD	University College Dublin
UK	United Kingdom
US	United States
USA	United States of America
UV	Ultraviolet
v/v	Volume per Volume
VAN	Vancomycin
VFDB	Virulence Factor Database
w/v	Weight per Volume
WEBRIBO	Web-based repository of electronic *Clostridioides difficile* PCR ribotyping data
WGS	Whole Genome Sequencing
WHO	World Health Organisation

1 General Introduction

Chapter 1

1.1 The genus Clostridium

The genus *Clostridium* are rod-shaped, Gram-positive, anaerobic bacteria, and are a member of the *Peptostreptococcaceae* family. *Clostridium butyricum* is the type species for the *Clostridium* genus, and historically it was the general repository for any Gram-positive, spore-forming, anaerobic organisms (Lawson and Rainey, 2016). With the advancement of molecular biological methods, the phylogenetic diversity of the *Clostridium* genus was revealed. As a result of 16S rRNA gene sequencing, the *Clostridium* genus was acknowledged as *Clostridium sensu stricto* and contained the type species *Clostridium butyricum* and related species. This had ramifications for *Clostridioides difficile*, as phylogenetic analysis showed that it was genetically distinct from other members of the *Clostridium sensu stricto* genus (Lawson et al., 2016). It was originally proposed by Yutin and Galperin (2013) to rename the genus *Peptoclostridium*, but there were concerns that the name change would have international ramifications, as the name "*C. difficile*" or "*C. diff*" was colloquial in both clinical and commercial settings worldwide (Lawson et al., 2016, Rupnik, 2016). Changing the initial letter from "C" would have wider implications including, but not limited to, changing government guidelines, brand names and trademarks, and education materials (Lawson et al., 2016). It has been recommended to rename the genus as *Clostridioides*, and the type species as *Clostridioides difficile* but both names, *Clostridioides difficile* and *Clostridium difficile* can currently be used (Oren and Rupnik, 2018). In addition, this name change was appropriate as it minimises misunderstandings when both names are used interchangeably, particularly in the medical field where inconsistent terminology can create risks (The Lancet, 2019). However, *Clostridioides difficile* is the recommended terminology and will be used henceforth in this thesis.

1.1.1 History of *Clostridioides difficile*

C. difficile is a spore forming bacterium which has been recognised as an important emerging pathogen in both humans and animals. This bacterium is accepted as a frequent cause of nosocomial diarrhoea and the most common cause of pseudomembranous colitis in healthcare facilities (Salen and Stankewicz, 2017). There

were several steps in the discovery of *C. difficile* as an important causative agent of antibiotic-associated colitis (Figure 1.1). First identified as a bacterium from neonatal faeces by Hall and O'Toole (1935), *C. difficile* was initially given the name *Bacillus difficilis* due to the difficulty associated with its culture and isolation *in vitro*. It was recognised as a potential enteric pathogen in the late 1970s, when cytotoxin was found in the faeces of patients with pseudomembranous colitis after antibiotic treatment with the antibiotic clindamycin (Larson et al., 1977). George and co-workers discovered that the organism was responsible for a large percentage of antibiotic-associated diarrhoea cases (George et al., 1978). The group reported that *C. difficile* was responsible for pseudomembranous colitis and that susceptibility to *C. difficile* infection (CDI) increases after antimicrobial treatment because of an alteration in the bacterial flora of the gut. Finally, it was demonstrated that vancomycin therapy had a positive clinical outcome for patients with pseudomembranous colitis as it eliminates toxin-producing *C. difficile* from the colon and it was thereafter recommended as treatment for CDI (Keighley et al., 1978).

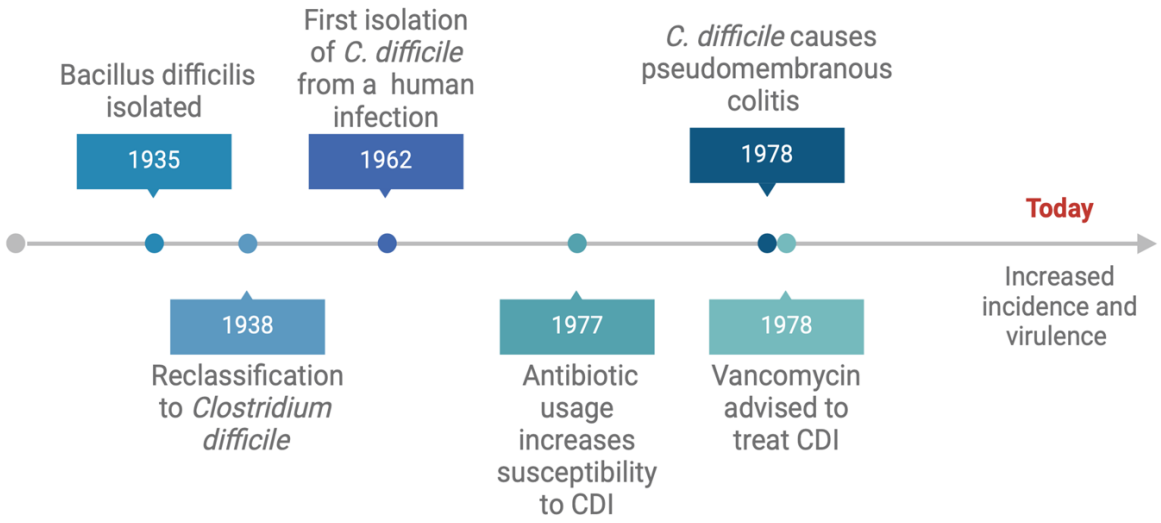


Figure 1.1 A summary of the early history of *Clostridioides difficile* in humans.
CDI: *C. difficile* infection; PMC: Pseudomembranous colitis. Adapted from Rodriguez et al. (2016b).

1.2 *C. difficile* infection (CDI)

C. difficile requires anaerobic conditions to grow, meaning that it exists in conditions without oxygen, and is ubiquitous in the environment and the gastrointestinal tract of humans and animals (Czepiel et al., 2019). This pathogen can cause severe gastrointestinal illness and, having been associated with CDI in 1977 after clindamycin usage, *C. difficile* is now established as the primary bacterial cause of antibiotic-associated diarrhoea accounting for 15-25% of all cases globally (Depestel and Aronoff, 2013, McFarland et al., 2016). There has been a distinct increase in the incidence of CDI since the turn of the 21st century, with *C. difficile* now being among the most common etiological agents of nosocomial illness (Gupta and Ananthakrishnan, 2021). Nosocomial infections, also referred to as hospital-acquired (HA) infections, are infections that are acquired from 48 hours after admission to a healthcare facility, and are not present at the time of admission (Lim et al., 2020b, Slimings and Riley, 2021). The incidence rate of *C. difficile* has now surpassed that of Methicillin Resistant *Staphylococcus aureus* (MRSA) as the leading cause of nosocomial infections, with studies from European hospitals revealing that the incidence of nosocomial CDI is twice as high as the incidence of nosocomial MRSA (both colonisation and infection) (Meyer et al., 2012).

1.2.1 Clinical features of CDI

Typical clinical features of CDI include watery diarrhoea, abdominal pain and cramps, fever, hypotension, and leucocytosis (Gerding et al., 2016). Currently, the accepted criterion for diarrheal symptoms and the subsequent diagnosis of CDI is at least three or more unformed stools within a 24-hour period (van Prehn et al., 2021). Manifestations of CDI can range from mild diarrheal illness to severe disease, including pseudomembranous colitis, sepsis, and in some cases, CDI can lead to death. Pseudomembranous colitis is a condition that is most commonly caused by CDI, and causes acute inflammation of the colon (Salen and Stankewicz, 2023). One of the major problems associated with CDI is the high risk of recurrence, which occurs among approximately 13% to 30% of cases following an initial episode, and among 60% of cases after multiple prior recurrences (Song and Kim, 2019, Leffler and Lamont, 2015). Recurrent CDI (rCDI) is defined by European guidelines as a CDI that occurs within eight

weeks of a previous infection, once the original symptoms have resolved and the treatment regimen has been completed (van Prehn et al., 2021, HPSC, 2021).

1.2.2 The epidemiology of CDI in Ireland

In May 2008, new cases of CDI became a notifiable disease in Ireland (HPSC, 2021). Until then, there was limited information on CDI in Ireland. However, there was potential underreporting of the true burden of the disease from 2008 to 2012, as recurrent cases of CDI were not captured. In August 2009, the national enhanced surveillance system was established, which records the origin, onset and severity of CDI cases in Ireland (HPSC, 2021). In 2012, both recurrent and new cases of CDI in Ireland became notifiable to the HSE as part of the Infectious Diseases Regulations 1981 and subsequent amendments (HPSC, 2021).

1.2.2.1 Annual data from the Health Protection Surveillance Centre (HPSC)

This data was retrieved from the Annual Epidemiological report produced by the HPSC (2021) on CDI in Ireland. There was no annual report for 2019 because of the impact of the COVID-19 pandemic on infection surveillance in Ireland. From 2020 to 2021 there were 3,530 cases of CDI reported to the enhanced surveillance scheme. Of these, 3,055 (86.5%) were new cases and 230 (6.5%) were recurrent cases, with the remainder (n=245) being of unknown case type. Healthcare-associated (HA) infections occurred in 1,957 (55.4%) of cases and 1,100 (31.1%) were community-associated (CA). Severe cases of CDI, where the patient required ICU admission or colectomy, occurred in 86 patients (2.5%) between 2020 and 2021. The crude incidence rate (CIR) for new and recurrent CDI in 2020 to 2021 ranged from 30.7 to 32.8 per 100,000 of the population, respectively. The average female to male infection ratio was 3:2.

1.2.2.2 Quarterly data from the *C. difficile* National Reference Laboratory (NRL)

The most recent data on CDI in Ireland relates to the period from quarter 4 (Q4) 2022 to quarter 1 (Q1) 2023 and may be retrieved from the Q1 National Report on the Enhanced Surveillance of *Clostridioides difficile* Infection in Ireland (HSE-HPSC and NRL, 2023). The National Reference Laboratory (NRL) for *C. difficile* was established in 2021, and offers whole genome sequencing (WGS), with 59 acute hospitals (public and private) now participating in the National Enhanced CDI Surveillance (HSE-HPSC and NRL, 2023).

During Q4 of 2022 to Q1 of 2023, there were 891 cases of CDI reported to the enhanced surveillance scheme in Ireland. Of these, 776 (87.1%) were new cases and 80 (8.9%) were recurrent cases, with the remainder (n=35, 3.9%) being of unknown case type. HA infections occurred in 515 (57.8%) cases and 293 (32.8%) were classified as CA-CDI. Severe cases of CDI occurred in 28 (3.14%) of total CDI cases between Q4 of 2022 and Q1 of 2023. Most patients were aged over 65 years (67%), which is an important risk factor for the development of CDI.

1.2.3 Risk factors for CDI

The US Centers for Disease Control and Prevention (CDC) has listed *C. difficile* as an urgent antibiotic resistance threat, with "urgent" being the highest level of concern to human health (CDC, 2019). This brings to the fore the influence of these bacterial infections on both patients, healthcare systems, and the economy. Prevention of CDI is imperative to reduce the burden on healthcare facilities. Infection control measures together with antimicrobial stewardship can help to decrease incidence rates and transmission events (Davies et al., 2020). Although antibiotic stewardship is more about reducing the risk of CDI in individual patients, it is particularly important to identify patients who are at heightened risk of infection and implement the appropriate interventions to reduce the clinical burden on patients and healthcare facilities.

C. difficile is found in the human gut and animals, and is considered part of the normal intestinal microbiota (Bien et al., 2013). It is estimated that 2-5% of healthy adults are colonised with *C. difficile* without any clinical symptoms, although not always with toxigenic strains (Schaffler and Breitruck, 2018). Although the prevalence of CDI is low in healthy adults, this can rise dramatically when exposed to certain risk factors. There are several risk factors for developing CDI including advanced age (patients aged over 65 years), antibiotic usage, prior admission to healthcare facilities, underlying medical conditions, use of proton pump inhibitors (PPIs) and gastrointestinal co-morbidity (Bagdasarian et al., 2015). The production of spores also contributes to the persistence of *C. difficile* in the gut and the environment and recurrence of infection because spores are resistant to antibiotic therapy (Song and Kim, 2019). Once the *C. difficile* spores have germinated to vegetative cells, they are susceptible to antibiotics (Song and Kim, 2019).

1.2.3.1 The gut microbiota

The gut microbiota (also known as the microbiome) is a collection of bacteria, archaea and eukarya that colonise the GI tract (Thursby and Juge, 2017). The gut microbiota provides many benefits to the host in a wide range of physiological functions such as maintaining gut integrity, providing protection against pathogenic bacteria, ensuring homeostasis of host immunity and metabolic functions (Thursby and Juge, 2017, Kho and Lal, 2018). Growth of the bacterium is suppressed by competition from other anaerobic bacteria in the gut, but when the normal microbiota is disturbed by antibiotics *C. difficile*, an opportunistic pathogen, can begin to proliferate, as illustrated in Figure 1.2 (Pilmis et al., 2020). Thus, antibiotics are a major instigator in CDI. Broad spectrum antibiotics such as clindamycin, broad-spectrum beta-lactams including cephalosporins, and fluoroquinolones are commonly associated with gut dysbiosis and consequently are implicated in predisposition to CDI (Bagdasarian et al., 2015).

1.2.3.2 The ageing gut microbiota and CDI

Ageing is linked with physiological changes that contribute to immune senescence, or failure of the immune system response (Davies et al., 2020, Jump, 2013). Advancing age is associated with less colonisation resistance, which could be as result of a decreased bacterial diversity in the gut in comparison to younger individuals (Calder et al., 2022, Rea et al., 2012). For example, the abundance and diversity of anaerobic gut microbiota such as *Bacteroides* spp. and *Bifidobacteria* spp. decreases with ageing, while the age-related increase in certain facultative anaerobes, such as *Enterococcus* spp. is widely reported (Biagi et al., 2013, Claesson et al., 2011). CDI disproportionally affects older populations, and in 2023, the majority of patients (67%) in Ireland were aged 65 years or older (HSE-HPSC and NRL, 2023). The elderly are at a heightened risk of infection due to intrinsic physiological factors associated with ageing, and this risk is increased further when they are exposed to healthcare facilities and antibiotics (Donskey, 2017a).

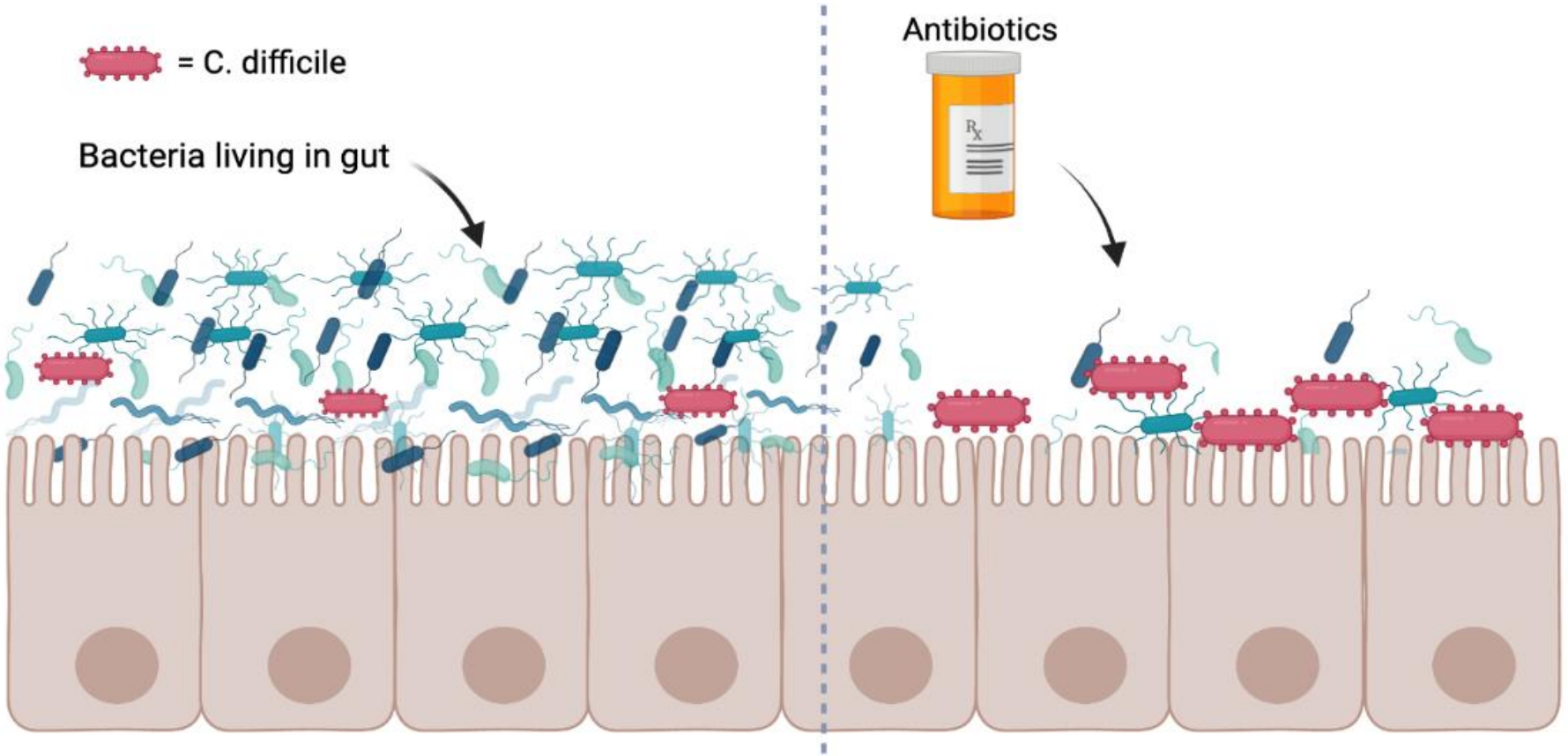


Figure 1.2 The effect of antibiotics on the intestinal microbial community.
The gut microbiota plays a crucial role in inhibiting the overgrowth of pathogenic bacteria (Pilmis et al., 2020). Antibiotics kill bacteria, both good and bad, in the body. This allows opportunistic pathogenic bacteria resistant to antibiotics, such as *C. difficile*, to begin to proliferate, a process called dysbiosis

1.2.4 Community acquired *C. difficile* infection (CA-CDI)

Historically, *C. difficile* has been associated with nosocomial diarrheal illness in older, immunocompromised patients, as discussed in section 1.2.3. However, CDI cases in the community are increasingly being reported (Pituch, 2009, Al Assaad et al., 2020). The emergence of WGS techniques and increased surveillance have indicated dramatic changes to the epidemiology of CDI and the proportion of CDI that is arising from clinical settings and healthcare facilities has shifted. This could be attributed to the rise in antibiotic consumption worldwide, especially with the increased use of last-resort antibiotics (Van Boeckel et al., 2014). The changing epidemiological patterns of *C. difficile* raises serious concerns for public health due to the development of antibiotic resistance and its impact on antibiotic efficacy.

Members of the public in the community, who were previously believed to be low risk for the development of CDI, are now acquiring the infection (Rupnik et al., 2009). CDI affecting community populations, with no recent healthcare exposure, is referred to as community acquired CDI (CA-CDI), and is defined by the onset of symptoms within 48 hours of admission to hospital, or more than 12 weeks after discharge from an inpatient healthcare facility (Kim and Zhu, 2017). The incidence and severity of CA-CDI in healthy adults and children is increasing. The introduction of the *C. difficile* NRL by the Health Service Executive (HSE) has enabled the enhanced surveillance of CDI in Ireland, and the latest figures from Q1 of 2023 reported 31% of total CDI cases as CA-CDI (HSE-HPSC and NRL, 2023). However, underdiagnosis of CDI in the community is likely, and the burden of CA-CDI is probably much higher than estimated (Maisa et al., 2019).

By definition, individuals with CA-CDI have not had any recent overnight stay in a healthcare facility. However, healthcare facility exposure is associated with a large proportion of CA-CDI cases (Skally et al., 2023). A study performed by Chitnis et al. (2013) in the United States found that 82% of patients with CA-CDI had a recent outpatient or inpatient healthcare exposure without an overnight stay. These facilities include general practitioners' offices, dental clinics, and emergency departments, which can be the source of acquisition because of contaminated environmental surfaces and antibiotic prescriptions. Ear, sinus, and upper respiratory tract infections are common reasons for the inappropriate use of antibiotics (Murphy et al., 2012). This misuse of antibiotics is

an important factor in the threat of antimicrobial resistance worldwide but is also considered a driver of CDI in the community (Chitnis et al., 2013).

It has been suggested that the 48 hour cut-off to define a CA-CDI case may not be appropriate as it is not reflective of the characteristics of *C. difficile* as a pathogen and ultimately could result in the overestimation of the true burden of HA-CDI (Skally et al., 2024). The rates of outpatient attendance and day case care has increased by 11% between 2013 and 2022, and these cases are currently classified as CA-CDI (Department of Health, 2024). Skally et al. (2024) have proposed a new category of "health-exposure" CDI (HE-CDI), which considers prior hospital day admittance separate to CA-CDI, which could be used to monitor and track for infection prevention and control purposes, and as a support for antimicrobial stewardship programmes.

In contrast to healthcare-associated CDI (HA-CDI), where risk factors are well established, there are very few risk factors recognised for the development of CA-CDI (Gould, 2010). For example, a study performed by Fawley et al. (2016) in England found that CA-CDI cases are almost twice as likely as HA-CDI cases to have had no prior antibiotic exposure before the onset of infection. This finding suggests that there are additional risk factors that contribute to the development of infection. It is not clear as to where CA-CDI patients become colonised or infected with toxigenic *C. difficile* strains, which raises the possibility that some individuals acquire toxigenic *C. difficile* from their home, the environment or animal sources including foodstuffs (Kim and Zhu, 2017).

Close contact with children younger than 2 years of age is associated with a higher risk of CA-CDI (Gupta and Khanna, 2014). Clinical manifestations in children under 2 occur only in rare cases, and healthy infants can be asymptomatic carriers of *C. difficile.* The carrier rate in children under 2 years of age varies widely, between 1% and 84%, but this rate decreases to less than 5% by 8 years of age (Shim, 2014). Susceptibility to asymptomatic colonisation may be because of the lack of protective intestinal microbiota in children below 2 years of age (Krutova et al., 2022a). However, protective factors in breast milk and the absence of cellular pathways necessary for *C. difficile* pathogenicity such as host toxin receptors may help to prevent the development of clinical infection (Borali and De Giacomo, 2016). Thus, nappy changing may be a risk factor for the development of CA-CDI in parents, and those who work in childcare.

C. difficile was previously believed to be a transmitted through healthcare settings, but a study by Eyre et al. (2013) found that 45% of cases of CDI were genetically distinct from all previous cases. This suggests that patients may be colonised with individual strains of *C. difficile* prior to hospitalisation, and that genetically diverse sources, such as animals or the environment may also play a key role in the transmission of *C. difficile*. The incidence of CA-CDI in members of populations that were previously considered to be low risk is an ever-evolving problem as there has been a dramatic paradigm shift – *C. difficile* can no longer be viewed solely as a healthcare-associated infection.

1.2.5 *C. difficile* virulence factors

Toxin production by *C. difficile* is the primary determinant of disease pathogenesis and is responsible for the development of symptoms during CDI. Therefore, CDI is correlated with virulent, toxin-producing strains of *C. difficile*, and the presence of some toxin genes is significantly associated with the risk of all-cause mortality in patients (Berry et al., 2017)

After colonisation of the large intestine, two clostridial toxins A (TcdA) and B (TcdB), which are the main virulence factors of *C. difficile*, are released (Kachrimanidou and Tsintarakis, 2020). These clostridial toxins are exotoxins, and cause damage to colonic epithelial cells by altering host cell physiology (Popoff, 2018). *C. difficile* toxins act synergistically and can alter the intestinal epithelial cell barrier, increase intestinal permeability, and in turn, cause colonic epithelial cell damage and intestinal inflammation (Chumbler et al., 2016).

The genes encoding for the toxins A and B (*tcdA* and *tcdB*, respectively) are positioned in a pathogenicity locus (PaLoc), a 19.6-kb genetic locus, along with three more accessory genes, *tcdC*, *tcdD*, *tcdR* (Figure 1.3) (Aktories et al., 2017). These accessory genes are involved in the regulation of *tcdA* and *tcdB*. It has been suggested that variations in the pathogenicity locus also account for hypervirulence in strains of *C. difficile* (Lewis et al., 2017).

The pathogenicity of *C. difficile* varies and depends on the strain possessing the *tcdA* and *tcdB* genes. Many studies have investigated the virulence of toxins TcdA and TcdB, but the TcdB toxin is the more potent virulence factor of *C. difficile* and is associated with

more severe infection (Paparella et al., 2021). Studies in animal models have shown reduced virulence in $TcdA^+$ $TcdB^-$ mutants, whereas both the wild-type strain ($TcdA^+$ $TcdB^+$) and $TcdA^-$ $TcdB^+$ mutants remained virulent (Carter et al., 2015). Strains of *C. difficile* that do not produce either toxin is referred to as non-toxigenic and are not pathogenic. The PaLoc is absent in non-toxigenic strains (Smits et al., 2016). The increased virulence of some strains is believed to be as result of a deletion in *tcdC*, which leads to a truncated TcdC protein and is associated with higher toxin production because of a lack of regulatory control from *tcdC* (Goorhuis et al., 2008).

In addition, it is estimated that 20% of strains produce another toxin, binary toxin CDT, which may play a role in the adherence to colonic epithelial cells and colonisation of *C. difficile* by forming microtubule-based protrusions (Carter et al., 2015). This toxin is encoded by *cdtA* and *cdtB* genes which are located in a 6.2 kb region called the "CDT locus". The role of CDT in CDI is not well established, but it is believed that although it may not be essential for disease, it may be important for colonisation of *C. difficile* during an infection and be involved in the upregulation and enhancement of TcdA and TcdB toxicity and be related to more acute disease in patients (Martinez-Melendez et al., 2022, Lyon et al., 2016).

1.2.5.1 CDI and non-toxigenic strains of *C. difficile*

Asymptomatic colonisation by non-toxigenic *C. difficile* strains appears to be associated with a lower risk of subsequently developing CDI (Smits et al., 2016). In addition, patients colonised by non-toxigenic strains have significantly reduced risk of rCDI after receiving standard-of-care treatment (Gerding et al., 2015). However, it is important to note that *C. difficile* is continuously evolving and toxin producing strains of *C. difficile* have been shown to convert non-toxigenic strains to toxin producers *in vitro* by horizontal gene transfer of the PaLoc, although it is unclear if this can occur in a human host (Brouwer et al., 2013).

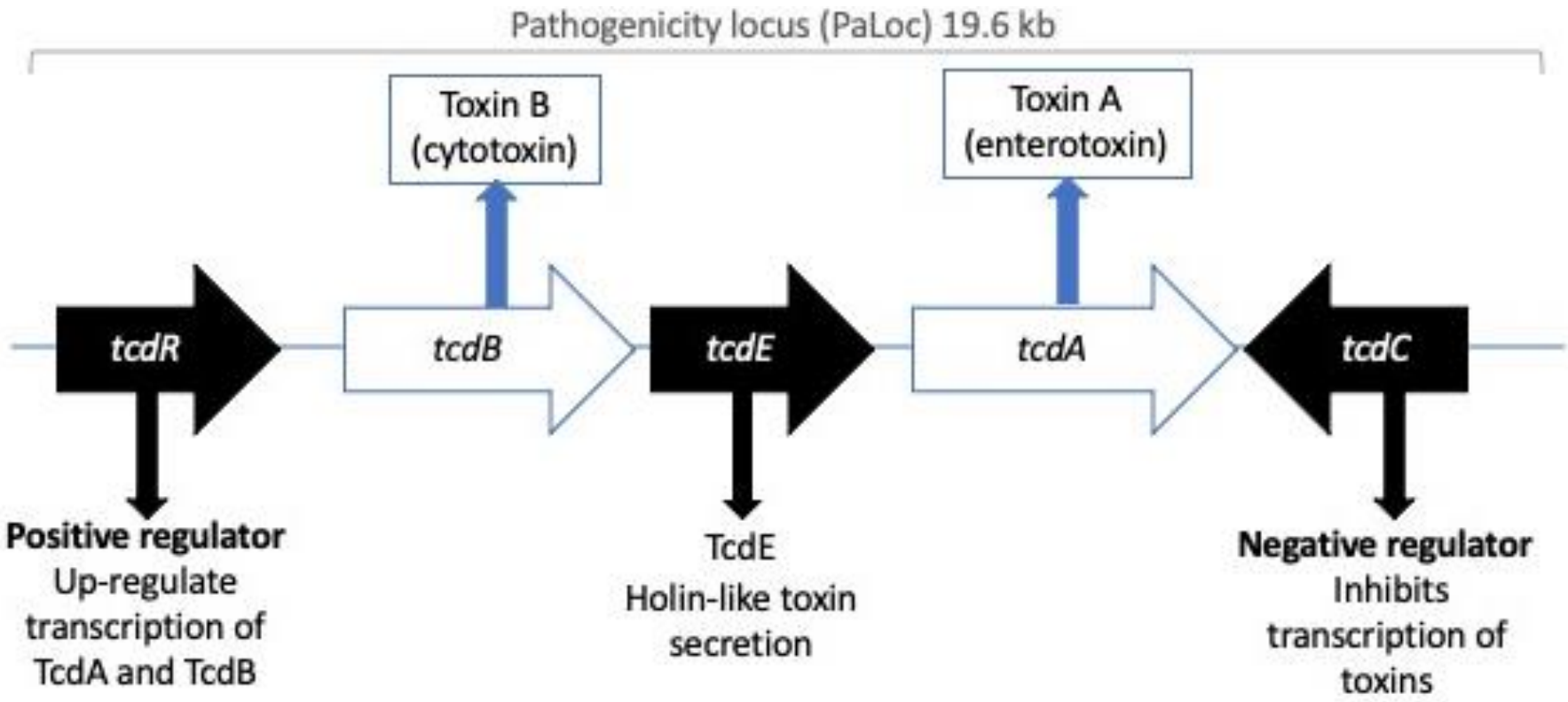


Figure 1.3 The pathogenicity locus of *C. difficile*

This encodes for toxin A (TcdA) and toxin B (TcdB). Two accessory genes (*tcdR* and *tcdC*) are involved in the regulation of *tcdA* and *tcdB*. The *tcdE* gene facilitates toxin release by the lysis of the bacterial cytoplasmic membrane. Adapted from (Martinez et al., 2012).

1.2.6 Sporulation and germination

C. difficile can exist as both a vegetative cell and in spore form (Smits et al., 2016). Spore formation is essential for bacterial survival in aerobic conditions, resistance to extreme environmental conditions, and for the dissemination and persistence of CDI (Lawler et al., 2020). *C. difficile* is ubiquitous in the environment and transmission to humans occurs via the faecal-oral route through the ingestion of spores, which are excreted through the faeces of an infected or colonised individual (Liubakka and Vaughn, 2016). The manifestation of CDI is dependent on spore germination to vegetative form, which occurs in the gut where conditions for *C. difficile* are favourable.

The spore structure (Figure 1.4) allows the bacterium to resist chemical and physical treatments with disinfectants and antibiotics (Lawler et al., 2020). This resistance poses a particular challenge for healthcare facilities, such as hospitals and nursing homes, as standard disinfection procedures are ineffective in the prevention and control of *C. difficile*. Transmission of CDI in healthcare settings commonly occurs through contaminated hands (health care professionals, visitors) and contamination of medical instruments (e.g., thermometers, stethoscopes, blood pressure cuffs) (Vonberg et al., 2008a). *C. difficile* can be spread when CDI patients come in contact with surfaces, such as in bathrooms and bedrooms, and transmission occurs when other individuals, such as visitors or healthcare workers, are exposed to contaminated environmental surfaces (Jullian-Desayes et al., 2017). These individuals can either spread the bacteria further after contact with other surfaces, or patients, and/or become infected by eating food with contaminated hands (Schoyer and Hall, 2020). For this reason, it is recommended to implement appropriate infection control measures once a case of CDI is confirmed to prevent the further spread of infection throughout the facility (Barker et al., 2018). This involves early diagnosis of infection, education of staff and visitors, isolation of patients in single rooms with designated toilets, meticulous soap and water handwashing, environmental disinfection, the use of disposable materials, when possible (including gloves and gowns), and dedicated medical instruments.

Some members of the gut microbiota are involved in bile acid metabolism (Kachrimanidou and Tsintarakis, 2020). These can convert primary bile acids to secondary bile acids such as deoxycholate, which have been shown to inhibit *C. difficile*

growth *in vitro* by inhibiting spore germination and thus, suppressing vegetative growth (Theriot and Young, 2014). In spore form, *C. difficile* can survive in the environment for long periods of time and can withstand the acidic conditions of the stomach. If the gut microbiota has been disrupted, this can allow the *C. difficile* spores to germinate into vegetative cells in the colon and proliferate (Kachrimanidou and Tsintarakis, 2020).

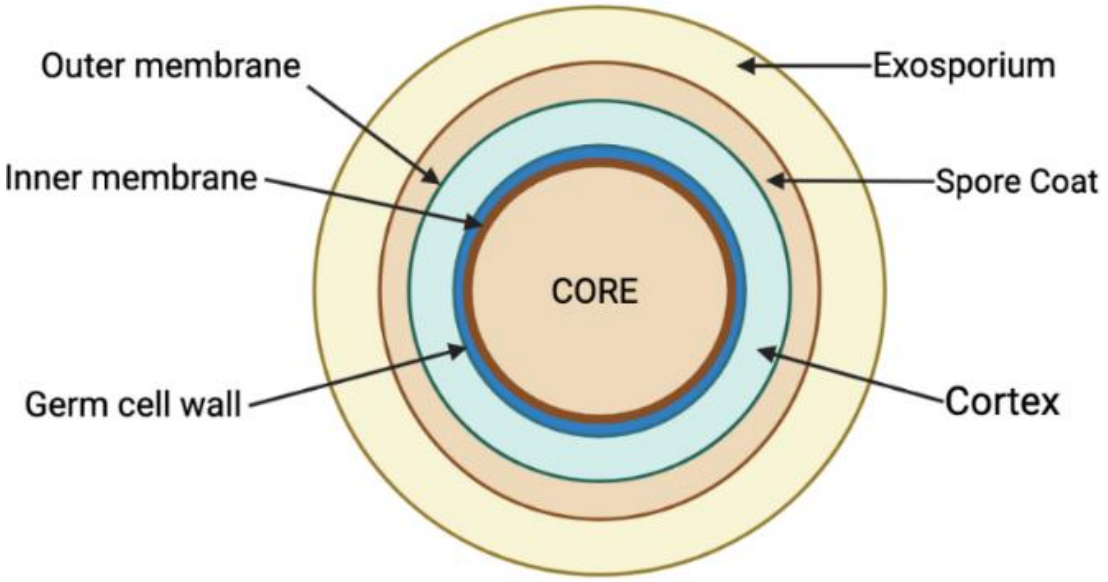


Figure 1.4 Cross-section of *C. difficile* spore layer structure.
Spore structure contributes to spore resistance to damage, heat, chemicals, and UV. Spore core (centre) contains DNA, RNA, ribosomes, and enzymes essential for the return to metabolic activity during germination. The inner membrane is a permeability barrier that prevents entry of compounds that damage DNA. The inner membrane is surrounded by a peptidoglycan cell wall. Adapted from Lawler et al. (2020).

1.2.7 Proton-pump Inhibitors (PPIs) as a risk factor for CDI

C. difficile must be in vegetative cell form to cause CDI symptoms, and secondary bile acids provide protection against CDI as they provide unfavourable conditions in the gastrointestinal tract, which in turn prevents *C. difficile* spore germination to vegetative cells. The use of PPIs is a potential risk factor for the development of CDI, as they induce altered bile acid metabolism (Tawam et al., 2021). PPIs are a commonly prescribed medication to treat gastric acid-related disease, such as gastroesophageal reflux disease (GERD) and peptic ulcer disease, and in 2017, an Irish audit found that PPIs represented 20% of all prescriptions issued (O'Mahony and Yelverton, 2019). PPI therapy inhibits gastric acid secretion, which removes the acidic barrier that prevents germination of *C.*

difficile spores to vegetative form (Trifan et al., 2017). In addition, PPIs are commonly prescribed for long-term use (>6 months) after discharge from a healthcare facility, which may contribute to the development of CDI in the community (Inghammar et al., 2021, Haastrup et al., 2021). *C. difficile* sporulation frequency is lower under acidic conditions, and is increased in high gastric pH (more alkaline) environments (Wetzel and McBride, 2020). The alteration of the acidic environment of the stomach with acid suppressive therapies, such as PPIs, may facilitate *C. difficile* survival and growth, as a higher pH in the gastrointestinal tract is strongly associated with CDI (Wetzel and McBride, 2020, Tawam et al., 2021).

1.2.8 Diagnosis of CDI

The development of CDI requires both the acquisition of *C. difficile* and disruption of the normal gut microbiota, which typically occurs after antibiotic treatment, as discussed in section 1.2.3. Early intervention and accurate diagnosis are imperative to achieve the best clinical outcome for the patient, but also to implement effective infection control measures. The incidence of *C. difficile* has increased rapidly with the emergence of hypervirulent strains in the previous decade and so too has the burden on healthcare facilities (Gerding et al., 2016).

Diagnosis of CDI requires the input of both clinical and laboratory findings. Firstly, characteristic clinical manifestations are used to identify a patient with a potential CDI (van Prehn et al., 2021). Clinical symptoms of diarrhoea, and a history of antibiotic exposure indicates a possible case of CDI. There are variations in diagnostic methods used, which includes detection of toxin B (TcdB) by rapid enzyme immunoassay (EIA) and detection of toxin genes by nucleic acid amplification tests (NAATs) (Humphries et al., 2013, van Prehn et al., 2021). In Ireland, it is recommended to use a two-step strategy to correctly diagnose CDI and to prevent overdiagnosis ((NCEC), 2014). Firstly, a screening test is performed by using either *C. difficile* toxin enzyme immunoassays (EIAs) to detect glutamate dehydrogenase (GDH), which is an enzyme produced by *C. difficile*, or a NAAT to detect toxin gene *tcdB*. If the first test is negative, then a second test is not required. If the first test is positive, then the second test used is either an EIA to detect the toxin, or a NAAT to detect toxin genes.

1.2.9 Clinical management of CDI

The severity of disease, rate of recurrence of CDI episodes, and treatment failures have all increased in recent years (Bagdasarian et al., 2015). Changes to CDI severity could be because of a complex interplay of many factors including host health status, increasing antibiotic treatment failure, and bacterial pathogenicity, as discussed in section 1.2.5.

CDI severity is classified by the presence of diarrhoea and other symptoms. Mild disease is defined as diarrhoeal symptoms only, whereas severe disease is defined as CDI with additional symptoms as listed in Table 1.1. Severe-complicated CDI, which is also referred to as fulminant disease, imposes a considerable burden on healthcare facilities. Early intervention is imperative – practitioners must promptly assess the severity of infection to adjust the medical management of the patient or to anticipate surgery (Fujitani et al., 2011).

Table 1.1 The criteria for severity of CDI
Guidelines as per European Society of Clinical Microbiology and Infectious Diseases (ESCMID) in van Prehn et al. (2021)

Definition of CDI	**Patient characteristics**
Mild to moderate	Diarrhoea
Severe	Fever Serum albumin (<30 g/L) Leucocytosis (Leucocyte count >15 × 10^9/L) Elevated serum creatinine (>50% above the baseline) Large intestine distension
Severe-complicated (or fulminant)	Hypotension Septic shock Elevated serum lactate Toxic megacolon Bowel perforation

1.2.10 The economic burden of CDI

With the increased incidence of recurrent infections and need for hospitalisation, there is a considerable economic impact associated with CDI. Data from a personal communication by Boyle and Drew (2015) found that the average hospitalisation cost of CDI in a large tertiary referral centre in Ireland was €31,680, which was €15,680 higher than a non-CDI case. By increasing the length of hospital stay, which in turn incurs additional treatment costs, CDI places a large financial burden on healthcare (Eze et al., 2017). For rCDI patients there is a significantly higher risk of death within 6 months after initial treatment than for patients without an infection recurrence (p=0.001) (Olsen et al., 2015). The heightened risk for rCDI patients is believed to be most often because of a relapse caused by the original strain, as opposed to reinfection from a new strain (Mac Aogain et al., 2015b). This raises important clinical concerns, and basic preventative measures are crucial for the control of rCDI.

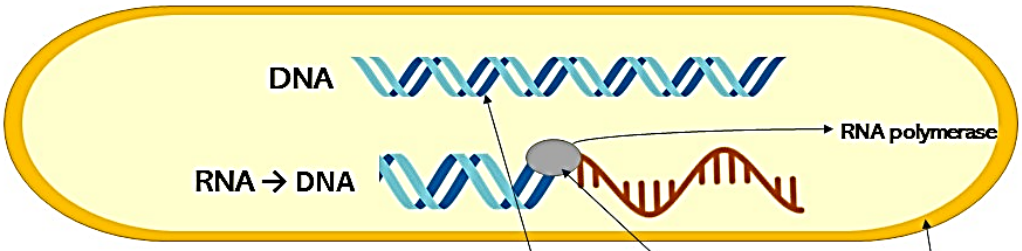
1.2.11 Therapeutic options for CDI

The treatment of CDI worldwide is limited in options to three agents administered orally: metronidazole, vancomycin and fidaxomicin (van Prehn et al., 2021). Choosing which antibiotic to use to treat CDI is dependent on the severity of disease and whether it is a recurrent infection. The main pharmacological properties of the three major antibiotics used in the treatment of CDI are detailed in Figure 1.5.

In the upper GI tract, oral metronidazole is almost fully systemically absorbed (90%), where it is then secreted across the gut mucosa into the large intestine (Krutova et al., 2022b). Oral metronidazole is effective for use in the treatment of mild to moderate CDI, but vancomycin is recommended for the treatment of more severe infections. In Ireland and across the world, the use of oral vancomycin tapering/pulse therapy is recommended, which comprises a tapered regimen (gradually decreasing doses) of vancomycin, followed by a pulsed dosage regimen (intermittent high dosing, with dose-free periods) to enhance the therapeutic effect of the drug ((NCEC), 2014).

Fidaxomicin, previously known as OPT-80, has emerged recently as a third drug option for the treatment of CDI (Louie et al., 2011). It is the active ingredient in Dificlir®, which is a macrocyclic narrow spectrum antibiotic. Fidaxomicin has bactericidal activity against

C. difficile, and acts by inhibiting RNA polymerase, thus preventing bacterial RNA synthesis and transcription (Venugopal and Johnson, 2012). After oral administration, fidaxomicin exhibits low serum concentration levels and high faecal concentration levels (Zhanel et al., 2015). In contrast to metronidazole and vancomycin, fidaxomicin has a limited effect on the microbiota diversity and has a prolonged post-antibiotic effect which allows for reduced dosing, fewer side effects and also results in a lower rate of recurrent infections in comparison to patients treated with metronidazole or vancomycin (Babakhani et al., 2011).



	Properties	Metronidazole	Fidaxomicin	Vancomycin
	Systemic Absorption	High	Low	Low
	Concentration in stool	Low	High	High
	Effect on microbiome diversity	Reduced	Preserved	Reduced
	Decline in *C. difficile* shedding in stool	Slow	Sharp	Fast
	Environmental contamination	High	Low	Low
	Inhibits sporulation	No	Yes	No

Figure 1.5 Pharmacokinetic properties of metronidazole, fidaxomicin, and vancomycin. These are the three first-line antibiotics recommended for use in the treatment of CDI. Adapted from Krutova et al. (2022b).

Increasing rates of clinical failure and inferior treatment performance in comparison to vancomycin, has resulted in the removal of metronidazole as a recommended first-line treatment for mild to moderate CDI in North America and Europe (McDonald et al., 2018, van Prehn et al., 2021). The Infectious Diseases Society of America (IDSA) and the European Society of Clinical Microbiology and Infectious Diseases (ESCMID) now recommends vancomycin and fidaxomicin for the initial episode of both mild and severe

cases of CDI. However, the Australasian Society of Infectious Diseases (ASID) still recommends metronidazole as the treatment for mild to moderate cases of CDI (Trubiano et al., 2016).

1.2.11.1 Faecal Microbiota Transplantation (FMT)

Therapies other than antimicrobial treatment are in development, such as faecal microbiota transplantation (FMT), otherwise known as faecal biotherapy (Hvas et al., 2019). This is a last resort treatment for rCDI and ulcerative colitis in patients who have not otherwise responded to previous interventions and is gaining increasing importance in both clinical and research settings. In CDI, the indigenous microbiota is disrupted or depleted – the concept of FMT is to replace the intestinal microbiota composition and function (Borody et al., 2014). A healthy stool donor is identified, and the stool sample is administered into the patient's intestinal tract with the aim of normalising the microbial diversity. By restoring the normal faecal microbial community, it is hypothesised that *C. difficile* is suppressed by nutrient competition, restoration of secondary bile-acid metabolism, immune-mediated colonisation resistance, or a combination of these (Khoruts and Sadowsky, 2016). However, the safety of this method has to be considered, as normal faecal bacteria from healthy donors still have the potential to contain harmful pathogens, or FMT therapy could cause other negative side effects such as weight gain and/or obesity (Reeves et al., 2011, Alang and Kelly, 2015).

1.3 Management of outbreaks in healthcare settings

Strategies to prevent the spread of infection should not only be implemented in cases of confirmed CDI but in every suspected case of CDI. Given the multiple risk factors that are associated with developing CDI, there are many infection control strategies in place to prevent the spread of infection in healthcare facilities, including antimicrobial stewardship, hand hygiene, contact and outbreak precautions, appropriate environmental cleaning and disinfection, and education.

1.3.1 The importance of antimicrobial stewardship for the control of CDI

The overuse of all antibiotics contributes to the spread of antimicrobial resistance globally, but also poses a risk for the development of CDI in patients. Broad spectrum antibiotic classes are associated with heightened risk of CDI including clindamycin,

fluoroquinolones, and cephalosporins (Turner and Anderson, 2020). The implementation of an antimicrobial stewardship programme is an effective way of reducing the overall rates of CDI. Such a programme should be aimed at optimising reduced frequency of prescribing and shortening the duration of antibiotic use (Stuart et al., 2019b). It is also important to promote the choice of antibiotics that are effective as targeted treatment without being unnecessarily broad-spectrum.

In agriculture, antimicrobials are commonly used prophylactically or metaphylactically, and are administered to food-production animals orally, through medicated feed or water, or by injection (O'Neill et al., 2020). A “One Health” approach to the control of antimicrobial resistance has been proposed, which recognises that the health of humans, animals, and the environment are interconnected (European Commission, 2017). Resistance may be transferred between animals and humans through direct contact with animals, the environment, or the food chain (Martin et al., 2020). Many of the same antimicrobials that are used to treat human infections are used in animals, such as cephalosporins and macrolides, which are listed as critically important antimicrobials for human use by the World Health Organisation (WHO) (WHO, 2019). In an effort to combat antimicrobial resistance, the European Union (EU) has restricted the use of some antimicrobials in veterinary medicine (e.g. cephalosporins and fluoroquinolones), with an outright ban on some antimicrobials in food-production animals (e.g. vancomycin and rifampicin) (European Medicines Committee, 2020).

1.3.2 Management of patients with CDI

C. difficile spores are resistant to disinfectants and can persist in the environment for long periods of time. Good hand hygiene is an essential practice for the prevention and control of pathogen transmission to ensure maximum safety of patients and staff in healthcare facilities (Kociolek et al., 2023). Alcohol based hand rubs should not be relied upon solely as they are ineffective against *C. difficile* spores (Turner et al., 2020). While soap and water are also not effective at killing *C. difficile* spores, the friction of rubbing hands together can help to reduce the spore burden.

Patients with diarrhoeal symptoms (three or more loose stools within a 24-hour period) should immediately be placed in a single room that has ensuite toilet facilities, if available (Stuart et al., 2019b). Personal protective equipment (PPE) like gowns and

gloves should be worn by healthcare workers and visitors prior to entry to rooms with suspected or confirmed cases of CDI to reduce contamination on clothes and hands. As a precautionary measure, patients should be isolated in the room at all times, if possible, but if they are moved to another clinical area then all staff must be aware to take contact precautions and all wheelchairs and trolleys should be appropriately cleaned (Vonberg et al., 2008a).

1.3.3 Cleaning of *C. difficile* contaminated patient care areas

The healthcare environment is an important source of CDI as *C. difficile* can be recovered from environmental samples in hospitals nearly 100% of the time, with high touch surfaces such as toilets and sinks carrying the highest organism load (Schoyer and Hall, 2020). As the economic burden associated with *C. difficile* is high, environmental cleaning and decontamination has been recognised as the most cost-effective strategy for preventing CDI (Louh et al., 2017). It is recommended that *C. difficile* contaminated patient care areas be cleaned, firstly with a neutral detergent, followed by a solution containing hypochlorite (5000 ppm) or another sporicidal solution, such as vaporised hydrogen peroxide (Stuart et al., 2019b, Schoyer and Hall, 2020). Disinfection of a room contaminated with *C. difficile* using hypochlorite-based cleaners or hydrogen peroxide can reduce the environmental spore burden, but can also reduce the risk of new and recurrent CDI cases, as acquisition from an exogenous source is essential for *C. difficile* colonisation (Barbut, 2015, Macleod-Glover and Sadowski, 2010). Once a patient has been discharged or transferred from the room, terminal cleaning should commence before it is reoccupied. This includes laundering of all linen, discarding of disposable items (e.g., toilet roll), and deep cleaning of all surfaces, with special care taken of items such as bed rails, light switches, and door handles.

1.4 Tools used for the routine surveillance of *C. difficile*

Different geographic regions across the globe report differences in the dominant strain type of *C. difficile*. With the enhancement of molecular typing methods over the last decade, genomic analysis of the pathogen has emerged as a critical tool enabling in-depth exploration of the evolutionary trajectory of different strains of *C. difficile*. The United Kingdom has a routine typing programme, with the *Clostridioides difficile*

Ribotyping Network (CDRN), which is based in Leeds, offering PCR ribotyping on request (Wilcox et al., 2012). In Ireland, the *C. difficile* NRL was established in 2021, and is based in the Public Health Laboratory, Cherry Orchard Hospital. Here, WGS is performed on *C. difficile* isolates submitted from 61 participating hospitals across the Republic of Ireland (HSE-HPSC and NRL, 2023).

1.4.1 PCR Ribotyping

PCR ribotyping was developed by Stubbs et al. (1999) and involves the amplification of the heterogenous 16S-23S ribosomal DNA (rDNA) intergenic spacer region (ISR) using conserved primers (Collins et al., 2015). The ISR is variable in both base pair (bp) length and copy number between different ribotypes of *C. difficile*. PCR ribotyping is based on genetic variation and can be used to distinguish *C. difficile* strains belonging to distinct ribotypes and may be used to identify hypervirulent strains of interest, such as RT078. This method involves amplification of the region which generates various amplicons, then the amplicons are separated by gel electrophoresis which generates a DNA fingerprint and the banding patterns generated are referred to as PCR RTs (Knetsch et al., 2013). PCR ribotyping is the most frequently employed *C. difficile* typing method in Europe.

1.4.2 Pulsed-field Gel Electrophoresis (PFGE)

Pulsed-field gel electrophoresis (PFGE) is more commonly used in North America, than in Europe, for routine molecular characterisation of *C. difficile* strains (Abad-Fau et al., 2023). PFGE involves the digestion of chromosomal DNA using a restriction enzyme, and the resulting fragments are separated by electrophoresis which produces a DNA fingerprint (Collins et al., 2015). *C. difficile* pulsetyping uses the prefix “NAP” (North American pulsed-field), followed by a number that denotes the pulsetype group.

1.4.3 Multilocus Sequencing Typing (MLST)

While PCR ribotyping has been commonly used for the typing of *C. difficile*, there are sequence-based methods that allow a more in-depth insight into the molecular diversity of *C. difficile*, such as Multilocus Sequence Typing (MLST). MLST is a typing technique that was first developed for *Neisseria meningitidis* by Enright and Spratt (1999), and has since been widely employed to study the epidemiology of other microorganisms, such

as MRSA (Earls et al., 2018). MLST is used to classify bacterial isolates in a comparable global context, and allelic sequences are stored in curated databases (Larsen et al., 2012). The PubMLST site collects this sequence data to make it easily accessible across the world (Public databases for molecular typing and microbial genome diversity, [https://pubmlst.org/]). The most common nomenclature used to describe the phylogenetic relatedness between bacterial isolates is described in Table 1.2.

The MLST typing scheme was first applied to *C. difficile* by Griffiths et al. (2010). Sequence types (STs) are assigned to isolates based on the nucleotide sequences of the seven housekeeping genes present in the genome: adenylate kinase (*adk*), adenosine triphosphate (ATP) synthase subunit alpha (*atpA*), 1-deoxy-D-xylulose 5-phosphate reductoisomerase (*dxr*), serine hydroxymethyltransferase (*glyA*), recombinase A (*recA*), superoxide dismutase (*sodA*) and triose phosphate isomerase (*tpi*) (Figure 1.6). Different STs have a unique combination of alleles, which are assigned an ST number. The *C. difficile* phylogenetic tree is based on MLST and consists of five different clades (Baktash et al., 2022). The majority of STs belong to clade 1, whereas ST11 is a genetically distinct outlier and belongs to clade 5.

Table 1.2 Nomenclature used to describe the phylogenetic relationships between bacterial strains

Nomenclature	Definition
Clade	A group of microorganisms that have evolved from a common ancestor
Clone	Bacterial strains that are genetically identical
Phylogeny	The evolutionary relationship between microorganisms

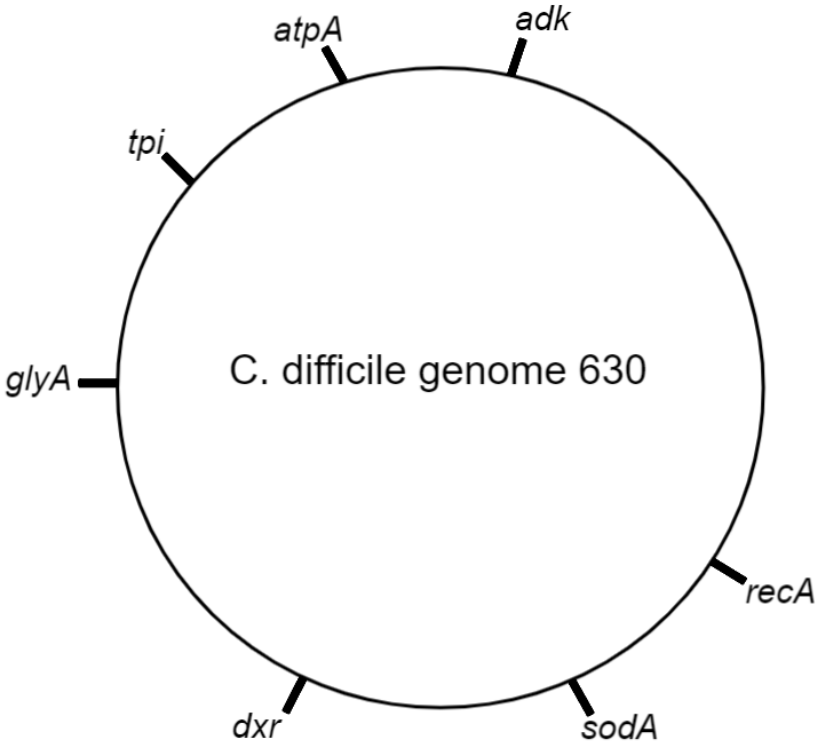


Figure 1.6 The seven housekeeping genes used for MLST typing and their relative positions on the *C. difficile* genome.
Adapted from (Griffiths et al., 2010).

1.4.4 Core genome MLST (cgMLST)

There are two commonly applied methods that utilise WGS technology to identify genomic variation. The first method is core genome MLST (cgMLST), which is a useful MLST technique that uses sequencing to detect variation in the genes of the core genome of *C. difficile*. This approach is generally applicable and has been used to study the epidemiology of a number of bacterial pathogens in Ireland, including methicillin-resistant *Staphylococcus aureus* (MRSA), *Neisseria meningitidis*, and *Mycobacterium tuberculosis* (Earls et al., 2017, Mulhall et al., 2019, Griffin et al., 2023). This technique is a variation of MLST, but more genetic information is included in cgMLST analysis, which provides more discrimination between closely related *C. difficile* strains, and makes it an important tool in outbreak investigations (Abad-Fau et al., 2023). There are several user-friendly schemes that are available for cgMLST typing of *C. difficile*, including commercial platforms such as SeqSphere^{+} software (Ridom GmbH, Germany), which has been used for the analysis of *C. difficile* in this thesis, and the freely available online database from Enterobase (University of Warwick, UK)(Frentrup et al., 2020, Junemann et al., 2013).

1.4.5 Single Nucleotide Polymorphism (SNP) analysis

Another WGS method analyses single nucleotide polymorphisms (SNPs), which are single base-pair variations in the DNA sequence, and are also called single nucleotide variant (SNV) sites (Janezic and Rupnik, 2019). This approach involves mapping the short reads of a bacterial genome to a reference genome to determine nucleotide differences. The number of SNP differences can then be used to determine the genetic relatedness between isolates (Didelot et al., 2012). SNP analysis has been used to differentiate closely related strains and to understand transmission networks (Abad-Fau et al., 2023). SNP typing is useful as it provides the most resolution and discrimination in comparison to other available tools. However, SNP analysis is time consuming and requires advanced bioinformatic tools and expertise for data interpretation (Janezic and Rupnik, 2019, Eyre et al., 2019).

1.5 Antimicrobial resistance in *C. difficile*

In addition to enabling the high-resolution genomic analysis of *C. difficile*, WGS is a valuable tool in patient care as it can be used to detect genes that confer antimicrobial

resistance (AMR), which can help medical professionals to devise an appropriate treatment plan for CDI (Jon et al., 2021).

A key feature of *C. difficile* is that it flourishes when there is dysbiosis in the gut microbiota, which is an important defence against *C. difficile*. Exposure to antimicrobials is an important risk factor for the development of CDI, as described in section 1.2.3. Changes in the gut microbiota have been associated with several health conditions, including CDI. Strong inducers of CDI include antimicrobials that target the anaerobic gut microbiota (for example, *Bacteriodes* spp. and *Bifidobacterium* spp.) and facilitate the increase of certain facultative anaerobes (for example, *Enterococcus* spp.) (Baines and Wilcox, 2015). A change to the abundance of these bacteria is also strongly linked with the physiological changes that are associated with ageing, as described in section **Error! Reference source not found.**.

Antibiotic resistance mechanisms in *C. difficile* include the presence of antimicrobial resistance genes (ARGs), mobile genetic elements, changes to metabolic pathways and antimicrobial targets, and the formation of biofilms (O'Grady et al., 2021). Resistance to antimicrobials can be conferred by genes that are located on mobile genetic elements, such as transposons (Imwattana et al., 2020). Transposons are segments of the DNA that are known as “jumping genes”, as they can move their position on the genome, and can also be transferred within, and between species by horizontal gene transfer (Pray, 2008, Imwattana et al., 2020). Common resistance mechanisms found in *C. difficile,* and their relevant loci are detailed in Table 1.3. Increasing numbers of strains are now showing multidrug resistance (MDR), which is defined as resistance to three or more antimicrobial agents (Knight et al., 2019). *C. difficile* is one of the most prevalent MDR bacteria, and this is considered to be a key contributor to the emergence and spread of the hypervirulent strains of *C. difficile* (Jane, 2021).

Table 1.3 Common Antimicrobial Resistance (AMR) mechanisms in *C. difficile* and the relevant loci associated with these resistance mechanisms. This table was compiled from information provided Imwattana et al. (2020) and O'Grady et al. (2021).

Antimicrobials	Resistance mechanisms	Relevant resistance loci		Plasmid mediated (Smits et al., 2022)
		Accessory genes families	Mutations in existing genes	
Fluoroquinolones	Mutations of GyrA/B in the quinolone resistance-determining regions (QRDR)	-	*gyr*	-
Vancomycin	Mutations to peptidoglycan biosynthesis proteins	-	*vanS, vanR*	pX18-498
Fidaxomicin	Mutations		*rpoB*	-
Tetracyclines*	Encodes a protein that protects the ribosome	*tet*	-	-
Metronidazole^	Alteration of metabolic pathways Biofilm formation	-	-	pCD-Metro
Rifamycin	Mutations in bacterial RNA polymerase	-	*rpoB*	-
MLS_B*	Methylation of rRNA to protect the protein from antimicrobials*	*erm*, *cfr*	-	-
Aminoglycosides	Produce enzymes that inactivate aminoglycosides	*aph, aac, ant, sat*	-	-
Linezolid and phenicols	Mutations in ribosomal proteins	*cfrB, cfrC*	*rplC*	-

*genes are often carried on transposons, which are segments of DNA known as “jumping genes” as they are capable of moving positions within a genome (Pray, 2008)

1.5.1 Antimicrobial resistance to first-line therapy options for CDI

The development of resistance to first-line antimicrobials recommended for the treatment of CDI is of particular concern, and although rare, treatment failure has been reported in certain ribotypes (Saha et al., 2019). A meta-analysis performed by Sholeh et al. (2020) found a resistance rate, using the EUCAST breakpoint, to vancomycin of 3.7% (416 out of 11,188 isolates) in 58 studies, and to metronidazole of 3.2% (190 out of 5,900 isolates) in 32 studies. Resistance to fidaxomicin was found in 0.08% (1 out of 1,184 isolates) in six studies.

The ClosER study by Freeman et al. (2020) monitored the antimicrobial susceptibility and geographical distribution of 3499 *C. difficile* isolates from 51 sites in 28 European countries between 2011 and 2016. There was 1 isolate, from a recurrent case of CDI in year 5 of the study, that was resistant to fidaxomicin. In this study, RT027 consistently demonstrated resistance or reduced susceptibility to metronidazole, rifampicin, moxifloxacin, and imipenem. It was also found that the Minimum Inhibitory Concentrations (MICs) for metronidazole and vancomycin, which is the lowest concentration of antimicrobial agent that prevents *C. difficile* growth, increased over the five years of the study, which could be attributed to the reduced use of those antibiotics, or greater strain diversity.

The emergence of antibiotic resistant ribotypes plays an important role in the dissemination of epidemic *C. difficile* strains (Kuijper et al., 2001, Donskey, 2017b). A study by Dingle et al. (2017) in the UK found that restrictions in the prescription of fluoroquinolones played a significant role in the control and decline of CDI due to fluoroquinolone-resistant *C. difficile* RTs, including RT027. This was also observed in a study at St James's Hospital between 2006 and 2012, where a restriction on ciprofloxacin prescription for treating gram negative infections coincided with a reduction in CDI cases at the hospital (O'Connell et. al., unpublished data). It is surprising that vancomycin remains effective at achieving clinical cure of CDI, as *C. difficile* often coexists in the colon with vancomycin-resistant enterococci (VRE), which carry vancomycin resistance operons on transferable elements (Greentree et al., 2022).

1.6 Hypervirulent strains of *C. difficile*

There has been an increased incidence and severity of CDI globally, which is believed to be as a result of the emergence of hypervirulent strains such as RT027/NAP1 and RT078/NAP7/8 (Depestel and Aronoff, 2013, Freeman et al., 2010). Highly virulent strains have reduced susceptibility to fluoroquinolone antibiotics, have been shown to produce more toxin *in vitro*, for longer periods of time, and are implicated in more complicated disease outcomes. Strains are coined "hypervirulent" as they are linked with higher-than-expected rates of morbidity and mortality in patients compared to typical endemic strains (Yakob et al., 2015). There are several microbial characteristics that have been associated with this hypervirulence including polymorphisms in the toxin production regulatory gene, *tcdC*, and the presence of genes *cdtA* and *cdtB* which encode for the binary toxin CDT (Lessa et al., 2012).

The earliest recorded RT027 isolate was in 1985 in a Parisian Hospital, when strain CD196 was isolated from a patient with CDI (Popoff et al., 1988). There was a large outbreak of CDI in Canada in 2003-2004, with over 30 hospitals involved and many patients experienced remarkably severe CDI (Labbe et al., 2008). Over 20,000 nosocomially acquired cases were reported in the region to the Quebec provincial surveillance program by July 2007. The same ribotype had also been isolated in several regions of the USA between 2000 and 2002 (Warny et al., 2005). During 2005, a study by the European Study Group on *Clostridium difficile* (ESGCD) found that RT027 was circulating in Ireland, The Netherlands and Belgium (Barbut et al., 2007), and by 2008 it had spread to sixteen European countries causing severe CDI (Kuijper et al., 2008). RT027 was designated an "epidemic" strain due to its tendency to cause outbreaks, and its association with causing worldwide nosocomial disease.

Since the emergence of outbreaks of CDI associated with RT027, there has been a shift in the diversity of *C. difficile* ribotypes causing CDI. There has been a decline in the number of cases of CDI that are caused by RT027, and in 2021 there were no cases caused by RT027 reported in Ireland (HPSC, 2021). This could be because of the emergence of other hypervirulent ribotypes, such as RT078, which has been associated with CDI as severe as infections caused by RT027 (Stabler et al., 2012). Patients with CDI caused by RT078 have a similar attributable mortality as those infected with RT027, but

patients are younger and infections are more frequently community associated (Goorhuis et al., 2008, Bauer et al., 2011a). In the last decade, RT078 has remained as the most common circulating RT in Ireland, whereas RT027 has generally been absent (Skally et al., 2023). CA-CDI is commonly associated with RT078, which is also the most common ribotype found in animals, including pigs (Debast et al., 2014, Alvarez-Perez et al., 2018). The similar epidemiological patterns in pigs and humans regarding the prevalence of RT078 indicates a zoonotic link, as explored in section 1.7 (Moloney et al., 2021).

1.7 Farm animals as a reservoir for CDI and *C. difficile* RT078

Farm animals have been cited as a potential reservoir for isolates of *C. difficile* causing human CDI (Knetsch et al., 2014). *C. difficile* was first reported in animals in the 1980s, but it was not confirmed to be associated with enteric disease in animals (Rodriguez-Palacios et al., 2013). *C. difficile* is acknowledged as a gut coloniser in different domestic animals and livestock including pigs, cattle, and chickens, and is ubiquitous on Irish farms (McElroy et al., 2015, Weese et al., 2010b).

The gastrointestinal tract of humans and animals is the preferred point of colonisation for both toxigenic and non-toxigenic *C. difficile* strains (Lim et al., 2020b). Toxigenic *C. difficile* RT078 is commonly found in neonatal animals, with prevalence rates ranging from 20% in calves to 100% in piglets (Knight et al., 2019, Uzal et al., 2023, Magistrali et al., 2015). The intestinal colonisation rates of *C. difficile* in animals dramatically decline over the first two months of life (Weese et al., 2010b). It is not clear why *C. difficile* colonisation rates are highest in neonatal animals, but it is hypothesised to be as a result of the consumption of a diet that helps to form a well-developed protective microbiota in adult animals (Lim et al., 2020b). Once the neonatal animal begins to consume an adult diet, various microbial species colonise the gastrointestinal tract which outcompete *C. difficile*, a mechanism known as colonisation resistance, by inhibiting germination and vegetative growth, which in turn prevents toxin production (Britton and Young, 2014).

The sustainability of animal production systems is largely reliant on animal feed, as approximately 70% of farm costs are attributed to the cost of feed (Ronquillo and

Hernandez, 2017). Veterinary antimicrobials have been used since the 1940s as growth promoters, and for infection control and prevention (Patel et al., 2020). Growth promotion utilises antibiotics at subtherapeutic concentrations to increase feed utilisation efficiency, and to promote weight gain in food production animals. From January 2006, the Feed Additives Regulation enforced an EU-wide ban on the use of antibiotics as growth promoters in animal feed (EU, 2003). Farmers also utilise antibiotics prophylactically in healthy animals for infection prevention, which can prevent the manifestation of disease in stressful conditions, for instance during transportation, or in crowded living conditions (Rodriguez-Palacios et al., 2011, Alvarez-Perez et al., 2018). However, Regulation (EU) 2019/6 came into effect in 2022 and prohibits all routine antibiotic use in animals in the EU, including prophylactic use (EU, 2022). The primary driver of antibiotic resistance and the emergence of hypervirulent strains is overuse of antibiotics, and pig production is the highest consumer of veterinary antimicrobials in Ireland (Patel et al., 2020, O'Neill et al., 2020). Due to the high risk of antimicrobial resistance, the WHO has defined macrolides, fluoroquinolones and cephalosporins as the highest priority, critically important antimicrobials for human medicine, which should be restricted as part of antimicrobial stewardship programmes (WHO, 2019). However, these antibiotics are widely used on Irish pig farms (O'Neill et al., 2020). As a result, food production animals have become an important reservoir for *C. difficile*.

C. difficile is typically isolated from healthy pigs, but clinical infections can also occur in pigs resulting in diarrhoea, colonic oedema and CDI can sometimes lead to death (Susick et al., 2012). *C. difficile* is commonly identified as the infectious agent causing diarrhoea and enteritis in neonatal pigs in Ireland (DAFM et al., 2021, McElroy et al., 2015). Limited longitudinal studies have found varied prevalence of *C. difficile* in pigs, yet most have noted a higher prevalence (>70%) at birth which decreases to less than 10% at slaughter stage (Stein et al., 2017, Weese et al., 2010b, Rodriguez et al., 2016a). Various ribotypes, such as RT078, 014, 001, and 126, have been isolated in the environment including soil from recreational parks and gardens, water from rivers and beaches, and sewage effluent (Janezic et al., 2016, Moono et al., 2017). *C. difficile* in pig faeces may contribute to environmental contamination, and similarly, environmental contamination of the

piggery environment with *C. difficile* spores may contribute to the acquisition of *C. difficile* in pigs, and the subsequent dissemination of CDI to humans in the community (Squire et al., 2013).

A variety of *C. difficile* ribotypes can be found in pigs with the most prevalent ribotype being 078, as mentioned previously (Weese, 2020). With the enhancement of molecular techniques, WGS studies have found the potential for identical strains to share human and animal origins, and have defined genomic relationships between pig isolates and CDI cases, both in HA-CDI and CA-CDI (Knetsch et al., 2014, Moloney et al., 2021). There is evidence that the same strain of *C. difficile* can cause symptomatic infections in both animals and humans, which indicates plausible zoonotic and/or anthroponotic transmission between pigs and humans, either directly or via an unidentified intermediate vector.

C. difficile prevalence in piglets (less than 1 week of age) has been widely reported in the literature, but only a limited number of longitudinal investigations of *C. difficile* shedding in pigs have been undertaken (Hawken et al., 2013, Weese et al., 2010b, Susick et al., 2012). The increase in CA-CDI in humans highlights the importance of investigation into possible non-human sources or reservoirs. Studies have reported varied prevalence of *C. difficile* in piglets between countries, with 74% positivity reported in Canada, 77% in Ireland, 29.6% in the United States, 39.5% in Australia, and 56.7% positivity in the Czech Republic (Figure 1.7) (Weese et al., 2010b, Stein et al., 2017, Susick et al., 2012, Knight et al., 2014, Goldova et al., 2012).

1.8 Foodborne emergence of *C. difficile*

Although the transmission of *C. difficile* between humans is well established as being via the faecal-oral route, the establishment of *C. difficile* as a foodborne disease remains inconclusive. In Ireland, the incidence of CA-CDI is increasing (HPSC, 2021) Historically, it was widely accepted that *C. difficile* was transmitted to patients from healthcare environments only (Freeman et al., 2010). However, in recent years the proportion of CA-CDI has increased, affecting patients who have no immediate exposure to a healthcare facility. CA-CDI is now affecting populations that previously were considered as low risk, such as younger cohorts and patients with no recent history of antibiotic

treatment. This suggests that there are additional risk factors at play in CA-CDI and there is a paradigm shift from the risk factors that are linked with HA-CDI (Kim and Zhu, 2017).

The presence of *C. difficile* in non-human reservoirs has been widely reported as detailed in section 1.7, and studies have detected *C. difficile* in retail meat products, water, and vegetables (Weese et al., 2009, Schaffler and Breitruck, 2018, Tsuchiya et al., 2022). With the recognition of *C. difficile* in food production animals intended for human consumption, there has been concern that *C. difficile* may be a foodborne and zoonotic pathogen. To date, however, there has been no definitive proof of environmental or foodborne transmission of *C. difficile* (Uzal et al., 2023).

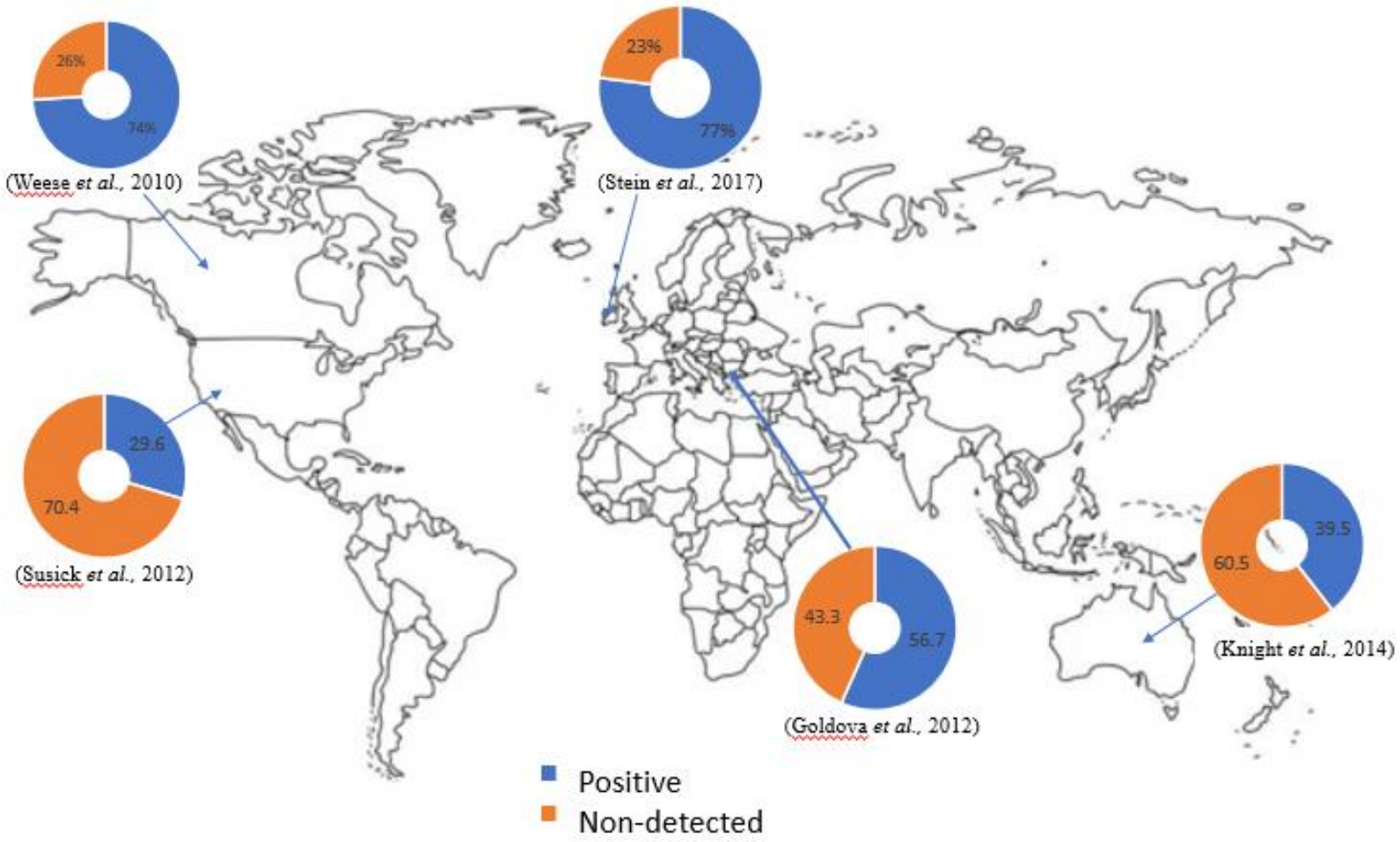


Figure 1.7 Geographical variations in *C. difficile* percentage positivity rates from piglet litters.
Data obtained from studies by (Weese et al., 2010b, Stein et al., 2017, Susick et al., 2012, Knight et al., 2014, Goldova et al., 2012)

1.9 Aims and objectives of the thesis

This work presented in this thesis is part of a collaborative research project funded by the Department of Agriculture, Food, and the Marine (DAFM), exploring the foodborne emergence and epidemiology of *C. difficile* in Ireland, and involved four research groups from different Irish Universities.

The epidemiology of *C. difficile* infection is poorly understood. The overall aim of the work presented in this thesis has been to help to identify the gaps in our knowledge of *C. difficile*, and to increase the understanding of the relationship between *C. difficile* isolates from pig origins with those from humans. This was carried out by completing the following objectives:

- To determine the prevalence of *C. difficile* in pigs at slaughter.
- To determine the prevalence of *C. difficile* in pig meat products
- To obtain *C. difficile* isolates from pigs and pig meat products for comparison with human isolates.
- To characterise the epidemiology of *C. difficile* in Irish pig farms in terms of farm-level prevalence in sows, piglets, weaners, finishers and slaughter pigs and risk factors affecting same.
- To prospectively collect laboratory detected isolates of *C. difficile* from patients attending St James's Hospital with a confirmed clinical diagnosis of CDI.
- To ribotype all the above isolates (target no. of 400) to identify those belonging to RTs 078, 020, 027 and 014.
- To conduct whole generation sequencing of isolates belonging to RTs 078, 020, 027 and 014 (target no. is 100).
- To investigate the genetic relatedness of clinical and food associated isolates by performing bioinformatic analysis on clinical isolates together with *C. difficile* ribotypes 078, 020, 027 and 014 from animal, food, and environmental origin.

Collection and characterisation of *Clostridioides difficile* isolates in Irish pigs, pork meat products, and from clinical cases of *C. difficile* Infection

2 Chapter 2

2.1 Introduction

Historically, CDI has been regarded as a nosocomial infection. It is now believed that there are diverse sources for acquisition of *C. difficile* that play a role in the development of CDI in the community, as the incidence of CA-CDI has increased globally (Martinez-Melendez et al., 2020). Patients who develop CA-CDI do not have the traditional risk factors that are associated with HA-CDI, such as contact with a healthcare facility, prior antibiotic exposure, or advanced age. In Ireland, one-third of all CDI cases occur in the community (HPSC, 2021). In addition, certain toxigenic strains such as RT078 are more likely to be implicated in CA-CDI in younger patients and can be carried asymptomatically (Knight and Riley, 2019).

C. difficile has been detected in food animals, including pigs, cattle and poultry, pork slaughterhouses, and in a range of meats and fresh produce (Rodriguez et al., 2013, Hawken et al., 2013, Weese, 2020, Rodriguez et al., 2016a). Moreover, similar PCR-ribotypes are found in humans and animals (Hensgens et al., 2012, Herbert et al., 2019). The RT078 strain has established significant reservoirs in Europe and North America and has been well documented as the dominant strain in food chain animals including pigs irrespective of age, diarrhoeal status, and other farm-specific factors (Rodriguez et al., 2016a). Toxigenic *C. difficile* has been identified in piglets with typhlocolitis but has also been found in clinically healthy animals (McElroy et al., 2015, Keessen et al., 2013a). In addition, WGS studies have revealed high level clonality between porcine and human isolates even where human cases are separated by long distances (Knight et al., 2016, Moloney et al., 2021).

C. difficile prevalence levels are generally much higher in farmed pigs than other species, although the reasons for this disparity are not fully understood. Commercial pig production is more intensive than other commercial farming sectors and this can present more opportunities for pathogen transfer (Uzal et al., 2023). Livestock accounts for a large proportion of global antimicrobial use, and the pig sector are a major consumer of veterinary antimicrobials nationally and internationally (O'Neill et al., 2021). The majority of antimicrobials (89.2%) that are used in pig production are administered in medicated

feed to prevent or treat disease (O'Neill et al., 2019). Antimicrobials are administered in feed to piglets during the weaning stage, when piglets are separated from their mother's milk (the sow) and transitioned to solid feed (Campbell et al., 2013). The level of antibiotic use on farms are associated with a number of factors, such as herd size, biosecurity practices, such as provision of boots to visitors, and socio-economic factors, such as age, education and years of experience, as antibiotic use is often considered as a cost-effective mitigation of disease by pig farmers (O'Neill et al., 2021).

The aim of this study was to investigate and characterise the true prevalence of *C. difficile* in piglet litters in Ireland, in comparison to a previous Irish study (Stein et al., 2017) which mainly focused on pooled samples. The objectives of this study were to determine the prevalence, ribotype, antimicrobial susceptibility and genomic sequences of *C. difficile* in pigs at selected Irish farms with different antimicrobial usage throughout different stages of production to slaughter, to determine the prevalence of *C. difficile* on porcine carcases in abattoirs and in retail meat products of porcine origin, and to compare porcine *C. difficile* isolates with isolates from human clinical *C. difficile* cases to investigate the genetic relatedness.

2.2 Materials and Methods

2.2.1 Materials

Table 1.1 provides summary details of the materials used and their respective suppliers. Section 2.2.1 provides details of the bacterial strains used and their growth conditions, and section 2.2.2 provides details of the agars and broth media prepared and used in various experiments detailed in this chapter.

2.2.1.1 Reference strains and growth conditions

C difficile reference strains were obtained from the National Collection of Type Cultures (NCTC) (Public Health England, UK). *C. difficile* NCTC 11209 (Type strain, PCR RT001), NCTC 13366 (PCR RT027) and NCTC 11204 (PCR RT001) were the reference strains chosen for use in this project. Each strain was inoculated in 10 ml of nutrient broth in sterile tubes, which were placed in a rack in a Genbox with Anaerogen sachets and incubated at 37 ^{o}C for five days. A 666 ml aliquot of broth was added to 333 ml of 50% glycerol in a cryovial and stored at -80^{o}C.

Resuscitation of the reference strains involved partial thawing of the frozen stock culture and streaking onto Columbia blood agar using a 10 ml inoculation loop. These plates were incubated at 37 ^{o}C for 48 hours under anaerobic conditions. The anaerobic conditions were created using Genboxes and anaerobic sachets or using the Whitley A25 anaerobic workstation in the main microbiology laboratory at St James's Hospital (SJH).

Table 2.1 Materials used and their suppliers.

Material	Supplier
Nutrient broth	VWR International
Columbia blood agar base	SLS (Scientific Laboratory Supplies)
Oxoid Anaerogen sachets	SLS
Genbox®	Biomerieux UK Ltd
C. difficile Agar Base (Oxoid)	Fisher Scientific Ltd.
C. difficile selective supplements	SLS
Gibco Bacto Yeast extract	SLS
Thermo Scientific Brain Heart Infusion (BHI) broth	Fisher Scientific Ltd
Maximum Recovery Diluent (MRD)	VWR International
CDMN selective supplements (Oxoid)	Sparks Lab Supplies
Defibrinated horse blood (E&O Labs)	SLS
Sodium taurocholate	VWR International
Glycerol	Fisher Scientific Ltd
Primers	Integrated DNA Technologies (IDT)
Hotstar Mastermix	Qiagen
Nuclease-free water	Bio-Sciences Ltd.
MicroAmp® PCR reaction plate	Bio-Sciences Ltd.
1.5 ml Eppendorf tube	Fisher Scientific Ltd
Sterile 30 ml tubes	Sparks Lab Supplies
2 ml Microcentrifuge tubes	Fisher Scientific Ltd
Instagene matrix	Accuscience Ireland Limited
Sterile inoculation loop	Fisher Scientific Ltd
Thermo Scientific Nalgene Cryogenic Tubes 2 ml	Fisher Scientific Ltd
0.2 ml PCR strips with lids	SLS
5 ml reagent reservoir	Fisher Scientific Ltd
GeneScan™ 1200 LIZ® Size Standard	Bio-Sciences Ltd
Hi-Di™ Formamide, 25 ml	Bio-Sciences Ltd
POP-7™; Polymer for 3130⁄3130xl Genetic Analyzers	Bio-Sciences Ltd
Fisherbrand L-Shaped Cell Spreaders	Fisher Scientific Ltd.
Oxoid Anaerobic Indicator Reazurin	SLS
Petri dish 90mm Triple Vented Classic Sterilin	SLS
Agarose	Sigma
6X loading dye	New England Biolabs (NEB)
1kb plus ladder	NEB

2.2.1.2 Preparation of growth media

All agar was prepared in-house using an automated Mediaclave 10/30 media steriliser (Integra Biosciences AG, Zizers, Switzerland) and Mediajet (Integra Biosciences AG, Switzerland) to ensure consistent and high-quality media. All broth was prepared in-house using a C40 Benchtop Autoclave (Priorclave, London, UK). Agar and broth preparations were autoclaved at 121°C for 15 minutes.

2.2.1.3 *C. difficile* Moxalactam Norfloxacin (CDMN) agar

C. difficile selective agar (CDMN agar) was prepared according to the manufacturer's instructions and autoclaved at 121°C for 15 minutes. CDMN selective supplements were prepared according to the manufacturer's instructions (1 vial per 500 ml of agar). Once cooled to 45-50°C, prepared CDMN selective supplements and defibrinated horse blood (5% w/v) were added. Agar was dispensed into petri dishes using an IBS Integra Biosciences Mediajet (Integra Biosciences AG, Zizers, Switzerland).

2.2.1.4 Brain Heart Infusion (BHI) broth

To make 1 L of BHI broth, 37 g of BHI broth powder was added to 1L of sterile distilled water (SDW) and autoclaved at 121°C for 15 minutes. When cooled, *C. difficile* selective supplements were prepared according to the manufacturer's instructions, and 2 vials were added to the broth (1 vial per 500 ml of broth), along with sodium taurocholate (0.1% w/v). The broth was mixed and dispensed into sterile 30 ml universal tubes.

2.2.1.5 Brain Heart Infusion-Supplemented (BHIS) broth

To make 1 L of BHIS broth, 37 g of BHI broth powder and 5 g of yeast was added to 1L of SDW and autoclaved at 121°C for 15 minutes. The broth was allowed to cool to room temperature before dispensing into sterile 30 ml universal sterile tubes.

2.2.1.6 Maximum Recovery Diluent (MRD)

MRD was prepared according to the manufacturer's instructions and autoclaved at 121°C for 15 minutes. The medium was allowed to cool to room temperature before use.

2.2.1.7 Glycerol (50% v/v)

Glycerol was diluted 1:1 with SDW and autoclaved at 121^{o}C for 15 minutes. The medium was allowed to cool to room temperature before use.

2.2.2 Methods

2.2.2.1 Pig farms and antibiotic usage

Pig faecal samples were collected from 10 pig farms by investigators from the Antimicrobial Use and Resistance in Animal Production (AMURAP). The AMURAP project is a collaboration between Teagasc, the University College Dublin (UCD) School of Veterinary Medicine, and Department of Agriculture, Food, and the Marine (DAFM), and is funded by DAFM project 15/S/676. The faecal samples were then aliquoted into sterile universal containers by Rachel Grimes, who was a previous postgraduate student undertaking this project. Due to GDPR and agreements made with farmers by project, the geographical locations of the individual farms cannot be disclosed, but they were distributed across 6 counties.

The details of the antibiotics used on each of the different farms are detailed in Table 2.2. High in-feed antimicrobial (HAU) farms use in-feed antibiotics through to the weaner stage, whereas moderate in-feed antimicrobial use (MAU) farms stop in-feed antibiotic use before the weaner stage. Low antimicrobial use (LAU) farms do not administer in-feed antibiotics, but antibiotics may still be administered to animals with clinical infections. Farms 1, 2, 8 and 10 were categorised as LAU farms; farms 5, 6 and 7 were categorised as MAU farms; farms 3, 4 and 9 were categorised as HAU farms. It was originally intended to study the 10 different pig farms in detail. Pig faecal sample collection began for Farm 1 during summer 2019, but sample collection on the final five farms was not fully completed due to restrictions from the COVID-19 pandemic. Therefore, five farms could be studied in detail – two LAU farms, one MAU farm and two HAU farms. Sow and piglet samples from a second MAU farm was also included in the analysis.

2.2.2.1.1 Medicated feed

Data on in-feed antimicrobial use in Irish farms was retrieved from O'Neill et al. (2020). The authors of this study provided the aliquots of pig faecal samples from different farms

for the longitudinal surveillance of *C. difficile* on Irish pig farms. Antimicrobial usage on farms alludes to the level of medicated feed use on the farms, which pertains to a diet containing antimicrobial oral premixes. This medicated feed may also contain other types of medication such as anthelmintics, anti-inflammatories and zinc oxide. Pig diets are specific to the stage of production, so the levels of antimicrobial oral premixes in this feed varies throughout each stage.

2.2.2.1.2 Ethics approval and consent to participate

The participating farmers gave informed written consent for the use of their data in this study. Thus, formal approval by an ethics committee was not required under Irish regulations.

2.2.2.1.3 Pig faecal sample collection

Approximately 10 g of fresh pig faecal samples was collected from pig farms in 6 different locations across Ireland between 2019 and 2020. The faecal material samples were collected from six different stages of pig life (Figure 2.1). The faecal material was collected immediately after defecation with gloved hands and placed in sterile screw-cap tubes. Gloves were changed in between sample collection to avoid cross contamination. Approximately 2g of these samples were then aliquoted into sterile universal containers in a biological safety cabinet. Samples were immediately transported to the DAFM laboratories in Backweston, Co. Kildare and stored at -80^{o}C until further processing. For piglet samples, as much faecal material as possible was collected due to the lesser amount (in grams) compared to adult pigs on average.

Table 2.2 In-feed antimicrobial use in different sampling farms across Ireland

HAU			
Farm[1]	**Diet**	**Medication[2]**	**Approx. duration**
3	creep[3]	apramycin (100 mg/kg)	1.5 weeks
	link[4]	trimethoprim & sulfadiazine (TMS) (300 mg/kg)	1.5 weeks
	weaner[5]	trimethoprim & sulfadiazine (300 mg/kg), tylosin (2nd stage)	TMS - 1 week Tylosin - 4 weeks
4	creep	amoxicillin (300 mg/kg) & chlortetracycline (405 mg/kg)	2 weeks
	link	amoxicillin (300 mg/kg) & chlortetracycline (405 mg/kg)	2 weeks
	weaner	chlortetracycline (400 mg/kg)	4 weeks
9	creep	amoxicillin (250 mg/kg) & tilmicosin (200 mg/kg)	3.5 weeks
	link	amoxicillin (250 mg/kg) & tilmicosin (200 mg/kg)	1.5 weeks
	weaner	chlortetracycline (300 mg/kg) amoxicillin (500 mg/kg)	4.5 weeks 3 weeks
MAU			
Farm	**Diet**	**Medication**	**Approx. duration**
5	creep	tylvalosin (85 mg/kg)	10 days
	link	tylvalosin (85 mg/kg)	10 days
	weaner	None	N/A
6	creep	trimethoprim & sulfadiazine (300 mg/kg)	10 days
	link	tylosin (100 mg/kg)	2 weeks
	weaner	None	N/A
7	creep	apramycin (100 mg/kg)	7 days
	link	apramycin (100 mg/kg)	10 days
	weaner	None	N/A

[1]Farms were group into three categories: High in-feed Antimicrobial Use (HAU), Moderate in-feed Antimicrobial Use (MAU) and Low in-feed Antimicrobial Use (LAU). [2]Medication weights (mg/kg) refers to mg of antibiotic per kg of feed. [3]Creep is given at approximately 12 days of age and helps the transition from sow's milk to solid food. [4]Link is a solid feed rich in macronutrients. [5]Weaner is a dry diet predominantly of vegetable origin. Only farms 3 and 4 (HAU) and 5 and 7 (MAU) were used in this study.

Note: Low in-feed antimicrobial use (LAU) (Farm 1, 2, 8 and 10) is another category of antimicrobial usage in this study but is not included in this table as is not applicable.

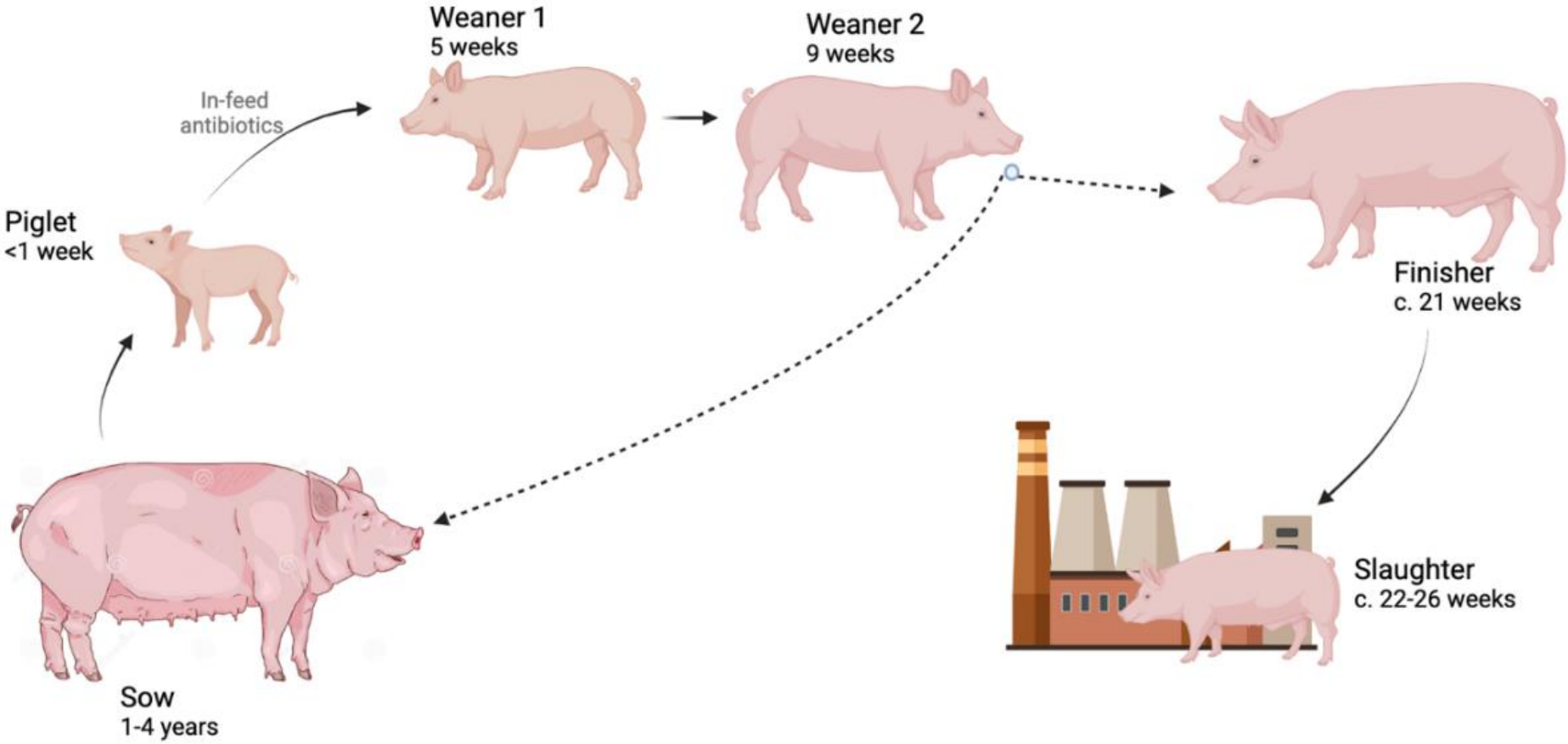


Figure 2.1 Different stages of pig rearing and the time that samples were taken from the animal.
Sows were between 1-4 years of age. Piglet samples were taken from pigs less than 1 week old. In-feed antibiotics are administered between the piglet and into the weaner stage and duration of antibiotic usage varies between different farms (between 1 week and 8 weeks), as described in Table 2.2. Weaner 1 samples were taken at 5 weeks of life, and weaner 2 samples were taken at 9 weeks. Finisher samples were taken at 21 weeks and slaughter samples were taken at 22-26 weeks of age.

2.2.2.2 Culture of *C. difficile* from pig faecal samples

Direct plating of samples to investigate *C. difficile* prevalence can be unreliable because of low numbers of spores that may be present in samples, so an enrichment step was used before plating onto selective agar, as previously described by (Alvarez-Perez et al., 2018). All samples were processed in DAFM laboratories in Backweston, Co. Kildare using the aforementioned enrichment step before plating on selective agar. In a biological safety cabinet, 1 g of each faecal sample was added to 9 ml of BHI broth in 30 ml sterile universal tubes using a disposable tongue depressor. Tubes containing the faecal-broth mix were placed in a rack in a Genbox with Anaerogen® sachets and incubated at 37 ^{o}C for five days, with the lids half open. After incubation, a 100 µL aliquot of the faecal-broth mix was plated onto CDMN agar and spread across the plate using an L-shaped cell spreader. The plates were placed in a Genbox (42 plates per box) with Anaerogen® sachets and incubated at 37 ^{o}C under anaerobic conditions for 48 hours. Suspected colonies were identified by colony morphology and characteristic smell, and isolate identification was confirmed using Matrix-Assisted Laser Desorption/Ionization-Time Of Flight (MALDI-TOF) Mass Spectrometry (MS), as detailed in section 2.2.2.5. Pure cultures were achieved by taking a single colony from the CDMN plate and streaking onto Columbia blood agar and were stored as detailed in section 2.2.2.6.

2.2.2.3 Culture, isolation, and identification of *C. difficile* from meat samples

2.2.2.3.1 Collection of meat products for *C. difficile* surveillance

DAFM are required to carry out surveillance for bacteria which are resistant to antimicrobials under Decision 652/2013/EU (EU, 2013). On a weekly basis, DAFM routinely conduct tests on chilled fresh pig meat for the presence of antimicrobial resistant *E. coli*. Pre-packaged chilled pork meat samples are collected by a DAFM sampling officer in their unopened original packaging from the chilled meat counter in supermarkets. In 2021, pig meat samples were taken randomly across the Republic of Ireland from retail premises in areas of high population density, based on Central Statistics Office (CSO) population data, and were transported, unopened, in a thermally insulated box to DAFM laboratories. Pig meat samples were aliquoted into a sterile universal container by a DAFM lab analyst and were processed within 2 hours of opening the packaging. This provided 100 samples from retailers across the Republic of Ireland.

To increase the sample size, fresh, non-frozen pork meat products in their unopened original packaging, taken from the chilled meat counter in the retailer, were purchased from five leading supermarkets in Dublin, Ireland– Aldi, Dunnes Stores, Lidl, Supervalu, and Tesco. A total of 80 products were collected, 40 of which were cooked pork meat products, and the remaining 40 were raw pork meat products. Each product was inspected to ensure that it carried the Bord Bia guaranteed Irish seal of approval. Pork products were kept cold in a cooler with ice packs during transport from the supermarket to the laboratory, and samples were processed within 2 hours of purchase. The supermarket name, product number, sell-by date, purchase date, and laboratory processing date were recorded for each pig meat sample that was processed for *C. difficile* culture. The availability of free-range pork meat in Ireland is limited and at the time of our study (2021), there were no free-range pork products in supermarkets that had the Bord Bia guaranteed Irish mark, so no free-range pork products were included in the sampling.

2.2.2.3.2 Culture of *C. difficile* from meat samples

To homogenise the meat, 5 g of each meat sample was added to 20 ml of BHI in a stomacher bag and blended using a Seward Stomacher® 400 (Seward, Worthing, UK), and the meat-broth mix was incubated under anaerobic conditions at 37 ^{o}C for five days. A 100 µL aliquot of the meat-BHI mix was then plated in duplicate onto CDMN agar, and plates were incubated under anaerobic conditions at 37 ^{o}C for 48 hours. For assurance, positive (*C. difficile* reference strain) and negative controls (blank) were included in each batch of sampling. Suspected colonies were identified by colony morphology and characteristic smell, and isolate identification was confirmed using MALDI-TOF MS, as detailed in section 2.2.2.5. Pure cultures were achieved by taking a single colony from the CDMN plate and streaking onto Columbia blood agar and were stored as detailed in section 2.2.2.6.

2.2.2.4 Culture, isolation, and identification of *C. difficile* from abattoir samples

2.2.2.4.1 Collection of abattoir (carcase and environmental) samples for *C. difficile* surveillance

Samples were taken from pork carcases in the chill fridge of three major abattoirs at three different locations in the Republic of Ireland. The slaughtered animals originated from 30 different pig herds from 15 different counties across the Republic of Ireland. This represents approximately 9.5% of the 316 commercial pig herds in Ireland with more than 500 pigs (DAFM, 2022b). As this was a convenience sample it represented herds of different size.

The slap mark was used to identify the pigs, as farms can send their animals for slaughter to any abattoir in Ireland. A slap mark is a "tattoo" (permanent inking) of the herd mark on the pig's skin that forms part of a tracing system implemented by DAFM, and allows the identification of all pigs that are moved across the country through a central movement database (DAFM, 2022a). For an individual to own or trade pigs, it is necessary to register with DAFM and to be issued with a valid pig herd number. The slap mark facilitated the identification of different counties that the carcases originated from.

The three abattoirs were pork-only factories with similar layouts and were each visited once in 2023. These are the largest pork-processing plants in Ireland with the slaughter capacity of each varying from 10,000 to 25,000 animals per week. Carcase swabs were individually collected from each of five pigs from the 30 randomly selected herds. Four areas from each carcase were swabbed to sample the medial hind limb, lateral abdomen (belly), mid-dorsal region (mid-back) and jowl in line with the DAFM sampling rules for *Salmonella* and Chapter III of Regulation 2073/2005 (Microbiological Criteria). Details of the abattoir, the sampling date and time, and the county of origin were all recorded. Gloves were changed between each carcase sampling and each site was swabbed using a pre-hydrated Whirl-Pak® Speci-Sponge® (B01423, Nasco) and one sponge was used per carcase. Two sites were swabbed with one side of the sponge, which was then turned over and the other side was used to swab the remaining sites. Pressure was applied onto the carcase and a scrubbing action was used 10 times vertically and 10 times horizontally to swab approximately 100 cm^2 per site. In each factory, fifty carcases were sampled. Before the pigs enter the slaughterhouse, they are kept in pens in a holding area called

the lairage for up to three hours. Environmental samples were taken from 10 pens from each abattoir. Once the pen was emptied, the ground was swabbed in four areas using the prehydrated sponge to sample approximately 100 cm^2 per area. Samples were transported in bags at 4°C to the laboratory and were processed within 3 hours of sampling.

2.2.2.4.2 Culture of *C. difficile* from abattoir samples

For each carcase, the sponge was added to 50 ml of BHI broth in a stomacher bag and blended using a Seward Stomacher® 400 (Seward, Worthing, UK). This was incubated under anaerobic conditions at 37 °C for five days. A 100 µL aliquot was then plated onto CDMN agar, and plates were incubated under anaerobic conditions at 37 °C for 48 hours. For assurance, positive (*C. difficile* reference strain) and negative controls (blank) were included in each batch of sampling. Suspected colonies were identified by colony morphology and characteristic smell, and isolate identification was confirmed using MALDI-TOF MS, as detailed in section 2.2.2.5. Pure cultures were achieved by taking a single colony from the CDMN plate and streaking onto Columbia blood agar and were stored as detailed in section 2.2.2.6.

2.2.2.5 *C. difficile* isolate identification confirmation using MALDI-TOF mass spectrometry

2.2.2.5.1 Sample preparation for MALDI-TOF MS

Colonies that were suspected to be *C. difficile* were presumptively identified by colony morphology and characteristic smell. *C. difficile* are characteristically flat, grey/white with a "ground glass appearance", and have a sickly-sweet smell that is compared to horse manure (Fordtran, 2006). In agar plates that were suspected to have mixed cultures of bacteria, each colony was confirmed by MALDI-TOF separately. A wooden toothpick was used to take the edge of a single colony which was then smeared in duplicate onto a MALDI-TOF MS steel anchor plate (MSP) 96 target (Bruker Daltonik) and allowed to dry. The dried bacterial smear was overlain with 1 µL of matrix solution, which consists of saturated α-cyano-4-hydroxy-cinnamic acid (HCCA) dissolved in a solution of 50% acetonitrile, 47.5% water, and 2.5% trifluoroacetic acid (Bruker Daltonik) and allowed to air dry at room temperature prior to analysis with the MALDI Biotyper

(Tsuchida et al., 2020). A bacterial test standard (BTS) (8290190; Bruker Daltonics) was used for instrument calibration. A positive control (*Staphylococcus aureus* ATCC 25923) and a negative control (2 µL of matrix) were included with each run. When dry, the plate was loaded into the Microflex LT (Bruker Daltonik) for MALDI-TOF MS. The MALDI Biotyper (MBT) Calibration Procedure was followed according to the manufacturer's instruction.

2.2.2.5.2 MALDI-TOF MS data processing and analysis

FlexControl version 1.4 and MBT compass IVD 4.2 software with default settings were used to perform identification and data analysis. The MALDI Biotyper output is two best-matches for each isolate. Cut-off scores ranged between 0 and 3 and were applied as recommended by the manufacturer (Table 2.3). Cut-off scores of more than 2.00 indicated identification to the species level, scores between 1.70 and 1.99 indicated identification to the genus level, and scores of less than 1.69 indicated no identification. Suspected *C. difficile* isolates that scored between 1.70 and 1.99 were repeated. When MALDI-TOF MS had the same identification to the species level (≥2.00) for both replicates, the identification was considered accurate.

Table 2.3 Bruker Daltonik MALDI-TOF Biotyper classification results, obtained from the Bruker Biotyper user manual.

Cut-off score Range	Interpretation	Symbols	Consistency	Colour
2.00 - 3.00	High confidence interpretation	(+++)	A	Green
1.70 - 1.99	Low confidence interpretation	(+)	B	Yellow
0.00 - 1.69	No organism identification possible	(-)	C	Red

Symbols refer to high, low, or no confidence interpretation. Consistency refers to the identification: A is a high consistency identification (all matches are a high confidence identification), B is a low consistency identification (the best match is high or low confidence, but further matches are not fully identified), and C means that none of the requirements for consistent identification are met.

2.2.2.6 Storage of confirmed pig *C. difficile* isolates

Once identification of *C. difficile* isolates was confirmed by MALDI-TOF MS, pure cultures were achieved by taking a single colony from the CDMN plate using a sterile inoculation loop and streaking onto Columbia blood agar (E & O Laboratories Ltd., UK) plates. If a plate was suspected to have different colonies of *C. difficile*, each colony was streaked, stored separately, and marked as an extra isolate for the sample. The CBA plates were placed in Genboxes with anaerobic sachets and incubated at 37 ^{o}C for 48 hours. An isolated colony was taken from each plate using a sterile inoculation loop and placed in BHIS broth. Tubes were incubated again under anaerobic conditions at 37 ^{o}C for 48 hours. A 666 µL aliquot of BHIS broth was then added to a cryovial with 333 µL of 50% glycerol and cryovials were stored at -20^{o}C for further processing.

2.2.2.7 Collection of clinical human clinical *C. difficile* isolates

Institutional ethics approval was provided for this research by St James's Hospital (SJH)/Tallaght University Hospital Joint Research Ethics Committee (Rec: 2021-06 Chairman's Action (11)). This detailed that the investigator was not permitted to handle patient faecal samples, and only *C. difficile* isolates from SJH were permitted to be used in this study. These ethics permissions detailed that *C. difficile* isolates must be irrevocably anonymised by medical laboratory scientists in the hospital enteric pathogens laboratory, prior to being handed over to investigators in TCD. Processing of samples and transfer of isolates in the enteric pathogens laboratory was performed by a St. James's employed Medical Scientist.

For initial diagnostic purposes in the EPL, *C. difficile* toxin gene *tcdB* had been detected by PCR on faecal samples from patients with diarrhoea at SJH, or from outpatients. Isolates were obtained from faecal samples and stored by medical laboratory scientists in the EPL. SJH is a tertiary referral hospital, so faecal samples are sent from general practitioners (GPs) to confirm a diagnosis of *C. difficile*. Isolates were obtained from faecal samples by SJH medical laboratory scientists for the purpose of further outbreak investigation, if required, by streaking onto Brazier's cefoxitin cycloserine egg-yolk (CCEY) agar.

These isolates were irrevocably anonymised by the SJH medical laboratory scientists in the EPL, and each tube was given an alphanumeric code for identification purposes. Isolates were initially stored at -80°C on cryovial beads (Gem Scientific, Batley, UK) in the EPL and later handed over to the investigator, where they were further stored at -80°C in the Sir Patrick Dun Laboratory (SPD), TCD, in the Central Pathology Laboratory of SJH. A total of 400 clinical isolates of *C. difficile* collected between 2018 and 2020 were stored pending further characterisation.

2.2.2.8 Ribotyping

2.2.2.8.1 DNA isolation for ribotyping

Bacterial DNA was extracted from pure cultures of all confirmed *C. difficile* isolates using the Instagene™ matrix. *C. difficile* was grown on Columbia blood agar for 48 hours at 37 °C under anaerobic conditions. An isolated bacterial colony was suspended in 1 ml of nuclease-free water in a 1.5 ml sterile microcentrifuge tube. The microcentrifuge tube was centrifuged (Fisherbrand Gusto Mini Centrifuge) for 1 minute at 10,000 X g and the supernatant was discarded. A 200 µL aliquot of Instagene matrix was added to the pellet and incubated at 56°C for 30 minutes. The microcentrifuge tube was vortexed at high speed for 10 seconds using a Stuart Vortex Mixer SA8 and was then placed in a 100°C heating block (Stuart Blockheater SBH200D) for 8 minutes. The tube was vortexed again and then centrifuged at 10,000 X g for 3 minutes. A 2 µL aliquot of the supernatant was used per 25 µL Polymerase Chain Reaction (PCR) reaction, and the DNA extract was stored at -20°C. When the DNA extract was reused after freezing, the microcentrifuge tube was vortexed for 10 seconds, centrifuged at 10,000 X g for 3 minutes and the supernatant was used.

2.2.2.8.2 Polymerase Chain Reaction (PCR) for ribotyping

The PCR Master Mix was prepared in a sterile 2 ml Eppendorf tube. Primers used are listed in Table 2.4. Primers were diluted to 10 pmol/ml by adding 10 µL of primer mix to 90 µL of nuclease-free water. A fluorescent label (6-Carboxyfluorescein (6FAM)) was added to allow the forward strand of PCR fragments to be detected on automated fragment analysis system (ECDC, 2018). Reagents were added in the following concentrations for one sample (and amounts were determined by the number of samples

to be tested): 12.5 µL Hotstar Master Mix, 0.5 µL 6FAM-16S forward primer, 0.5 µL 23S reverse primer and made up to 23 µL with nuclease-free water. The Master Mix was vortexed at high speed, centrifuged briefly and 23 µL was dispensed into 0.2 ml thin-walled PCR strips (SLS Analab, Northern Ireland). A 2 µL aliquot of *C. difficile* DNA was added to the strips and tubes were closed using PCR lids. Each ribotyping run used a negative control (nuclease-free water) and a positive control (*C. difficile* RT078, supplied from Teagasc Food Research Centre, Ashtown, Ireland). The PCR strips were centrifuged briefly using a microcentrifuge and placed on BioRad DNA Engine® Peltier Thermal Cycler. Cycling parameters were as per the ECDC ribotyping protocol as detailed in Table 2.5 (ECDC, 2018).

Table 2.4 List of primers used for amplification of DNA during PCR ribotyping (Bidet et al., 1999)

Name	Sequence (5'-3')	Notes
16s-for	GTGCGGCTGGATCACCTCCT	Fluorescent label (6FAM) was added to allow the forward strand of PCR fragments to be detected on automated fragment analysis system (ECDC, 2018)
23s-rev	CCCTGCACCCTTAATAACTTGACC	

Table 2.5 Cycling parameters for *C. difficile* ribotyping

Step	Temperature (°C)	Duration (minutes)	Number of cycles
Hot Start	95	15	1
Denaturation	95	1	24
Annealing	60	1	
Extension	72	1	
Final Extension	72	30	1

2.2.2.8.3 Gel electrophoresis

Initially, to view ribotypes after PCR a gel electrophoresis was performed. Agarose gel (3%) was prepared in 0.5% TBE buffer and PCR products were electrophoresed at 180V for 40 minutes. A 5 µL aliquot of PCR product was added to 1 µL 6X loading dye in a microcentrifuge tube and mixed gently by pipetting up and down 3-4 times. Gels were visualised under a Quantity One® (Bio-Rad) fluorescent imager.

2.2.2.8.4 Fragment denaturation and analysis

To automate the PCR ribotyping process, fragment analysis was performed using an Applied Biosystems (ABI) 3500 Genetic Analyzer to generate .fsa files, which are then used to assign PCR ribotypes. The reaction mix was prepared with 10 µL of HiDi Formamide and 0.5 µL of GeneScan™ 1200 LIZ® size standard per sample used. A MicroAmp 96-well reaction plate was prepared by adding 10 µL reaction mix to each well, and 2 µL of PCR product was added to each corresponding well using a VWR Ergonomic High Performance Multichannel Pipette (0.5-10 µL). This was mixed by pipetting up and down 3-4 times. The microplate was sealed using adhesive film and centrifuged for 10 seconds using an Heraeus Multifuge 3SR+ Plate Centrifuge. The plate was denatured in a BioRad DNA Engine® Peltier Thermal Cycler at 95°C for 2 minutes and then held at 4°C for 10 minutes. The plate was centrifuged briefly again using the plate centrifuge. The adhesive film was then removed, and a 96-well plate septum was attached, and the plate was added to a plate base and retainer.

Fragment analysis was carried out using the ABI 3500 genetic analyser (Thermo Fisher, Dublin, Ireland), with default settings for POP-7™ and 36 cm capillary length (ECDC, 2018). Raw data was obtained (.fsa files) and uploaded to the freely available WEBRIBO database (https://webribo.ages.at/).

2.2.2.9 Statistical analysis

All statistical analyses were carried out using the GraphPad Prism software unless otherwise stated, with a *P* value of ≤0.05 being considered significant. Due to the absence of some values from MAU farms, the data was analysed by fitting a mixed model, rather than by repeated measures ANOVA (which cannot manage missing values).

2.2.2.10 Generation of graphs and figures

Graphing was done using GraphPad prism software, and figures were created using Microsoft PowerPoint, Biorender or Mind the Graph.

2.3 Results

2.3.1 Prevalence of *C. difficile* in collected pig faecal samples

A total of 1400 pig faecal samples from 10 farms across Ireland was collected in this study, from sows, piglets, weaner 1, weaner 2, finisher and slaughter animals (Figure 2.1).

A total of 776 pig faecal samples were tested from 6 farms. *C. difficile* was isolated from 51.4% (398/776) of pig samples overall and its identity was confirmed to the species level by MALDI-TOF MS (Table 2.6). *C. difficile* detection rates varied between age of pig and farms with different category of antimicrobial use.

Table 2.6 Sample of results generated by a MALDI-TOF run on 3 samples from Farm 1, table is adapted from the Bruker Biotyper user manual.

Sample Name	Sample ID	Organism (best match)	Score value	Organism (second-best match)	Score value
A1 (+++) (A)	BTS	*Escherichia coli*	2.37	*Escherichia coli*	2.32
A2 (+++) (B)	P1	*Clostridium innoccuum*	1.88	*Clostridium innoccuum*	1.85
A3 (+++) (A)	P2	*Clostridium difficile*	2.18	*Clostridium difficile*	2.07
A4 (+++) (A)	P3	*Clostridium difficile*	2.17	*Clostridium difficile*	2.04

BTS (Bruker Bacterial Test Standard) is the control run (*E. coli* negative control). Bruker BTS is an extract of *E. coli* that covers the entire mass range of proteins used by MALDI-TOF Biotyper for precise microorganism identification.
Piglet 1 (P1) was confirmed as *Clostridium innocuum*, Piglets 2 and 3 (P2 and P3) were confirmed as *C. difficile*.

The average positivity rate of *C. difficile* at HAU farms was 35.33%, in comparison to an average positivity rate of 61.89% for LAU farms, and 57.33% for MAU farms (Table 2.7). To note, sampling of Farm 5 was not completed in entirety due to the impact of the COVID-19 pandemic, therefore the slaughter and finisher stage are not included, so the average values for MAU are skewed. There was a significant difference in *C. difficile* prevalence between HAU farms and LAU farms ($P < 0.0001$), which is observed in Figure 2.2.

C. difficile was detected in 100% (40/40) of sows tested in Farm 3, 4, 5, and 7, and in 75% (6/8) of Farm 1 sows and 80% (8/10) of Farm 2 sows. *C. difficile* was cultured from 124

out 142 piglets (87.3%) that were sampled, and positivity rates in piglets ranged from 68% (17/25) on Farm 4 to 96% (25/26) positivity on Farm 8.

C. difficile was cultured from 40 out of 125 weaner 1 pigs (32%). Overall, there was a significant difference between sows and weaner 1 ($P = 0.001$). In addition, considering all farms together, there was a significant difference between piglets and weaner 1 ($P = 0.0032$). On HAU farms, there was a significant difference in the prevalence of *C. difficile* in weaner 1 pigs in comparison to the sow stage ($P < 0.0001$). The prevalence of *C. difficile* in weaner 1 pigs was statistically significantly lower ($P = 0.0016$) in HAU compared to LAU farms, as observed in Figure 2.3.

C. difficile was detected in 35 out of 150 weaner 2 pigs (23.3%). Overall, there was a significant difference between sows and weaner 2 ($P = 0.001$). In addition, considering all farms together, there was a significant difference between piglets and weaner 2 ($P = 0.0032$). There is a difference in the prevalence of *C. difficile* in weaner 2 pigs between LAU and HAU farms, as observed in Figure 2.3, and this difference is statistically significant ($P = 0.0016$). On HAU and MAU farms, there was a significant difference in *C. difficile* positivity between sows and weaner 2 stages ($P < 0.0001$ and $P = 0.014$, respectively). In addition, there was significant difference in the prevalence of *C. difficile* between piglet and weaner 1 stages on HAU farms ($P = 0.0002$).

C. difficile was detected in 47 out of 125 finisher pigs (38%). Overall, there was a significant difference between sows and finisher ($P = 0.0003$). In addition, considering all farms together, there was a significant difference between piglets and finisher ($P = 0.006$). There is a significant difference between the prevalence of *C. difficile* in finisher pigs on LAU farms and HAU farms ($P = 0.02$). On LAU and HAU farms, there was a significant difference in the prevalence of *C. difficile* between sows and finisher stages ($P = 0.006$ and $P < 0.0001$, respectively). On LAU and HAU farms, there was a significant difference in the prevalence of *C. difficile* between piglet and finisher stages ($P = 0.006$ and $P = 0.0002$, respectively).

C. difficile was detected in 41 out of 124 slaughter pigs (33%). *C. difficile* prevalence was found to increase again at the slaughter stage in HAU farms (Figure 2.3). Overall, there was a significant difference between sows and slaughter ($P = 0.001$). Furthermore, considering all farms together, there was a significant difference in overall *C. difficile*

positivity over time as the pig grew from the piglet stage to the slaughter stage ($P = 0.004$). On LAU and HAU farms, there was a significant difference in the prevalence of *C. difficile* between sows and slaughter stages ($P = 0.02$ and $P = 0.0003$, respectively). On LAU and HAU farms, there was a significant difference in the prevalence of *C. difficile* between piglet and slaughter stages ($P = 0.007$ and $P = 0.003$, respectively).

Table 2.7 *C. difficile* percentage positivity (%) in samples
Taken from High in-feed Antimicrobial Use (HAU), Moderate in-feed Antimicrobial Use (MAU) and Low in-feed antimicrobial Use (LAU) farms, for pigs at different stages.

***C. difficile* percentage positivity (%)**							
Farm Number	**Sow**	**Piglet**	**Weaner 1**	**Weaner 2**	**Finisher**	**Slaughter**	**Total Average (All stages)**
LAU Farm							
1	75	92	48	40	24	28	51.16
2	80	85.7	76	84	56	54	72.62
Average LAU	77.5	88.9	62	62	40	41	**61.89**
MAU Farm							
5	100	95.6	40	8	*	*	**61***
7	100	90	24	4	72	32	**53.66**
Average MAU	100	92.8	32	6	72*	32*	**57.33***
HAU Farm							
3	100	92	4	0	4	32	38.66
4	100	68	0	4	0	20	32
Average HAU	100	80	2	2	2	26	**35.33**
ALL FARMS	**92.5**	**89.7**	**32**	**23.3**	**38***	**33***	**51.4***

*Sampling of Farm 5 was not completed in entirety due to the COVID-19 pandemic, so the slaughter and finisher stage are not included, this means that the average values for MAU are skewed.

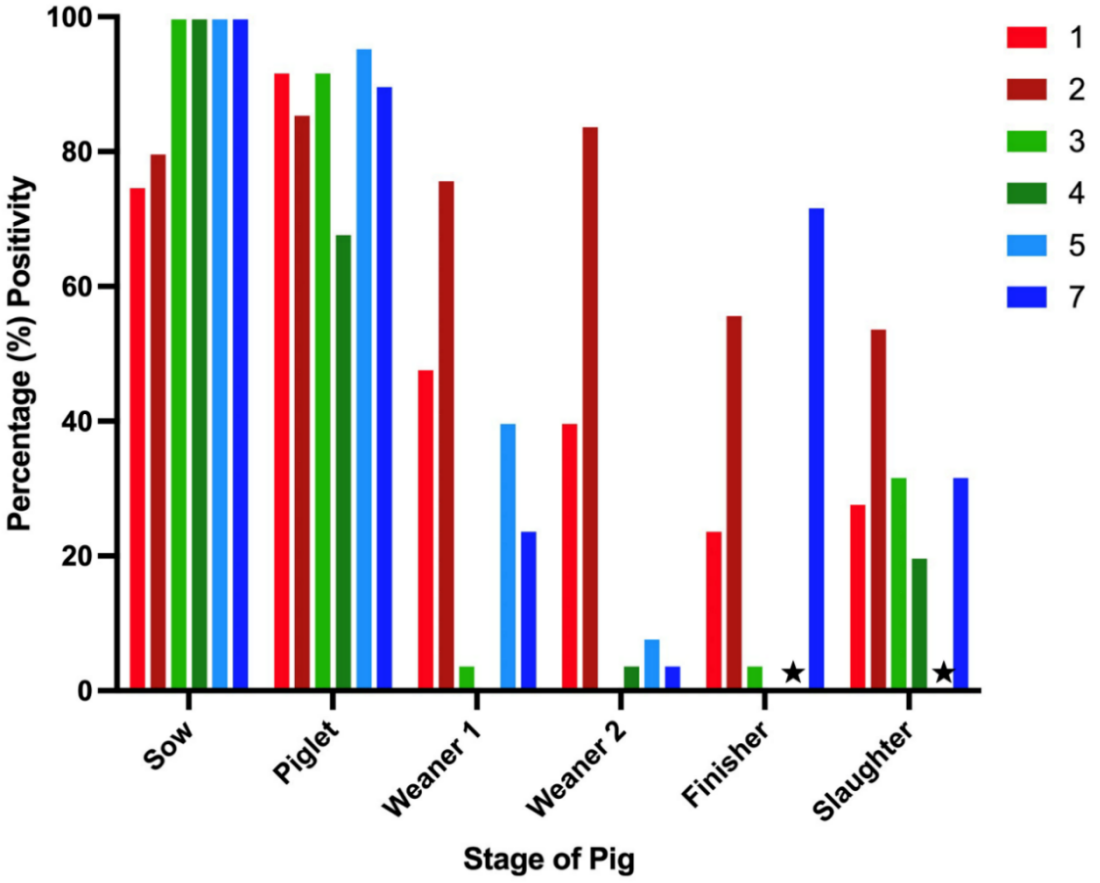


Figure 2.2 Bar chart demonstrating the percentage positivity of *C. difficile* on 6 different farms.

Red bars correspond to Low Antibiotic Use (LAU) farms, green bars correspond to High Antibiotic Use farms (HAU), and blue bars correspond to Moderate Antibiotic Use farms (MAU).

★ Sampling of Farm 5 was not completed in entirety due to the COVID-19 pandemic, thus the slaughter and finisher stage could not be included.

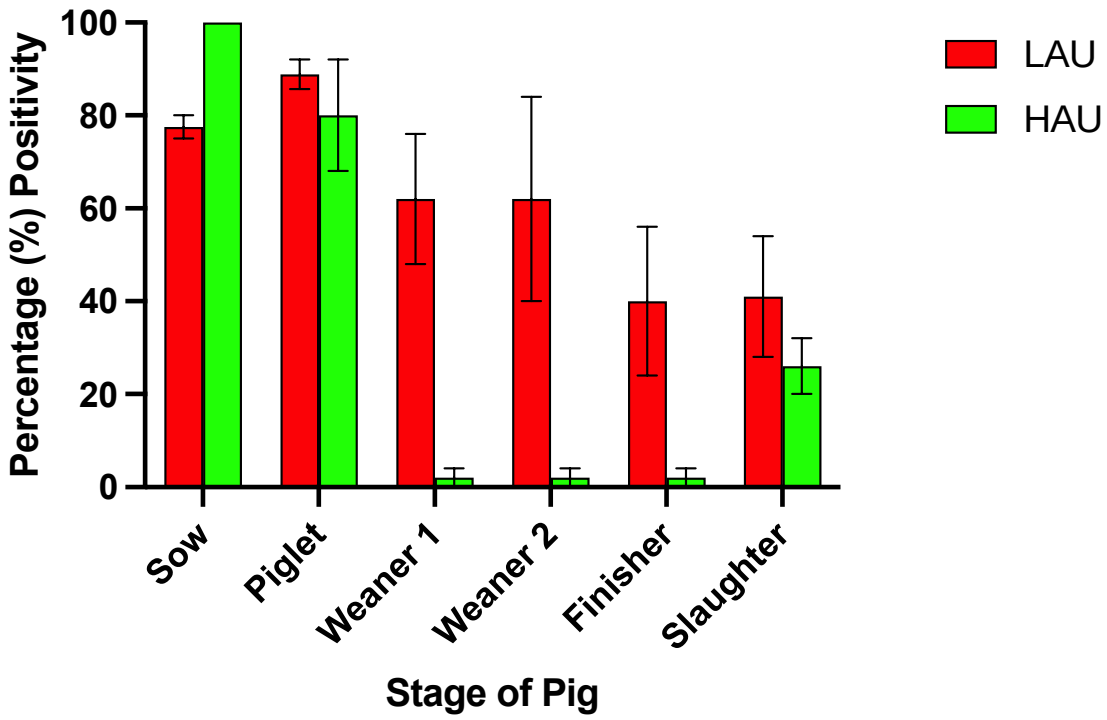


Figure 2.3 Bar chart demonstrating the average percentage positivity of *C. difficile* on High Antibiotic Use (HAU) farms and on Low Antibiotic Use (LAU) Farms.
The bars represent the average positivity on 2 LAU farms and 2 HAU farms, with legend shown to the right. Error bars represent the standard error of the mean (SEM).

2.3.2 Analysis of *C. difficile* isolates from pigs using PCR ribotyping

DNA extraction was successfully carried out for all pig *C. difficile* isolates. PCR ribotyping was performed on 223 pig *C. difficile* isolates. Eighteen different PCR ribotypes were identified from the analysis, as detailed in Table 2.8.

Table 2.8 Distribution (%) of PCR ribotypes of *C. difficile* isolates recovered from pig faecal samples from 6 farms across Ireland.

PCR Ribotype	Number of isolates	Percentage of isolates (%)
078	148	66.37
015	26	11.66
062	14	6.28
050	10	4.48
035	4	1.79
013	4	1.79
051	3	1.35
002	3	1.35
026	2	0.90
047	1	0.45
593	1	0.45
029	1	0.45
220	1	0.45
110	1	0.45
066	1	0.45
045	1	0.45
191	1	0.45
17	1	0.45
Total	**223**	100

2.3.3 *C. difficile* in collected meat product samples (n=180)

A total of 180 pig meat products were collected from supermarkets between September 2021 and December 2021. The majority (n=100) were submitted by the DAFM AMR surveillance programme, as described in section 2.2.2.3.1, and thus pork meat products were from supermarkets geographically distributed across the Republic of Ireland. *C. difficile* was not isolated from any of the pork products. Due to the negative result, the resulting data is not shown.

2.3.4 *C. difficile* recovery from abattoir samples

Clostridioides difficile was detected in 10 out of 30 (33%) environmental swabs from the lairage of the abattoirs (Table 2.9). *C. difficile* was detected on 1 out of 150 (0.66%) pig carcase swabs (Table 2.9). Six different ribotypes (RT078, RT413, RT066, RT015, RT002, and RT002) were found. RT078 and RT015 ribotypes were found to be prevalent and were both detected in 2 out of 3 abattoirs.

Table 2.9 Distribution of *C. difficile* in lairage (holding area pre-slaughter) and from carcases in the chill room of abattoirs.

Date of sampling	Positive swabs (total samples)		PCR ribotypes obtained (n)
	Lairage	Carcase	
05/04/2023	3 (10)	0 (50)	078 (2) 413
05/05/2023	3 (10)	0 (50)	078 066 015
09/05/2023	4 (10)	1 (50)	002 003 015 (2)

2.3.5 Clinical identification and data collection relating to *C. difficile* isolates

Clinical human *C. difficile* isolates (n=350) collected from laboratory-detected cases of CDI were stored by the Microbiology Department in the Central Pathology Laboratory in SJH. Although samples were processed on an anonymised basis, it was estimated, based

on national and local epidemiological data, that 25% of the 350 clinical isolates originated from the community. Isolates were collected between 2018-2020; 54 isolates were collected in 2018 (15.2%), 197 isolates were collected in 2019 (56.4%) and 99 isolates were collected in 2020 (28.4%).

2.3.6 PCR ribotype diversity of clinical isolates from SJH

Analysis identified 62 different PCR ribotypes, as detailed in Table 2.10. The four most prevalent ribotypes in our study account for 48.8% of all isolates, which is similar to prevalence numbers reported in Ireland by the HPSC (HPSC, 2021) (Figure 2.4). Over the three years, the most common ribotypes identified were RT014 (15.71%), RT002 (15.43%), and RT078 (12.57%). In 2018, the most common ribotypes were RT078 (15.1%), RT049 (11.3%), and RT014 and RT002 (both 7.5%). In 2019, the most common ribotypes were RT002 (19.3%), RT014 (16.8%) and RT078 (11.7%). In 2020, the most common ribotypes were RT014 (18.2%), RT078 (13.1%) and RT002 (12.1%).

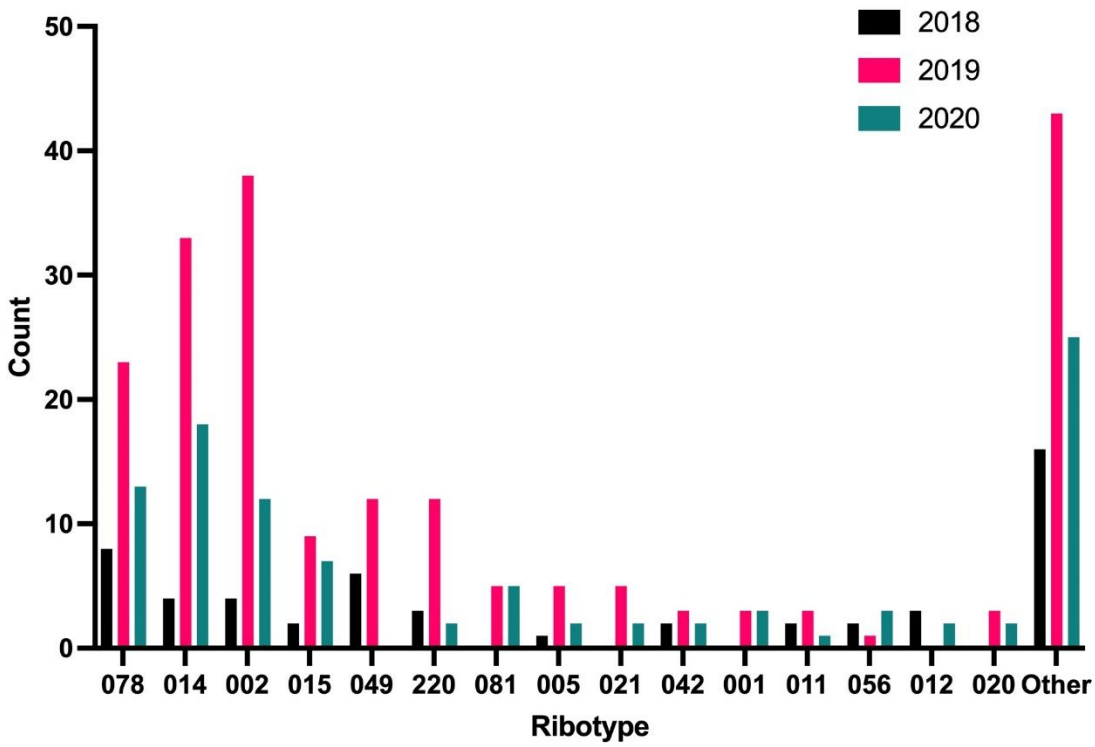


Figure 2.4 Distribution of the number of PCR ribotypes found in *C. difficile* isolated from human clinical samples during the three years of the current study

Table 2.10 PCR ribotypes of *C. difficile* isolates identified in confirmed CDI cases from SJH between 2018-2020

PCR Ribotype	Number of isolates	Percentage of isolates (%)
014	55	15.71
002	54	15.43
078	44	12.57
015	18	5.14
049	18	5.14
220	17	4.86
081	10	2.86
005	8	2.29
021	7	2.00
042	7	2.00
001	6	1.71
011	6	1.71
056	6	1.71
012	5	1.43
020	5	1.43
072	4	1.14
451	4	1.14
449	4	1.14
018	4	1.14
053	4	1.14
054	3	0.86
106	3	0.86
103	3	0.86
029	3	0.86
050	3	0.86
Other	49	14.00
Total	**350**	**100.00**

Table 2.11 The distribution of ribotypes found in human clinical *C. difficile* isolates between 2018 and 2020

Ribotype	2018		2019		2020		Total	
	n	%	n	%	n	%	n	%
078	8	15.1	23	11.7	13	13.1	44	12.6
014	4	7.5	33	16.8	18	18.2	55	15.7
002	4	7.5	38	19.3	12	12.1	54	15.4
015	2	3.8	9	4.6	7	7.1	18	5.1
049	6	11.3	12	6.1		0.0	18	5.1
220	3	5.7	12	6.1	2	2.0	17	4.9
081		0.0	5	2.5	5	5.1	10	2.9
005	1	1.9	5	2.5	2	2.0	8	2.3
021		0.0	5	2.5	2	2.0	7	2.0
042	2	3.8	3	1.5	2	2.0	7	2.0
001		0.0	3	1.5	3	3.0	6	1.7
011	2	3.8	3	1.5	1	1.0	6	1.7
056	2	3.8	1	0.5	3	3.0	6	1.7
012	3	5.7		0.0	2	2.0	5	1.4
020		0.0	3	1.5	2	2.0	5	1.4
Other	16	30.2	43	21.8	25	25.3	83	23.7
Grand Total	**53**	**15.1**	**198**	**56.3**	**99**	**28.3**	**350**	**100.0**

2.4 Discussion

This study investigated the longitudinal prevalence of *C. difficile* in pigs from farms across Ireland with different antimicrobial usages, in abattoirs, and in retail pork products, and investigated the genetic relationship to human clinical isolates.

Like what has been found with human CDI, piglets can be colonised with *C. difficile* yet remain free from overt disease. Culture-based methods in the current study found a higher positivity rate in piglets in Irish farms than a previous Irish study, with detection of *C. difficile* in 89.7% (146/165) of piglet faecal samples, compared to the previous report of 34/44 (77%) (Stein et al., 2017) in such samples. Stein et al. (2017) found a *C. difficile* prevalence rate of 33% (33/156) in sow faecal samples in Ireland, although this varied according to different gestational status, while in the current study *C. difficile* was found to be present in 95% (83/87) of sow samples. The sows included in the current study were within 1 week of parturition. These results are similar to a study by Hopman et al. (2011a), who found that all antepartum sows tested negative for *C. difficile*, and then 10-56 hours postpartum, the same sows all (100%) tested positive for *C. difficile* RT078. The reason for this colonisation is unclear, but it could be due to the physiological stress of pregnancy and parturition (Uzal et al., 2023, Weese, 2020).

The current study utilises an additional enrichment step before plating on agar, which could explain the higher *C. difficile* detection rates than previously reported (Lister et al., 2014, Connor et al., 2018). The addition of antibiotics and sodium taurocholate to BHI broth has been recommended for the culture of *C. difficile* from faeces as it improves the detection rate and allows for the recovery of spores at low concentrations (Connor et al., 2018). It is likely that the piglets may be colonized because of high environmental contamination rates in the piggery, based on the high prevalence of *C. difficile* that was isolated from sows. A study by Susick et al. (2012) found that the prevalence of *C. difficile* in the environment reciprocated with the prevalence of *C. difficile* in pigs.

Another possible explanation for high colonization rates among piglets could be that they lack toxin receptors or colonisation resistance by the undeveloped intestinal microbiota, in a similar manner to human neonates as described in section 1.2.3, whose microbiome is still contentious. The significant decrease in *C. difficile* colonisation as the

piglets get older mirrors what has been observed in humans. Historically, it was believed that the human uterus is sterile in healthy pregnancies, and recent studies have indicated no difference between negative controls and the placental microbiome (Theis et al., 2019, Leiby et al., 2018). However, other researchers have hypothesized that the human microbiome is developed *in utero*, with several studies finding bacterial DNA present in the meconium and amniotic fluid (Stinson et al., 2019, Shi et al., 2018). Some authors have further suggested that ingestion of amniotic fluid containing bacteria can lead to the colonization of the foetal gastrointestinal tract (Perez-Munoz et al., 2017).

C. difficile colonization was found to decline dramatically within the first five weeks of life, from piglet to weaner 1 stage, which has also been reported by a limited number of other cross-sectional studies (Susick et al., 2012, Hawken et al., 2013, Weese et al., 2010b). This reduction could be because of competition by other commensal bacteria in the pig gut as the piglets grow. There is a difference observed between weaner 1 stage animals in LAU farms and HAU farms. The rapid decrease in faecal shedding of *C. difficile* at weaner 1 stage in HAU farms may be explained by the impact of in-feed antibiotics on the bacterial community structure. The indigenous gut microbiota is important in mediating colonization resistance by pathogenic *C. difficile* (Reeves et al., 2011). The use of antibiotics may lead to a decrease in colonization resistance to pathogenic bacteria, as is observed in humans after antibiotic treatment (Schaffler and Breitruck, 2018). None of the antibiotics that were administered to pigs, as described in Table 2.2, would be expected to kill or inhibit *C. difficile* growth. However, some studies have reported that *C. difficile* is susceptible to tylosin, an MLS_B antibiotic that is administered on HAU and MAU farms in this study, which could explain the difference in *C. difficile* colonisation rates between HAU farms and LAU farms (Songer and Anderson, 2006, Post and Songer, 2004). During the weaner stage the animals are still being fed antibiotics, hence the low recovery levels of *C. difficile* in HAU farms.

Once the antibiotics are ceased an increase in *C. difficile* detection was observed in this study. Antibiotic therapy causes an alteration in gut microbial composition, thus allowing *C. difficile* colonization and susceptibility to toxin production and CDI. In HAU farms the animals receive antibiotics through to the weaner stage, in comparison to MAU farms where antibiotics are stopped after the link feed. The effect of different antibiotic use

regimens can be seen in Figure 2.3, where there is an increase in *C. difficile* colonization between the finisher and slaughter stage of HAU pigs.

The overall positivity at the slaughter stage was 33%. This finding is similar to that of a study by Alvarez-Perez et al. (2018) who found a 37.5% positivity in pigs at the slaughter stage, and also found high positivity (80%) in trucks transporting pigs to slaughter plants. In addition, Hawken et al. (2013) found *C. difficile* in 25% of samples from carcases and in the slaughter environment. The stress of transportation on the animals could be a possible reason for the increase in *C. difficile* shedding at slaughter compared to the finishing stage (Hain-Saunders et al., 2022, Baverud, 2004). An increase the prevalence of *C. difficile* at the slaughter stage may indicate a high contamination risk at evisceration and in abattoirs. This phenomenon has been observed in many studies for *Salmonella*, Enterobacterales and *E. coli* (Massacci et al., 2020, Fernandez et al., 2021). This indicates that slaughterhouses are a possible reservoir for *C. difficile* and could play a role in disseminating the pathogen in CA-CDI due to the environmental persistence of *C. difficile* by virtue of spore formation.

PCR ribotyping identified 18 different ribotypes from 223 *C. difficile* isolates from pig faecal samples (Table 2.8). The most common ribotype identified in pigs was RT078 (66.51%), which was not surprising as this is reported to be the most common ribotype found in pigs and other production animals such as calves (Weese, 2020, Alvarez-Perez et al., 2018, Keel et al., 2007). In humans, RT078 is the most commonly identified ribotype in Ireland, accounting for 16% of all CDI cases in 2021 (HPSC, 2021). *C. difficile* may be passed between sows and piglets through vertical transmission, but it is believed that it is more likely that piglets acquire the pathogen through contact with other animals or from the surrounding environment (Spigaglia et al., 2023, Moono et al., 2016). A study by Hopman et al. (2011a) supported this hypothesis as they found that piglets were negative for *C. difficile* RT078 at birth, but became positive by 48 hours of age. In the same study, animals that were born by caesarean section were kept in sterile incubators and remained negative for *C. difficile* after 21 days of age.

The diversification of ribotypes is seen to occur after the piglet stage, and 15 different ribotypes were observed in the latter stages of the pigs' lives in this study. The variety of PCR ribotypes identified in pigs at slaughter age is similar to that found by Hopman et

al. (2011a) and (Keessen et al., 2011). It is not clear as to why this occurs, but it could be due to the stress of transfer to the finishing stage, or the morphological and functional stage of the pig's intestine after the weaning stage (Zheng et al., 2021). The most common ribotypes identified in *C. difficile* isolates from slaughter pigs (n=25) in this study were RT050 (28%) and RT062 (16%). Notably, these rare toxigenic strains were also identified in a study by Clayton et al. (2012), who found these ribotypes in patients with inflammatory bowel disease in Ireland. In addition, RT062 was found in a healthy control group in a study by Burke et al. (2017) in Ireland. These patients had no recent exposure to antibiotics or any other risk factors associated with CDI, so acquisition from a community reservoir could be presumed.

There was no *C. difficile* detected in the retail pork and pork meat products tested in our study. These results are comparable to several studies performed in the EU, which also failed to detect *C. difficile* in pork and pork products (Bouttier et al., 2010, de Boer et al., 2011, Tkalec et al., 2020). Previous studies investigating the prevalence of *C. difficile* contamination in retail meats have found variable results. European studies found *C. difficile* in retail meats with levels ranging from 0.7% in Italy (Licciardi et al., 2021) to 4.7% in Belgium (Rodriguez et al., 2014). Studies performed in other countries showed variable isolation rates in pork and pork meat products, with rates in Canadian reports ranging from 1.2% (Tan et al., 2022) to 12% (Weese et al., 2009) and those in USA reports ranging from 11.5% (Varshney et al., 2014) to as high as 42% (Songer et al., 2009). This variation may be as result of sampling approaches, isolation procedures, slaughtering and handling practices, and transportation used in different jurisdictions. Low isolation rates in pork meat products across the EU may be related to strict EU food safety standards, hazard analysis critical control point (HACCP) principles and microbiological quality controls that help to identify and control hazards that can occur during food production (EU, 2021). The current study, along with other previous studies, indicates that contaminated meat is not an important source in the transmission of CDI to humans, and that other factors may serve as a source of CA-CDI.

Although higher isolation rates of *C. difficile* in food have been found in the USA and Canada, quantitative studies by Weese et al. (2009) have reported low levels of contamination with 20-60 spores/g of ground pork. However, these levels coupled with

the heat tolerance and persistence of spores could potentially facilitate foodborne transmission. In addition, the exact infectious dose is not known and presumably could vary greatly between healthy individuals and those with underlying health conditions or additional risk factors.

There could be environmental factors associated with CA-CDI, such as factory workers in slaughterhouses or farmers contracting *C. difficile*, which can then be spread into the community. This has been observed in a study by Keessen et al. (2013a), who found that people who had daily or weekly direct contact with pigs had high carriage rates (21%) of *C. difficile*, which were predominantly RT078, and these *C. difficile* isolates were genotypically and phenotypically related to pig isolates from the same farms. In comparison, the carriage rate of 11% found in asymptomatic adults, and half of these *C. difficile* isolates are toxigenic strains (Truong et al., 2017, Le Monnier et al., 2022). *C. difficile* has been found on farm floors, air and under the boots of workers and, similarly to what is observed in nosocomial settings, environmental contamination could play a role in the spread of *C. difficile* and, in particular, hypervirulent strains such as PCR RT078 (Hopman et al., 2011a).

If data were to be collected by the HPSC about the occupation of patients with a confirmed case of CDI, that could give an important insight into the epidemiology of *C. difficile* in Ireland. This has been observed in a study performed in the Netherlands by Knetsch et al. (2014), where they also found that farmers were asymptomatic carriers of *C. difficile* RT078 and these isolates were identical to isolates found at the farms where they worked.

The prevalence of *C. difficile* in pigs at slaughter ranged from between 20-54% (33.2% average) in this study, which is higher than in other studies (Susick et al., 2012, Weese et al., 2010b, Spigaglia et al., 2023). This difference could be due to geographical or methodological variations, or differences in sampling size. However, one study by (Hopman et al., 2011b) found *C. difficile* in 28% of pigs post-mortem, after they had been stunned and bled in the slaughterhouse. *C. difficile* contamination rates could be because of the stress of transportation or environmental contamination with spores in the trucks or the waiting areas of the abattoirs.

C. difficile appears to be a widespread environmental contaminant in the lairage of slaughter plants, as positive samples were obtained from the lairage of the three different abattoirs after it had been occupied by the pigs. The pathogen was isolated from between 30-40% of pens in the lairage, which are cleaned and disinfected several times a day using Virkon™ 1% w/v solution, indicating that the cleaning protocols are ineffective in eliminating *C. difficile* spores. Virkon™ is a strong oxidizing agent with the active compound potassium peroxymonosulfate but it has been found that it is not fully sporicidal against *C. difficile* spores on hard, non-porous surfaces (Lawley et al., 2010, Sjoberg et al., 2014). A similar conclusion was found in a study by Alvarez-Perez et al. (2018), who isolated *C. difficile* from trucks (80%) and the lairage (37.5%) post-disinfection. This highlights the potential risk of contamination by *C. difficile* from the lairage to the processing environment.

Notably, RT078 was recovered from the lairage of two of the abattoirs and it has also commonly been recovered from slaughterhouses in other studies (Alvarez-Perez et al., 2018, Hawken et al., 2013). In this study, one carcase (0.6%) from the cold-store post-processing was positive for *C. difficile*. Moreover, this carcase was contaminated with RT078. Although carriage is low, pig carcases are potentially exposed to *C. difficile* contamination through the slaughter line. This poses a risk to workers who potentially could be exposed to *C. difficile* during food handling and processing if they have more risk factors for becoming colonised by *C. difficile*. In addition, they are at risk of becoming asymptomatic carriers, thus spreading *C. difficile* in the community. However, there is no evidence that *C. difficile* from the lairage is the source of dissemination through the slaughter line, and carcase contamination may be from the farms as opposed to the processing environment (Hawken et al., 2013).

In the slaughtering process, there are several potential contamination points such as de-hiding, evisceration and handling the cutting equipment. As *C. difficile* spores can survive for long periods and different disinfection methods, as described in section 1.2.6, equipment can become contaminated and *C. difficile* can survive in the environment for long periods of time. This is also seen in hospitals, where *C. difficile* spores have been associated with hospital outbreaks with contaminated surgical equipment as a source point (Testore et al., 1988). A scalding process at 60°C can generally reduce enteric

pathogens, but *C. difficile* is the exception as this temperature does not kill spores, allowing them to survive harsh conditions. The water in the scalding tanks is not frequently replaced, so could be an important factor in carcase contamination in slaughterhouses (Wu et al., 2017). Contamination points after the lairage of each abattoir were not examined in the current project but might shed an important light on critical contamination points (CCP) during the farm-to-food process.

PCR analysis identified 62 different ribotypes in human clinical samples from St James's Hospital, spanning the years 2018 to 2020 (Table 2.10 and Figure 2.4) The most frequently identified ribotypes of human clinical *C. difficile* isolates in this current study were RT014 (15.71%), RT002 (15.43%) and RT078 (12.57%). The most prevalent ribotypes correspond closely to what was reported across Europe in 2011 (Bauer et al., 2011b), in 2016 (Davies et al., 2016), in 2018 (Freeman et al., 2018), in 2020 (Freeman et al., 2020). The persistence of the same ribotypes over a 10-year period is indicative of the stability of ribotypes over time. Yearly fluctuations in ribotype prevalence were found in the current study, but this is expected due to the endemic and epidemic spread of *C. difficile* (Freeman et al., 2010, Freeman et al., 2018).

The most common ribotype found in the current study was by RT014 (15.7%), which is a ribotype that is commonly implicated in human CDI. Notably, RT014 is well established in humans with CDI and pigs in Australia, accounting for 30% and 20% of *C. difficile* isolates, respectively (Lim et al., 2020b). In comparison to what has been found in Europe, where RT078 is the most common ribotype identified in neonatal pigs, RT014 is the most common *C. difficile* ribotype in neonatal pigs in Australia (Knight et al., 2015b). Genotypic relationships have been identified between RT014 isolates of human and pig origin, indicating a shared ancestry. Knight et al. (2016) identified that over 42% of RT014 isolates from human CDI had a clonal relationship (≤ 2 SNPs) with one or more pig strains, and more than 50% of these human CDI cases had no prior healthcare facility exposure. Clonal *C. difficile* isolates were also found on sites that were spread over a large geographical area (average distance between sites was 894 km), which suggests a continual environmental reservoir for long-distance dissemination, perhaps through the food chain, water, animals, or animal effluent by-products such as compost.

The second most common ribotype found in the current study was RT002 (15.4%). In 2019, there was a dramatic increase in *C. difficile* RT002, which was implicated in a nationwide outbreak of healthcare associated CDI in that year (HPSC, 2021). RT002 is similar in characteristics to the epidemic strain RT027, with a higher sporulation rate and higher rates of fluoroquinolone resistance and is associated with high morbidity and mortality (Hong et al., 2020, Wong et al., 2016). It is not clear what caused this outbreak in Ireland in 2019, but these virulence characteristics facilitate the nosocomial spread of *C. difficile* as spores remain infective and persist in the environment for many months (Verity et al., 2001). Infection control and appropriate antimicrobial stewardship helped to control hospital transmission of RT027 (Debast et al., 2009a, Vonberg et al., 2008b, Stuart et al., 2019a). Similarly, implementation of strict infection control measures in the hospital environment during the COVID-19 pandemic may explain the nationwide decrease in RT002 in 2020.

The diversity of PCR ribotype distribution in human samples is consistent with what has been observed in other studies (Freeman et al., 2018, Herbert et al., 2019). A study by Freeman et al. (2020) found that a higher ribotype diversity is associated with lower antimicrobial resistance rates. Increased awareness of CDI, antimicrobial stewardship and the introduction of infection control programmes may all have contributed to the decrease in the rates of endemic ribotypes. Notably, RT027, which has been associated with increased CDI severity in patients in the past, was only detected in 1 of the 350 *C. difficile* isolates in the current study (Bauer et al., 2011a).

Several ribotypes overlapped between pig and human *C. difficile* isolates, such as RT078, RT015, RT002, RT050, and RT018. Ribotypes RT078, RT002, and RT015 are commonly isolated from animals and humans, whether asymptomatic carriers of *C. difficile* or those with symptoms of CDI (Herbert et al., 2019, Kachrimanidou et al., 2019).

It is important to consider that the current study only sampled each farm once, so it cannot be definitively said that the prevalence of *C. difficile* is affected by the different levels of antibiotic usage on farms. In addition, only one sample was taken per animal in the current study, and it is possible that the cumulative prevalence would have been

higher if more than one sample had been taken per animal, as reported by Hopman et al. (2011a).

2.5 Conclusions

It is evident that *C. difficile* can be commonly found in pigs, and it is also found at a higher prevalence at different timepoints of the pigs' life, with environmental contamination being the likely route of acquisition. As observed in other European studies, *C. difficile* RT078 is the predominant ribotype in Irish pigs and was the third most common ribotype found in humans clinical *C. difficile* isolates in Ireland during the years of the current study. RT078 is frequently implicated in CA-CDI patients, who do not have the typical risk factors commonly associated with HA-CDI. This study reinforces evidence that zoonotic spread of *C. difficile* could be particularly relevant in Ireland (Stein et al., 2017, McElroy et al., 2015, Moloney et al., 2021). However, this study did not find evidence of pork products as a potential source of infection.

3 Characterisation of *Clostridioides difficile* isolates collected between 2018-2020 using Whole Genome Sequencing

Chapter 3

3.1 Introduction

Molecular tools, such PCR ribotyping, have been widely used for epidemiological investigation of CDI, particularly in relation to suspected hospital outbreaks. However, the discriminatory power of ribotyping is not sufficient to investigate the phylogenetic relationship between *C. difficile* strains with a view to tracing their origin. Ribotyping of *C. difficile* relies on the heterogeneity of the intergenic spacer region (ISR), located between the 16S and 23S rRNA genes, to generate a DNA fingerprint. An important limitation, however, is that there is no standardised type strain collection, and this has resulted in the development of different ribotyping classifications (Stubbs et al., 1999, Bidet et al., 1999). There have been calls for interlaboratory standardisation, and thus the nomenclature is constantly under review (Huber et al., 2013, Fawley et al., 2015).

In silico typing based on WGS, using MLST and Single Nucleotide Polymorphism (SNP) analysis, offers the highest level of *C. difficile* strain resolution, which helps in the study of transmission events (Baktash et al., 2022) MLST is a powerful typing method that can help to identify clonal relationships between different strains and relies on allelic variants between the seven housekeeping genes that are highly conserved in *C. difficile* (Knight et al., 2015a). WGS provides excellent discriminatory power to distinguish between isolates, making it a powerful tool to aid detection of patient-to-patient transmission events and outbreak investigations.

It has been suggested that the evolution of ISRs is likely as a result of homologous recombination (Janezic et al., 2014). Recombination of repeats in the ISR could lead to assignment of a new PCR ribotype with no apparent phylogenetic relationship to the closest PCR ribotype before recombination had occurred. In comparison, MLST is less impacted by recombination events as recombination in housekeeping genes would change the allelic profile on a single locus, but the phylogenetic links to the original MLST ST would be conserved (Knetsch et al., 2013).

The variation in repeat regions such as the ISRs cannot be accurately recorded by short read assemblies, but there has been some agreement on the correlation of MLST groupings and ribotypes (Janezic and Rupnik, 2015, Seth-Smith et al., 2021). WGS

comparison methods have found associations between lineages, PCR ribotypes and sequence types (e.g., hypervirulent clade 2, RT027, ST1) (Valiente et al., 2012).

C. difficile carriage has been well described in non-human sources such as animals and food products (Knight and Riley, 2019). In Chapter 2, PCR ribotyping was used to characterise *C. difficile* isolates of pig and human origin. However, these conventional typing methods do not provide the discriminatory power to differentiate between isolates of the same ribotype. In this chapter, whole genome analysis was used to investigate the genomic relationships between a selection of pig and human *C. difficile* isolates which were obtained and previously analysed, as detailed in Chapter 2.

3.2 Methods

3.2.1 Whole Genome Sequencing (WGS) of *C. difficile* isolates

3.2.1.1 Selection of isolates for WGS

Based on data obtained from PCR ribotyping results in Chapter 2, initially 100 *C. difficile* isolates from pigs and 100 *C. difficile* isolates from humans were chosen for sequencing. Additionally, towards the end of the project (with additional funding becoming available), an extra 22 clinical *C. difficile* isolates were sequenced. Ribotypes that were found in pig *C. difficile* isolates were correspondingly identified from among the human clinical *C. difficile* isolates, and the ribotypes that were found in both pig and human clinical *C. difficile* isolates were chosen for WGS. Ribotypes that were found in from both pigs and human *C. difficile* isolates were RT078, RT002, RT015, RT050, and RT018. Once these isolates were sequenced (75 isolates from pigs and 69 isolates from humans), there was still capacity to sequence more *C. difficile* isolates. Therefore, ribotypes that were commonly found in one source (e.g., RT014 in humans), and hypervirulent *C. difficile* ribotypes of clinical importance (e.g., RT027 in humans), were identified and chosen for sequencing.

The *C. difficile* ribotypes chosen for sequencing and their sources are detailed in Table 3.1 below. Sequenced pig and human clinical isolates of *C. difficile* comprised of 28 different ribotypes.

Table 3.1 Ribotypes chosen for Whole Genome Sequencing (WGS) and the host from which the *C. difficile* isolate was obtained

C. difficile Ribotype	Host		Total
	Porcine	Human	
078	60	40	100
014		28	28
002	1	19	20
015	7	6	13
062	9		9
050	6	3	9
013	4		4
001		4	4
005		3	3
011		3	3
056		3	3
035	3		3
021		2	2
129	2		2
103		2	2
046		2	2
220		2	2
018	1	1	2
026	2		2
320	1		1
191	1		1
027		1	1
091	1		1
016		1	1
051	1		1
733	1		1
106		1	1
020		1	1
Grand Total	**100**	**122**	**222**

3.2.1.2 DNA extraction for WGS

C. difficile isolates that had been stored at -20°C, as described in section 2.2.2.6, were removed from the freezer and streaked onto a blood agar plate, then incubated anaerobically at 37°C. 700 µL of PrepMan Ultra reagent was pipetted into a sterile tube containing a small quantity of glass beads, ensuring that the level of the beads was no more than 50% of the liquid level, and the tube was labelled accordingly. One loopful of pure *C. difficile* culture from blood plates was placed into the tube and swirled gently to mix the bacteria into the beads and the liquid. Bacterial cells were sheared in a BioSpec BeadBeater for 3 pulses of 40 seconds at 6.0 m/s. The tube was centrifuged at 10,000 X g for 10 minutes, and 450 µl of the supernatant was pipetted into a clean 1.5 ml Eppendorf DNA LoBind tube.

DNA was precipitated by adding 50 µl of 3 M sodium acetate (pH 5.5) and 1 ml of ice-cold 96% ethanol to the tube. The tube was vortexed and incubated at -20°C overnight. The tube was centrifuged at 10,000 X g for 15 minutes and the supernatant was pipetted off and discarded without disturbing the pellet. A 1 ml aliquot of 70% v/v ethanol was added to the tube and incubated for 1 minute, then centrifuged at 10,000 X g for 2 minutes. The supernatant was discarded without disturbing the pellet. The pellet was dried at 55 °C for 10-15 minutes, taking care that the pellet did not over-dry. The pellet was resuspended in 70 µl of nuclease-free water and mixed thoroughly. The tube was centrifuged at 5,000 X g for 2 minutes and the 60 µl of the supernatant was pipetted into a clean 1.5ml Eppendorf DNA LoBind tube.

Cleanup of DNA samples was performed by adding 50 µl of AMPure XP beads (0.7x) to the tube and mixing by pipetting up and down. The tube was then incubated for 10 minutes on a Hula mixer (Eppendorf Thermomixer comfort) at room temperature. The tube was centrifuged briefly and then placed on a magnetic stand to separate the beads from the supernatant. The supernatant was pipetted off and the beads were washed with 200 µl of freshly prepared 70% ethanol without disturbing the pellet on the magnet. The 70% ethanol was pipetted off and this wash step was repeated once more. The tube was centrifuged briefly and placed back onto the magnetic stand to separate the beads from any residual 70% ethanol, and the ethanol was pipetted off and

discarded. The tube was air dried for approximately 30 seconds and 35 µl of nuclease free water was added to the beads to elute the DNA. The supernatant was added to a clean 1.5ml Eppendorf DNA LoBind tube.

3.2.1.3 DNA quantification

Genomic DNA quantification was performed with a Qubit™ 4.0 Fluorometer (Invitrogen, California, USA) using the Qubit™ 1X dsDNA High Sensitivity (HS) Assay Kit (Thermo Fisher Scientific, USA). The instrument was calibrated before each use. All samples were prepared using thin-walled Qubit™ assay tubes, and each tube was labelled on the lid. Standards 1 and 2 were prepared by adding 10 µl of each Qubit™ standard to 190 µl of Qubit™ 1X dsDNA working solution in a Qubit™ tube. Using a P-2 pipette, 2 µl of DNA sample and 198 µl of Qubit™ 1X dsDNA working solution was added to the correspondingly labelled tube. The samples and standards were mixed by vortexing and incubated at room temperature for 2 minutes. Following the instructions from the "Read standards and run samples" function on the home screen of the Qubit™ 4.0 fluorometer, the instrument was calibrated, and the DNA concentration was measured. Samples containing less than 10 ng/µl of DNA were discarded and DNA extraction on these samples was repeated.

3.2.1.4 Performing WGS

For time and budgetary efficiency, it was opted to outsource the sequencing. WGS of selected *C. difficile* isolates was performed commercially by the company Novogene using a Novaseq. Sequencing data was returned as raw FASTQ files, which contains results of sequencing reads. Before FASTQ files were returned, Novogene performed quality control of raw FASTQ files. The quality control was assessed by Novogene at per base sequence quality and using FASTQC, and WGS was repeated for a selected *C. difficile* isolate if the sequence quality was not sufficient. A report was generated by Novogene before the FASTQ files were sent, which provided the detailed statistics on the quality of sequencing data. Raw FASTQ files of sequencing reads were downloaded from the Novogene cloud platform, then stored locally on the Linux computer in the Sir Patrick Dun (SPD) Laboratory (TCD) in the Central Pathology Laboratory (CPL) of SJH, and

backed up on an external hard drive that was stored in the postgraduate reading room in SPD.

3.2.1.5 *C. difficile* genome sequence analysis

The forward and reverse sequencing reads of each *C. difficile* isolate were uploaded as raw FASTQ files to the Ridom Seqsphere+ software version 9.0.10 (Junemann et al., 2013). The read data quality of the raw FASTQ files at per base sequence quality was checked again using FASTQC version 0.11.9 (Andrews, 2019), which is built into the Ridom Seqsphere+ software. The resulting FASTQ files were *de novo* assembled using the SKESA assembler, which is integrated in the Ridom Seqsphere⁺ software (Junemann et al., 2013, Souvorov et al., 2018). Paired-end reads were mapped to the *C. difficile* 630 reference genome (AM180355) (Sebaihia et al., 2006).

3.2.1.6 Multilocus sequence typing (MLST) target gene definition

The sequence types of the *C. difficile* isolates were obtained using the pubMLST database (Jolley et al., 2018), which is embedded into the Ridom Seqsphere Software.

3.2.1.7 Core genome multilocus sequence typing (cgMLST) target gene definition

The molecular epidemiology of the *C. difficile* isolates were identified by cgMLST based on a core genome of 2270 loci (Bletz et al., 2018a). To differentiate closely related strains, parameters for defining the threshold of allelic differences were set as published by Bletz et al. (2018b) for *C. difficile*. Following recommendations from the Bletz et. al. study, a cluster was defined based on the maximum allelic distance of ≤6 alleles. All strains within the defined threshold were grouped within the same cluster type (CT). The combination of all alleles in an isolate formed an allelic profile. For further analysis, a minimum spanning tree (MST) was generated using the parameter "pairwise ignore missing values" during distance calculation. Multiple samples can be grouped into the same node if *C. difficile* isolates were grouped together in the same clonal group if they are closely related.

3.2.1.8 SNP analysis

When isolates were grouped into the same node on the MST, the "Find group-specific SNPs" function on Seqpshere+ genome analysis tool was used to record all SNPs

between each isolate (Jünemann et al., 2013). This was used to generate a distance matrix comparison table, from which the distance between samples was visualised and calculated based on the nucleotides of the found SNP positions.

3.2.1.9 Toxin gene profiling

The virulence factor database (VFDB) program that is included in the Ridom Seqsphere+ software (version 9.0.10) was used to investigate the FASTQ files of the *C. difficile* isolates (n=222) for presence of toxin-encoding genes (Chen et al., 2016).

3.3 Results

3.3.1 Samples included in this study.

WGS was performed on *C. difficile* isolates from pigs and human clinical isolates recovered in Ireland between 2018 and 2020. The detailed statistics for the quality of sequencing data, as provided by Novogene, are shown in Supplementary Table S1. A total of 222 *C. difficile* isolates were included in the analysis: 100 isolates from pigs and 122 isolates from human clinical samples from SJH. There were no clinical details available for the *C. difficile* isolates from human clinical samples.

3.3.2 Genomic analysis of *C. difficile* isolated from different sources (pigs and human)

In silico MLST analysis identified 29 different sequence types (STs), with no novel STs represented in this group (Table 3.2). ST11 (45.05%), ST8 (9.01%) and ST2 (8.11%) were the most common STs identified from the 222 pig and human *C. difficile* isolates.

3.3.2.1 *C. difficile* isolates from pigs

There were 14 different STs identified in the 100 pig *C. difficile* isolates. In pigs, ST11 was the most common ST identified (n=60), followed by ST75 (n=9), ST44 (n=6) and ST16 (n=6) (Figure 3.1a).

3.3.2.2 *C. difficile* isolates from humans

Five STs that were found in pig *C. difficile* isolates were also found in human isolates (Figure 3.1b) There were 20 different STs identified in the 122 human clinical *C. difficile* isolates. In human *C. difficile* isolates, ST11 was the most common (n=40), followed by ST8 (n=19), and ST2 (n=18). The human clinical *C. difficile* isolates included in this study were distributed across three years, 2018-2020. There was not an even distribution of isolate sample numbers across the three years as some of the older *C. difficile* isolates (2018) were disposed of prior to commencing the project because of the lack of freezer space in SJH, and the lack of *C. difficile* isolates from 2020 is likely due to the impact of the COVID-19 pandemic. Of the 122 *C. difficile* isolates that were sequenced, 20 isolates were from 2018, 71 isolates were from 2019, and 31 isolates were from 2020 (Figure 3.2).

Table 3.2 STs identified in 222 pig and human *C. difficile* isolates

ST	Count of ST	Percentage	Pig and/or human host
11	100	45.05%	Pig, human
8	20	9.01%	Pig, human
2	18	8.11%	Human
44	11	4.95%	Pig, human
75	9	4.05%	Pig
16	8	3.60%	Pig, human
14	5	2.25%	Human
49	5	2.25%	Human
10	4	1.80%	Human
45	4	1.80%	Pig
3	4	1.80%	Human
107	4	1.80%	Pig
6	3	1.35%	Human
58	3	1.35%	Human
36	3	1.35%	Human
46	2	0.90%	Pig
17	2	0.90%	Pig, human
7	2	0.90%	Pig
56	2	0.90%	Human
53	2	0.90%	Human
13	2	0.90%	Pig
35	2	0.90%	Human
42	1	0.45%	Human
1	1	0.45%	Human
160	1	0.45%	Pig
571	1	0.45%	Pig
101	1	0.45%	Pig
18	1	0.45%	Human
133	1	0.45%	Human
Total	222	100.00%	

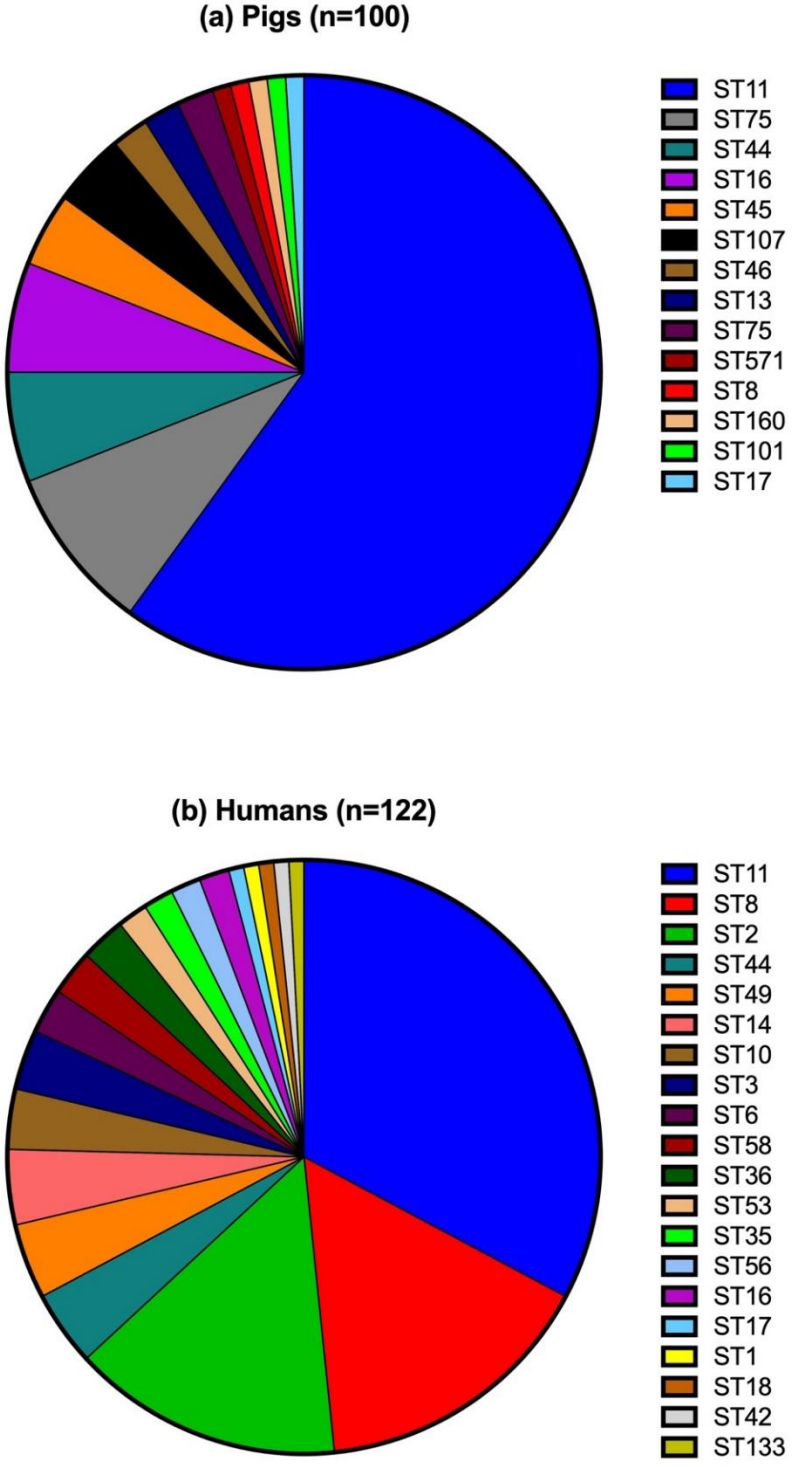


Figure 3.1 Pie chart of the distribution of STs identified in 222 *C. difficile* isolates from (a) pigs (n=100) and (b) human clinical samples (n=122).

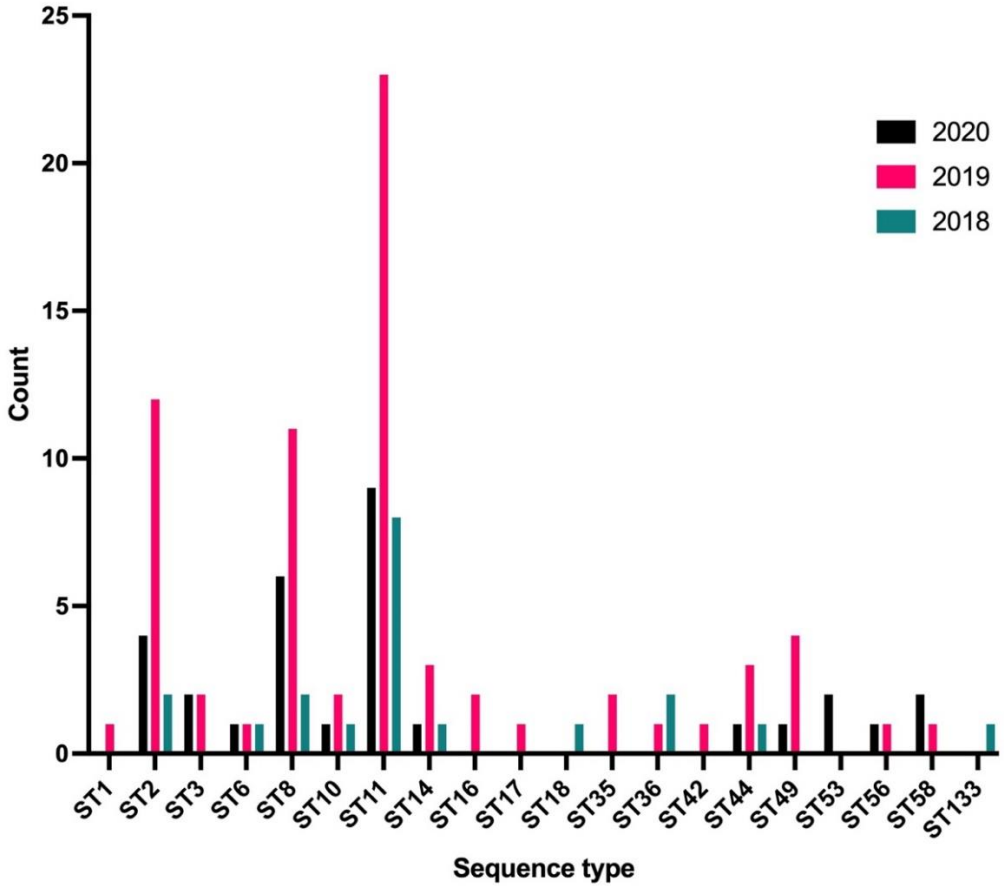


Figure 3.2 Distribution of each sequence type (ST) by the year during which the corresponding *C. difficile* isolate was originally isolated from human clinical samples (n=122).

There were 20 isolates from 2018, 71 isolates from 2019, and 31 isolates from 2020.

3.3.3 Analysis of PCR ribotype and sequence type associations

As mentioned in section 3.2.1.1, there were 28 different *C. difficile* RTs selected for sequencing and WGS revealed 29 different STs from pigs and human *C. difficile* isolates (n=222). Some RTs correspond to multiple STs (e.g. RT015 corresponds to ST10, ST44, and ST160), while some STs are also associated with multiple RTs (e.g. ST2 corresponds to RT014, RT020, and RT220), as which is detailed in Table 3.3.

The ribotypes and sequence types were matched manually, based on the ribotyping data which was obtained for each *C. difficile* isolate (sections 2.3.2 and 2.3.6), and the sequence data obtained from the same isolates, as detailed in this chapter. This was done by adding the RT number for the corresponding *C. difficile* isolate into Ridom Seqsphere+ as epidemiological data, so the RT was included in spreadsheets generated by Ridom Seqsphere+.

3.3.4 Multilocus Sequence Typing (MLST)

MLST was applied to the collection of 222 *C. difficile* isolates to identify the seven housekeeping genes (*adk, atpA, dxr, glyA, recA, sodA* and *tpi*) to classify the phylogenetic lineages in this study (Supplementary Table S2). A phylogenetic tree was constructed using the 222 *C. difficile* genomes (Figure 3.3) All isolates were successfully typed by MLST, and the genomes were divided into three different MLST clades: clade 1, 2 and 5. No isolates in this study belonged to clade 3 or 4. Clade 1 was assigned the most *C. difficile* isolates (n=121), one isolate belonged to clade 2, and 100 isolates belonged to clade 5 (Table 3.4). There were 27 different STs identified that belonged to clade 1, while clade 2 and clade 5 contained only one ST each.

Table 3.3 PCR ribotype and associated Sequence Types (STs) identified from *C. difficile* isolates in this study (n=222), and the host(s) from which each sequence type originated.

ST	Number (n)	Clade	PCR ribotypes	Pig and/or human *C. difficile* host
11	100	5	078	Pig, human
8	20	1	002	Pig, human
2	18	1	014, 020, 220	Human
44	11	1	015	Pig, human
75	9	1	062	Pig
16	8	1	050	Pig, human
49	5	1	014	Human
14	5	1	014	Human
107	4	1	035, 091	Pig
10	4	1	015	Human
45	4	1	013	Pig
3	4	1	001	Human
6	3	1	005	Human
58	3	1	056	Human
36	3	1	011	Human
56	2	1	021	Human
13	2	1	129	Pig
35	2	1	046	Human
7	2	1	026	Pig
53	2	1	103	Human
17	2	1	018	Pig, human
46	2	1	191, 320	Pig
101	1	1	051	Pig
133	1	1	016	Human
160	1	1	015	Pig
571	1	1	733	Pig
42	1	1	106	Human
1	1	2	027	Human
18	1	1	050	Human

Table 3.4 Phylogeny of pig and human *C. difficile* isolates (n=222)

Clade	Total number of isolates	Sequence type (ST)	Number of isolates within each ST
1	121	8	20
		2	18
		44	11
		75	9
		16	8
		49	5
		14	5
		107	4
		3	4
		45	4
		6	3
		58	3
		36	3
		56	2
		53	2
		10	4
		13	2
		17	2
		7	2
		35	2
		46	2
		101	1
		133	1
		571	1
		160	1
		18	1
		42	1
2	1	1	1
5	100	11	100

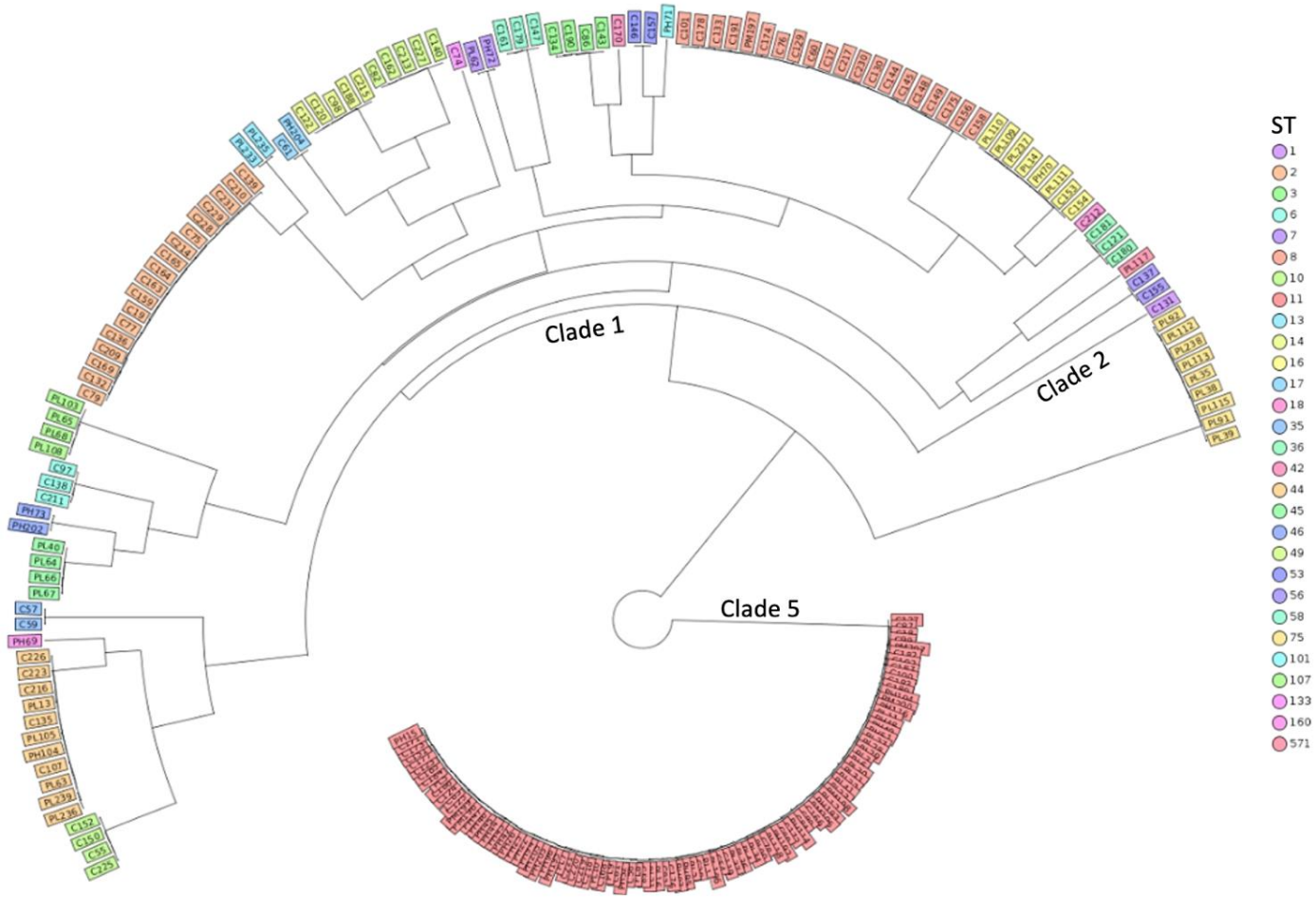


Figure 3.3 Neighbour-joining (NJ) circular tree of 222 *C. difficile* isolates

Constructed based on the Ridom Seqsphere+ core genome Multilocus Sequence Typing (cgMLST) scheme of the 7 housekeeping genes, ignoring pairwise missing alleles, coloured by sequence type (ST) (legend). The corresponding clades are marked on the branches of the tree.

3.3.5 Characterisation of pig and human clinical isolates of *C. difficile* using cgMLST

The mean percentage of good cgMLST target genes based on the core genome was 99.81%, and the median was 99.9%. Several researchers have considered a cgMLST stable if a minimum of 95% of the cgMLST target genes are present (Werner et al., 2020). The minimum spanning tree (MST) grouped *C. difficile* isolates by their STs, and clonally related clusters were identified (Figure 3.4). Each node (coloured circle on the MST in Figure 3.4) represents isolates that correspond to a single ST, with isolates that differ by 0-2 allelic differences being grouped in the same node. A cluster group was defined when isolates differed by ≤6 alleles.

The genomes clustered in groups according to their STs, and the largest clusters (containing five or more isolates) were ST11, ST8, ST75, and ST44. Within ST11 cluster A (n=90), which contains pig (n=54), and human (n=36) isolates and is shown at the ≈120° (≈11 o'clock) position and coloured in red, the maximum diversity between two genomes was 16 allelic differences. Within the ST11 cluster B (n=9), which contains pig (n=6), and human (n=3) isolates and is shown at the ≈120° (≈11 o'clock) position and coloured in red, the maximum diversity between two genomes was 5 allelic differences. Within the ST8 cluster (n=11), which contains only human isolates, and shown at the centre of the tree and coloured in salmon pink, the maximum diversity between two genomes was 9 allelic differences. Within the ST75 cluster (n=9), and contains only pig isolates, and is shown at the ≈0°/360° (≈3 o'clock) position and coloured in yellow, the maximum diversity between two genomes was 2 allelic differences. Within the ST44 cluster (n=7), which contains pig (n=5), and human (n=2) isolates and is shown at the ≈330° (≈4 o'clock) position and coloured in orange, the maximum diversity between two genomes was 6 allelic differences.

To further determine if *C. difficile* strains isolated from pig and human origins clustered together if they were phylogenetically related or distinct, a second MST was constructed with colour coding by the original host i.e. pig or human(Figure 3.5).

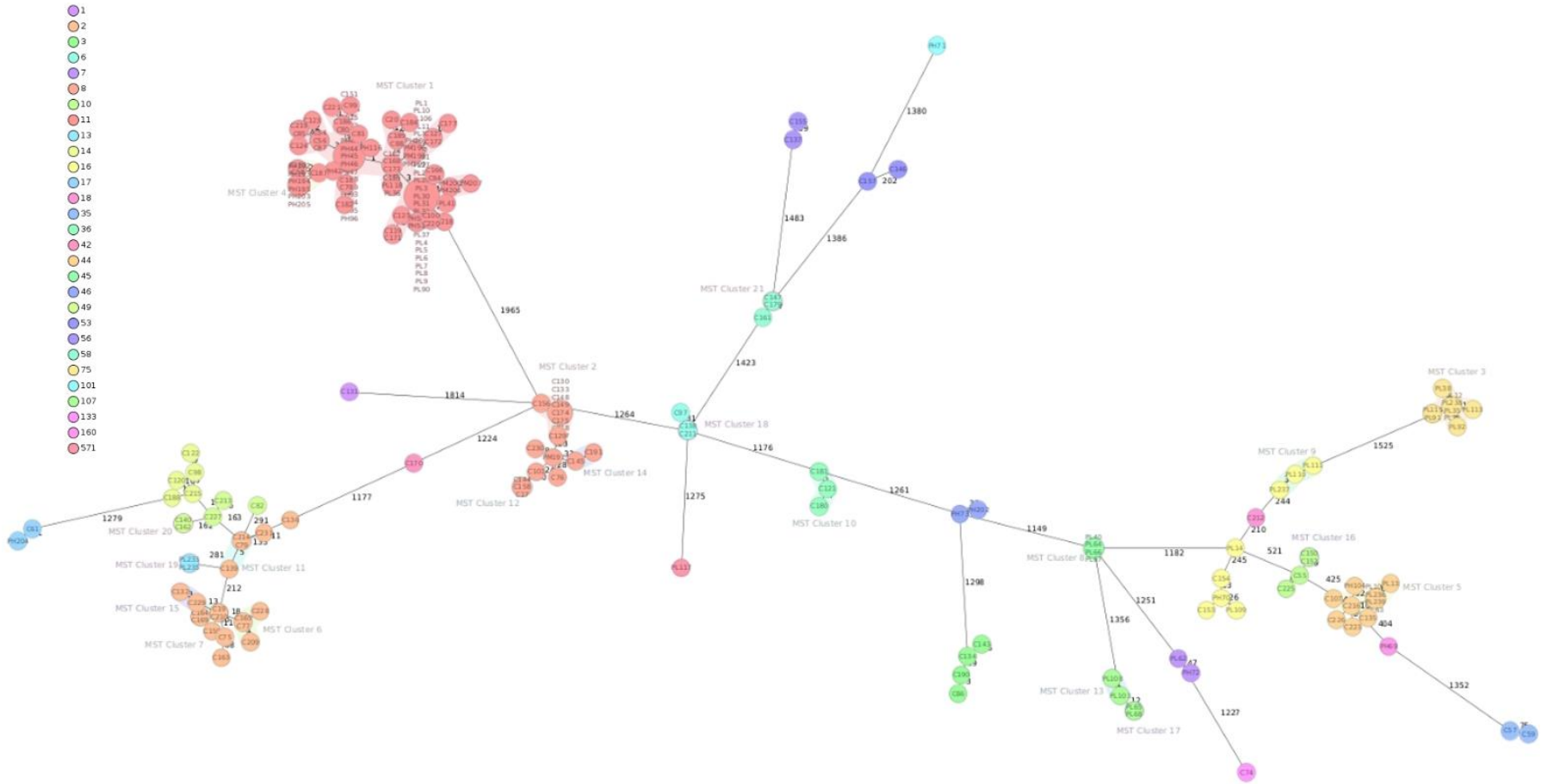


Figure 3.4 Minimum spanning tree (MST) 222 *C. difficile* isolates from pigs (n=100) and humans (n=122)

Generated by Ridom Seqsphere+ (2147 genes) pairwise ignoring missing values. Each circle (node) represents one or more isolates. The number on each branch between nodes represents the allelic distance. The MST cluster distance threshold is 6 allelic differences. *C. difficile* isolate names are present on the nodes of the tree. STs are contained in the same genomic clusters and are represented by assorted colours (legend left).

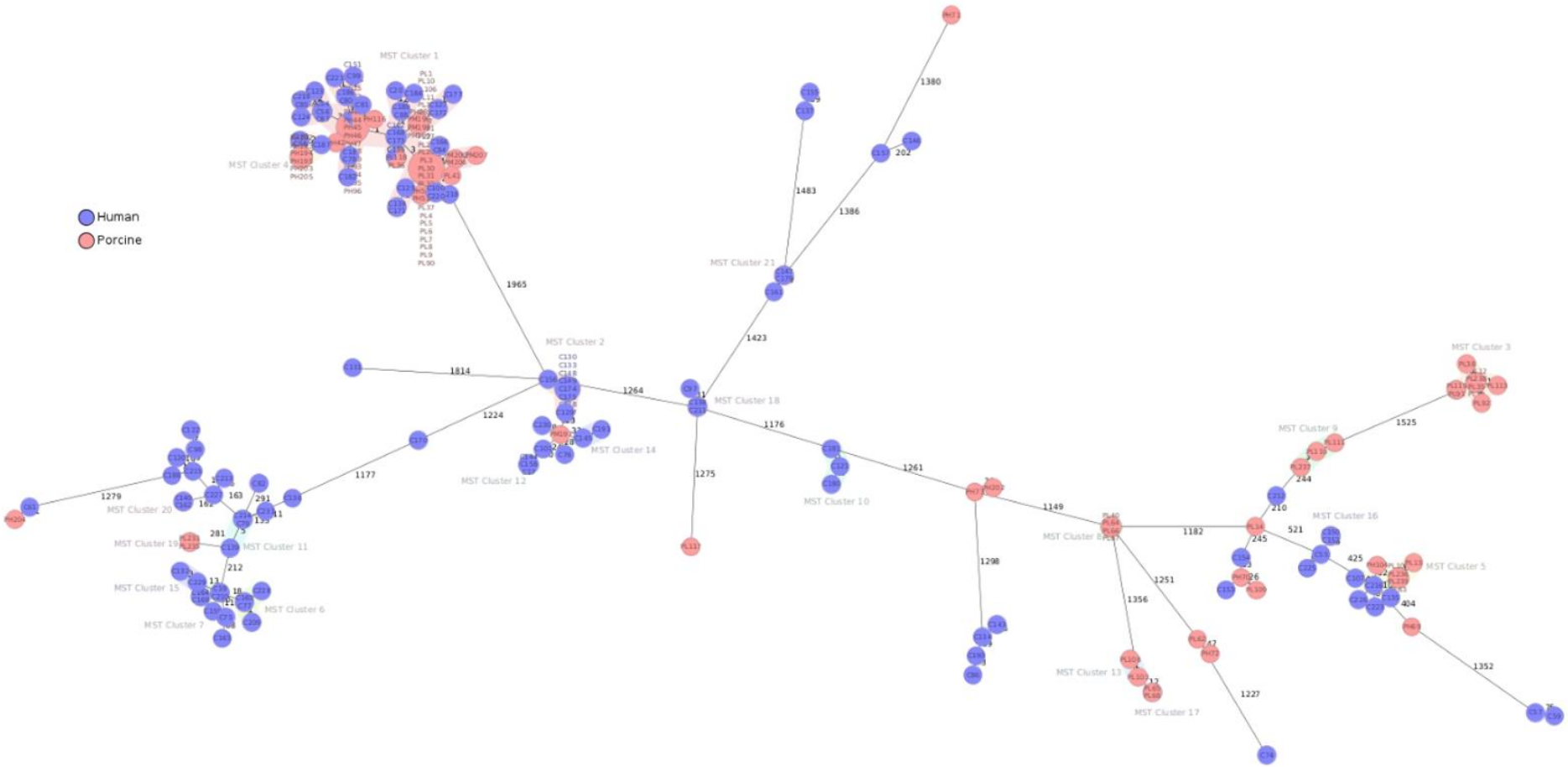


Figure 3.5 Minimum spanning tree for 222 *C. difficile* isolates from pigs (n=100) and humans (n=122)

Generated by Ridom Seqsphere+ v2 (2147 genes), pairwise ignoring missing values. MST cluster distance threshold is 6 allelic differences, and nodes are coloured by source of isolate (legend left). *C. difficile* isolate name is present on the nodes of the tree.

3.3.5.1 Identifying relationships between *C. difficile* isolates from different strains

Figure 3.4 and Figure 3.5 helped to identify strains from different host sources that were closely related. Three cluster groups (CGs) were identified that contain *C. difficile* isolates collected from both pigs and humans, two comprised of ST11, and the other comprised of ST44. These STs are magnified in Figure 3.6.

Following SNP analysis of a cluster of 100 *C. difficile* ST11 genomes, a heatmap of pairwise SNP differences was created and is shown in Figure 3.7. The pig *C. difficile* ST11 isolates originated from six different Irish farms that were geographically distinct from each other. Isolates were distributed independent of location as some isolates from different farms were grouped into the same node as they were closely related. The largest node (0-1 SNP differences in the core genome) contained 25 pig *C. difficile* isolates from 2 different farms. The second largest node contained 17 *C. difficile* isolate – 15 isolates of pig origin from two different farms and 2 of human origin. In this node, no SNP differences were identified between 15 pig isolates and one of the human *C. difficile* isolates, and one SNP difference was observed between the other 15 pig isolates and one human *C. difficile* isolate.

Following SNP analysis of 11 of the *C. difficile* ST44 genomes, a heatmap of pairwise SNP differences was created and is shown in Figure 3.8. This is made up of 6 pig *C. difficile* isolates and 5 human *C. difficile* isolates. Most (5/6) of the pig isolates originated from the same farm and showed a clonal relationship with one clinical isolate. The other pig *C. difficile* isolate was phylogenetically distinct to all the other ST44 isolates in this study. One clinical isolate (from 2019) was more closely related to the 5 pig *C. difficile* isolates (1-2 SNP differences in the core genome) than to the other clinical isolates.

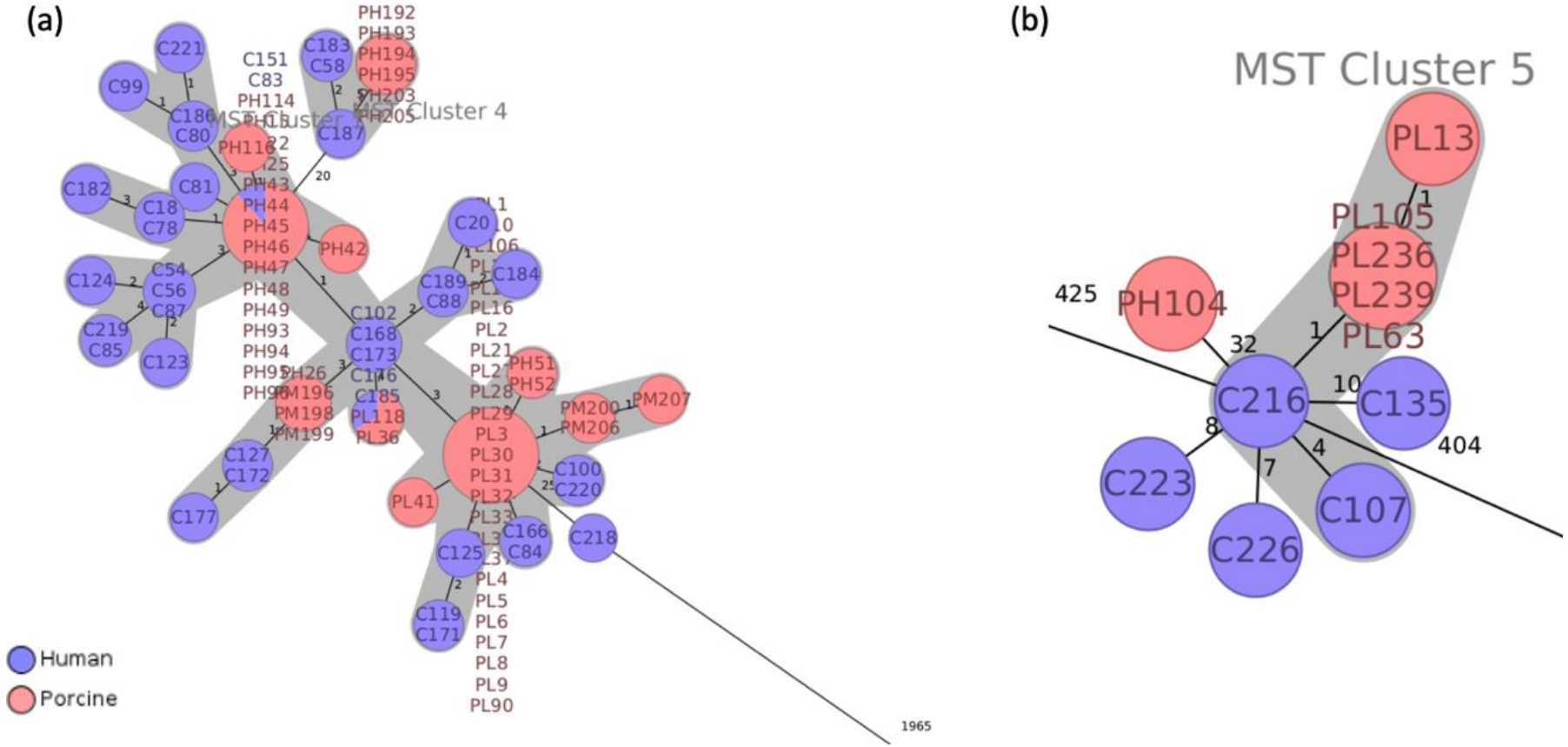


Figure 3.6 Snapshot of Two clusters from the Minimum spanning tree (A) represents a cluster containing ST11 isolates, (B) represents a cluster containing ST44 isolates.

Generated by Ridom Seqsphere+ v2 (2147 genes), containing both pig and human *C. difficile* isolates. Nodes are colour coded by source of *C. difficile* isolate – pig or human (Legend on the left). The grey shading represents the different cluster groups of *C. difficile*. *C. difficile* isolate name is present in each node of the tree

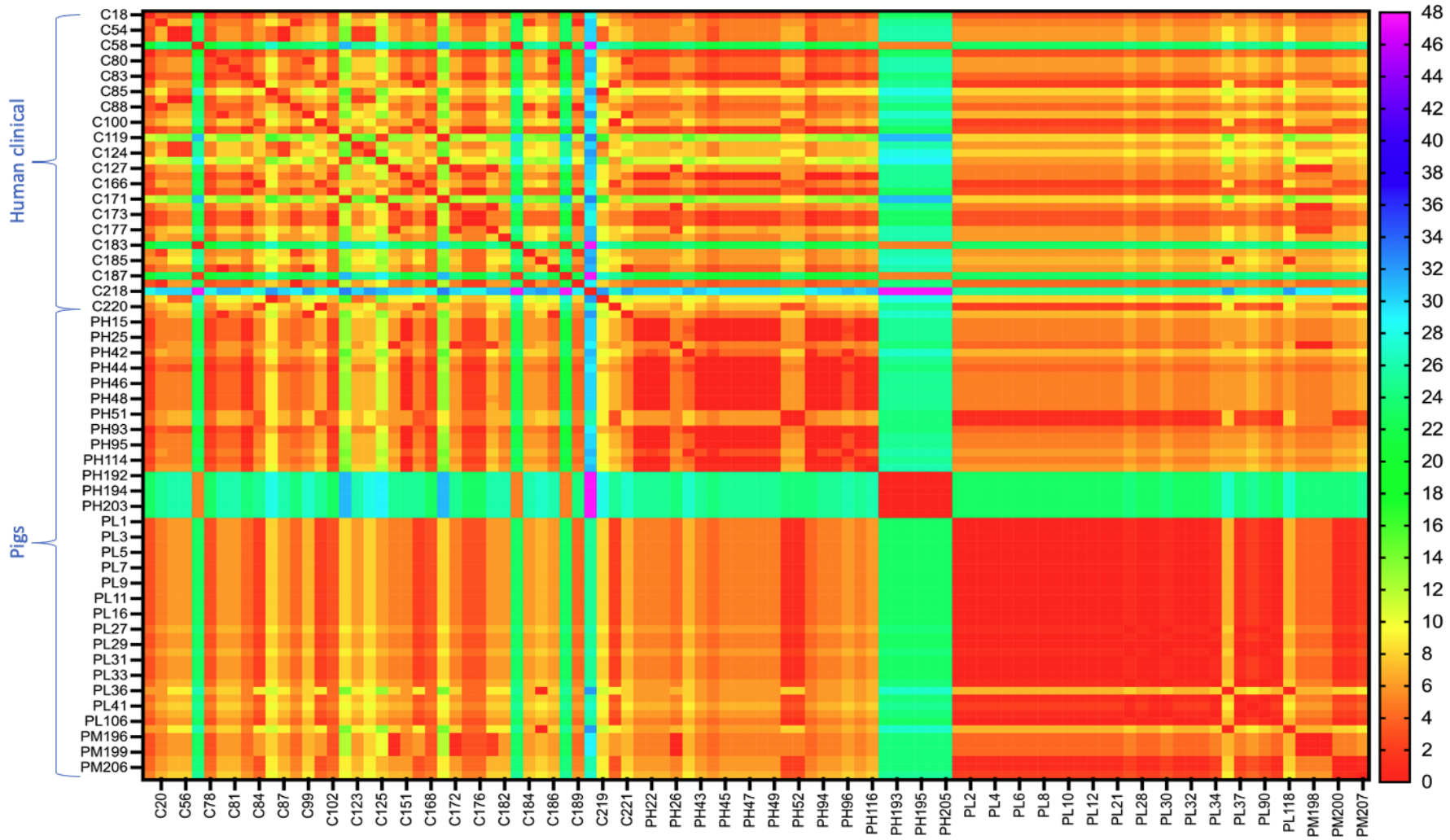


Figure 3.7 Heat map of SNPs between 100 *C. difficile* ST11 isolates of pig (n=60) and human (n=40) origin.

Isolate names are shown on the X and Y axis: PH represents HAU pigs, PL represents LAU pigs, and PM represents MAU pigs, and C represents Human clinical samples. To note: the label of each row/column alternates on the X and Y axis

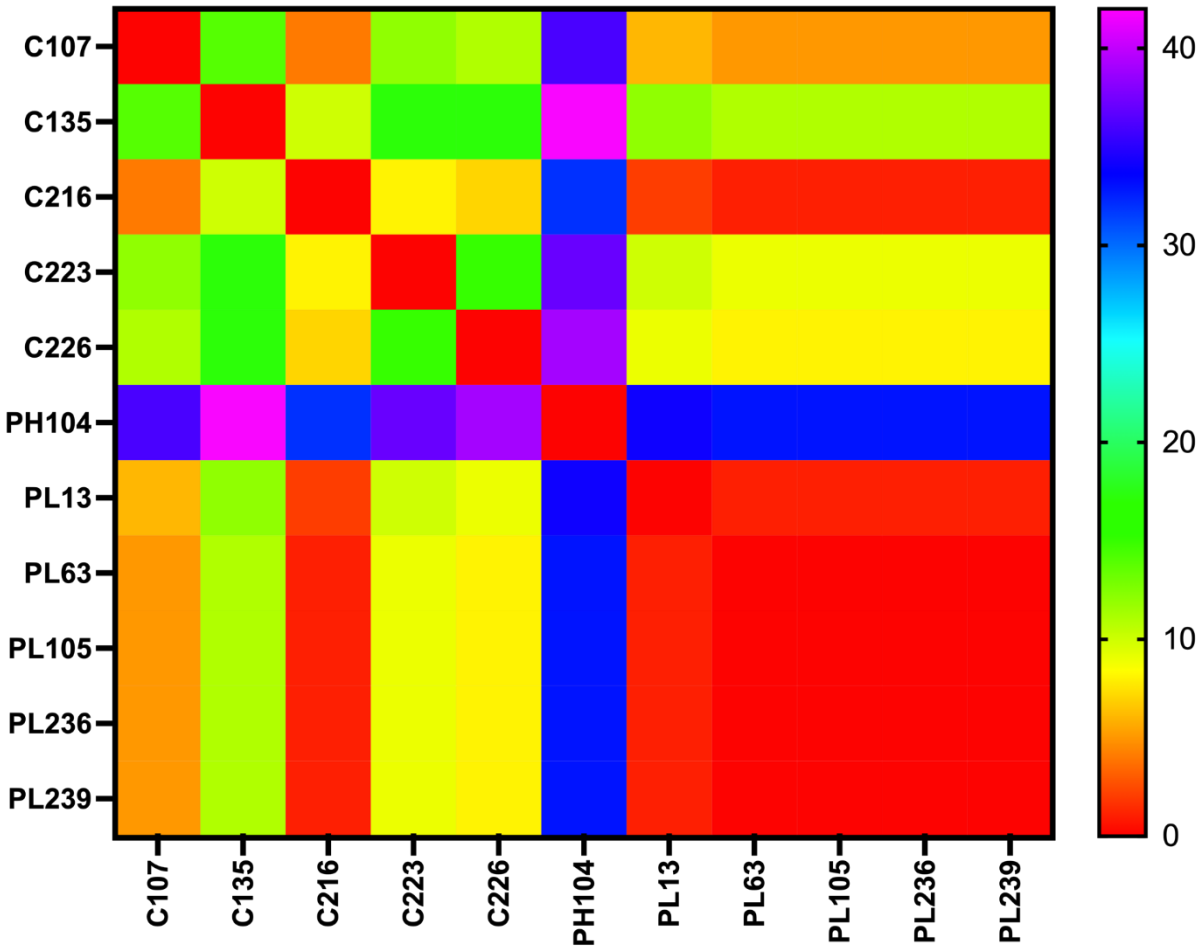


Figure 3.8 Heat map of SNPs between 11 *C. difficile* ST44 isolates of pig (n=6) and human (n=5) origin.

Isolate names are shown on the X and Y axis: PH represents HAU pigs, and PL represents LAU pigs, and C represents Human clinical samples. To note: in this graph, the label of each row/column does not alternate on the X and Y axis.

3.3.6 Toxin gene profiling

All 222 isolates were screened *in silico* for the toxin genes (*tcdA*, *tcdB*, *cdtA* and *cdtB*) and the regulatory genes (*tcdC*, *tcdR*, and *tcdE*). Both genes for the pathogenicity locus (*tcdB*, *tcdA*) were detected in 210 isolates (95%). The genes encoding for the binary toxin (CDT) (*cdtA*, *cdtB*), which is associated with more severe cases of CDI, were detected in 100 isolates (46%). There were 3 STs identified that were non-toxigenic, which accounted for 7 *C. difficile* isolates (all pig isolates). The toxin profiles of all isolates are shown in Figure 3.9. The predominant toxin profile was *cdtA-cdtB-tcdA+tcdB+* which was found in 100 *C. difficile* isolates. *C. difficile* isolates with this toxin profile are found in clade 1 and include 24 different STs. *C. difficile* strains with no toxin genes (*cdtA-cdtB-tcdA-tcdB-)* present were found in pig *C. difficile* strains only.

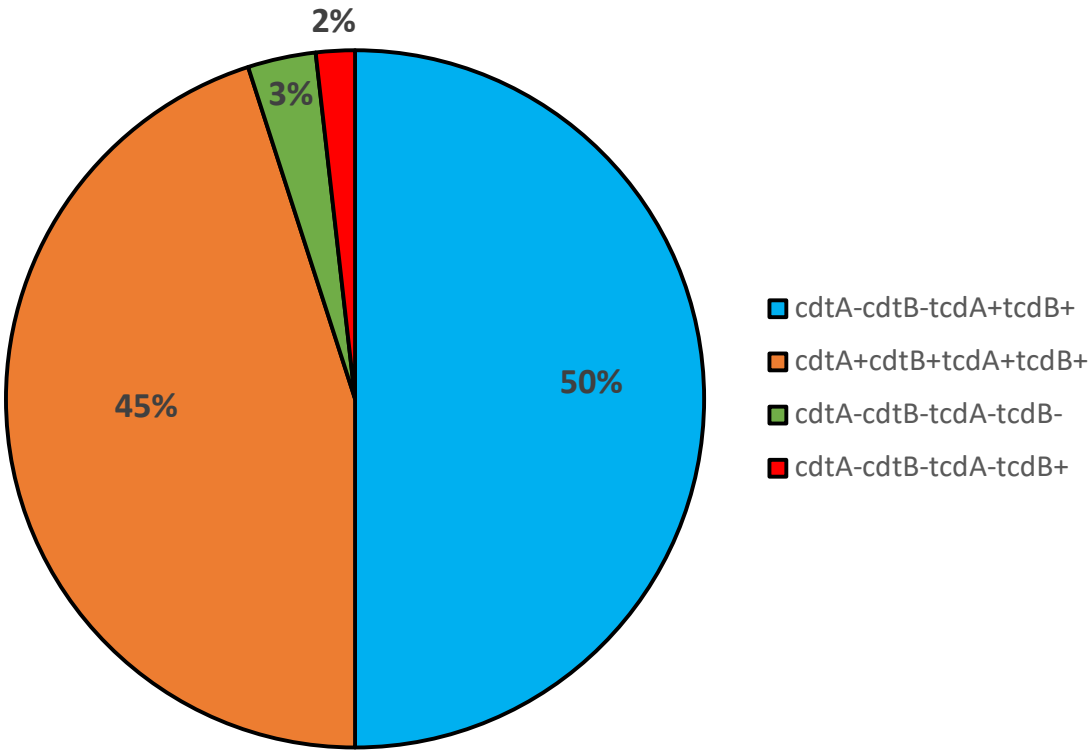


Figure 3.9 Toxin gene profiles from 222 *C. difficile* isolates from pigs (n=100) and humans (n=122).

3.4 Discussion

Human activity has driven the evolution of *C. difficile* as a diverse pathogen with the emergence of clonal clades, including RT078, as discussed in section 1.7. In this study, the molecular epidemiology of 222 *C. difficile* from pigs (n=100) and humans (122) were investigated using MLST and cgMLST.

MLST analysis identified three out of the five lineages of *C. difficile* identified in these isolates – clade 1, clade 2 and clade 5. From the 222 *C. difficile* isolates that were sequenced, 30 known STs were identified and the predominant STs identified were ST11, ST8, ST2 and ST44 (Table 3.2). There is some broad correlation observed between sequence typing and ribotyping such as ST11 corresponding to RT078, ST8 corresponding to RT002, ST2 corresponding to RTs 014, 020, 220 and ST44 corresponding to RT015. While sequence typing was not routinely used in Irish diagnostic laboratories at the time the isolates in this study were collected, these results are similar to previously reported data in Ireland (HPSC, 2021).

However, although MLST often correlates with a specific ribotype, a study by Seth-Smith et al. (2021) found that this should not be strictly relied upon as the resolution is not sufficient for some ribotypes. In Ireland, the National Reference Laboratory (NRL) for *C. difficile* was established in 2021, with 28 laboratories from across the Republic of Ireland participating in the National Enhanced CDI Surveillance. This is a welcome advancement, as WGS-based analysis is a superior method of exploring the epidemiology of *C. difficile* as it can explore patient-to-patient transmission, and provide more detailed epidemiological information than PCR ribotyping (Goyal et al., 2020, Seth-Smith et al., 2021) The annual report from 2023 demonstrates that ST11, ST8 and ST2 have persisted as the predominant *C. difficile* STs in Irish patients with CDI (HSE, 2023).

In this study it was found that clade 1 consisted of 123 isolates (54%) and 28 different STs, including both toxigenic, which possess toxin genes, and non-toxigenic strains, which do not possess toxin genes. The clade 1 lineage contains over 200 STs, including strains such as ST2 and ST8 which are prevalent strains associated with CDI worldwide (Knight et al., 2021). As described in section 1.2.5, genes encoding for the toxins TcdA (*tcdA*) and TcdB (*tcdB*) are located within the pathogenicity locus (PaLoc). In non-pathogenic strains of *C. difficile*, the toxin coding PaLoc is replaced by a highly conserved

non-coding region (Dingle et al., 2014). Non-toxigenic strains of *C. difficile* are commonly found in the environment and are also often isolated from asymptomatic individuals (Natarajan et al., 2013). Non-toxigenic *C. difficile* strains are often isolated from asymptomatic individuals, and studies by Villano et al. (2012) and Gerding et al. (2015) and have shown that prior colonization of patients with non-toxigenic strains could provide protection against CDI when exposed to toxigenic *C. difficile*. In addition, colonisation with non-toxigenic strains following vancomycin treatment is effective at preventing rCDI (Gerding et al., 2018). Non-toxigenic are more commonly found in the environment in comparison to humans, and this could explain why non-toxigenic *C. difficile* were predominantly detected in pigs and not in any of the human clinical isolates (Janezic et al., 2012, Weese, 2020).

WGS can provide higher discriminatory power than other molecular typing approaches such as ribotyping and can be used to investigate outbreaks and broader epidemiology with greater resolution (Kuijper et al., 2009). WGS is a valuable tool in the fight against *C. difficile* and the control of CDI as it can differentiate strains of the same ribotype, where ribotyping cannot. WGS has been used in this study to investigate the potential relatedness between *C. difficile* of pig and human origin and SNPs in the core-genome have been used to provide fine scale resolution of the *C. difficile* population.

Eyre et al. (2018) performed WGS analysis on clinical 1,211 isolates collected from patients between 2012-2013 from 482 different hospitals in 19 different European countries. In that study, it was found that, in comparison to healthcare-associated RTs (RT027 and RT001), *C. difficile* RTs, such as RT078, RT002, RT014, RT020, and RT015, had no epidemiological links, and strains that were genetically closely related were widely disseminated across Europe. The authors concluded that secondary transmission is likely through another vector, such as the food chain or the environment. These findings are consistent with what has been observed in our study with ST11 (RT078) and ST44 (RT015) isolates, where we have identified closely related strains that originated from different sources.

In addition, a study by Lim et al. (2022) found a high prevalence of RT014 in the hospital, as well as in environmental samples including isolates from lawns, compost, root vegetables and hospital grounds. This study also found extensive co-clustering of ST2

strains of environmental and human origin, and clonal clustering of RT014 strains from pigs and humans. The authors hypothesized that the environment is an important source of isolates causing CDI in hospital settings, potentially from contaminated shoe soles. A study by Lim et al. (2022) in Australia provided further evidence for long-range spread between humans and community reservoirs. Closely related strains that were identified in the cited study were collected 2-3 years apart, which suggests long-range transmission of *C. difficile* between the environment, animals, and humans, emphasizing the "One Health" aspect of this pathogen. Given the fecal-oral route of transmission of this pathogen, it is a reasonable assumption that the directionality of CDI transmission could be from the environment (including animal by-products) to humans.

There are currently several limitations to our study. Firstly, a limited diversity of *C. difficile* isolates was sequenced, as one of the objectives of the project was to identify the most common ribotypes found in pig and human *C. difficile* isolates and to investigate their genetic relatedness. There is no sequence data available for ST2 (RT014 and RT020) *C. difficile* isolates from pigs, and sequence data is available for only one ST8 *C. difficile* pig isolate, so the genetic relatedness between these RTs from pigs and humans could not be investigated. Secondly, the clinical *C. difficile* isolates in this study all originated from the same hospital (SJH). Finally, epidemiological data was not available for the human clinical CDI cases, as to whether they were community-associated or healthcare associated cases, or the occupation of the patient. The occupation of the patient may serve as a heightened risk factor for developing CDI as a study by Knetsch et al. (2014) revealed that farmers and their pigs, in close proximity, were colonized with identical *C. difficile* clones. If available, this data could greatly enhance our understanding of the complex epidemiology and transmission of *C. difficile*.

3.5 Conclusions

This chapter has highlighted the occurrence of genetically similar *C. difficile* isolates in pigs and in humans. It has also supported the hypothesis that *C. difficile* RT078/ST11 has persisted as a predominant strain in humans, but also is a predominant strain in pigs. Multiple clonal populations of ST11 *C. difficile* strains from pigs and humans are suggestive of recent transmission or a lack of strain diversity. In addition, genetic similarities have been revealed between isolates from pigs and humans in a limited

number of other strains such as RT015/ST44. These findings further support the hypothesis of community acquisition from environmental sources, but more robust epidemiological data including information such as occupation and dietary intake is needed to further support this. This highlights the need for a “One Health” approach, encompassing animal and human health, as well as the environment

4 Genomic analysis of *C. difficile* Ribotype 078 isolates from pigs, humans, and environmental samples

Chapter 4

4.1 Introduction

In the current work, by testing a large collection of isolates (n=222) of different ribotypes of *C. difficile* recovered from pigs and humans using a combination of ribotyping, MLST and cgMLST (reported in Chapters 2 and 3), it was confirmed that PCR ribotype 078 (clade 5, belonging to heterogenous ST11 lineage), i.e. RT078/ST11, is numerically dominant in both pigs and humans. In this chapter, RT078/ST11 will be investigated in further detail to gain new insight into its epidemiology in Ireland.

From a "One Health" perspective, certain *C. difficile* ribotypes are more important than others. The main ribotypes that have raised concern with respect to their zoonotic or food origins are contained in clade 5, in particular RT078 (Moloney et al., 2021). CA-CDI is increasing in incidence, but the sources of *C. difficile* in the community are poorly understood. RT078 is a common cause of CA-CDI. This ribotype is widely reported in production animals and is the most common ribotype recovered from pigs (Keessen et al., 2013a, Stein et al., 2017). In addition, further characterisation of *C. difficile* RT078 has shown indistinguishable isolates between pigs and humans which further supports the likelihood of zoonotic or foodborne transmission (Moloney et al., 2021, Knetsch et al., 2014, Keessen et al., 2013a, Debast et al., 2009b). Moloney et al. (2021) found close genomic relationships between RT078 isolates from Irish pigs and human cases of CDI from several European countries, suggesting broader epidemiological linkages.

It was decided to focus on *C. difficile* RT078/ST11 in this chapter because it forms its own phylogenetic lineage by MLST and WGS analysis and is the predominant ribotype in pigs and in humans, as discussed in Chapter 3. This chapter investigated the relationship between *C. difficile* isolates obtained from pigs, the environment, and from CDI cases from a selection of Irish hospitals by WGS analysis.

4.2 Methods

4.2.1 Collection of sequence data for *C. difficile* ST11 isolates

Sequence data (including data from collaborators) from a total of 186 *C. difficile* ST11 isolates were collated, from animal, human, environmental and food origins. The 186 *C. difficile* ST11 isolates included 100 isolates sequenced in the current study, as detailed in Chapter 3, 41 human isolates sequenced collected from a regional surveillance study between 2019 and 2020, and 43 isolates from a study performed at UCD by Pilar Marcos, as described in Marcos et al. (2021) and Marcos et al. (2023), between 2018 and 2020, who provided environmental and food *C. difficile* isolates. The number and percentage of *C. difficile* sequences included in this analysis by source are listed in Table 4.1.

Table 4.1 Number and percentage of *C. difficile* isolates by source

Source	Number of Isolates	Percentage (%) of Isolates
Human	81	43.5
Animal (pig)	60	32.3
Environmental	42	22.6
Food	3	1.6
Total	186	100

4.2.1.1 Sequence data from human *C. difficile* isolates

Sequence data from a total of 81 human *C. difficile* ST11 isolates were included in this analysis. This included sequence data from 40 *C. difficile* ST11 isolates from a tertiary referral hospital in Dublin, as described in Chapter 2, which is referred to as “centre 1” in this chapter. In addition, a tertiary referral hospital in Galway provided sequence data for 41 *C. difficile* ST11 isolates, which were obtained as part of a regional surveillance study that was carried out between 2019 and 2020 and is referred to as “centre 2” in this chapter. These *C. difficile* isolates were available to us irrevocably anonymised and given an alphanumeric code by laboratory scientists in the Galway hospital. This participating centre is located in the West/South-West of Ireland and is separated by over 200km from centre 1, which is located in the East of Ireland.

4.2.1.2 Sequence data from animal *C. difficile* isolates

Sequence data from a total of 60 animal *C. difficile* ST11 isolates were included in this analysis. This sequence data was from pig *C. difficile* isolates, as described in Chapter 2.

4.2.1.3 Sequence data from environmental *C. difficile* isolates

Sequence data from a total of 42 environmental RT078/*C. difficile* ST11 isolates were included in this analysis. These were obtained from abattoirs (n=2) in this study in 2023, and from collaborators on the DAFM project in UCD (n=40) between 2018 and 2020.

Using the results obtained from PCR ribotyping results presented in Chapter 2, two *C. difficile* RT078 isolates that were obtained in 2023 from the pig abattoir sampling were chosen for sequencing. These were excluded from Chapter 3, as the aim of that chapter was to focus on *C. difficile* isolates from pigs and humans from 2018 to 2020. There was still budgetary capacity to sequence more *C. difficile* isolates, so it was decided to sequence the *C. difficile* RT078 isolates from the abattoir sampling to add to the environmental samples that were provided by collaborators from UCD.

C. difficile isolates (n=40) from food and the environment were collected in a study in Ireland by Marcos et al. (2023), who surveyed the prevalence of *C. difficile* on cattle, sheep, and broiler farms, and in cattle, sheep and broiler abattoirs, and characterised the *C. difficile* isolates by PCR ribotyping and WGS. Samples from farms included environmental (water and soil) and faecal samples. In this analysis, faecal samples were characterised as environmental samples, as the investigator in UCD collected freshly voided faeces from the floors of pens and fields where animals were grazing. The investigators in UCD transferred the sequence data for ST11 isolates to the investigators in this study at TCD. Data was stored locally on the Linux computer in the Sir Patrick Dun (SPD) Laboratory (TCD) in the Central Pathology Laboratory (CPL) of SJH and backed up on an external hard drive that was stored in the postgraduate reading room in SPD. The breakdown of the origin of the *C. difficile* ST11 isolates that were provided by UCD are detailed in Table 4.2

.

Table 4.2 The number and origin of *C. difficile* ST11 isolates of environmental origin included in this study

These isolates were obtained from a study by Marcos et al. (2023), and were included in the analysis in the current work.

Environmental source	Type of farm			Total
	Beef	Sheep	Broiler	
Water	6	1	4	11
Soil	8	6	10	24
Faeces	1	1	1	3
Abattoir	-	2	-	2
Total	15	10	15	40

4.2.1.4 Sequence data from food *C. difficile* isolates

Sequence data from a total of 3 food *C. difficile* ST11 isolates were included in this analysis. This was provided by collaborators on the DAFM project from UCD. *C. difficile* isolates were collected in a study in Ireland by Marcos et al. (2023), who surveyed the contamination rate in food products. *C. difficile* isolates were cultured from ready-to-eat food products: coleslaw, cottage cheese, and wild rocket leaves.

4.2.2 Whole Genome Sequencing (WGS) of abattoir *C. difficile* isolates

4.2.2.1 Selection of *C. difficile* isolates

Using data obtained from abattoir sampling and PCR ribotyping results, as detailed in Chapter 2, two RT078 isolates were chosen for sequencing.

4.2.2.2 DNA extraction for WGS

Genomic DNA of *C. difficile* isolates was extracted as described in Chapter 3, section 3.2.1.2.

4.2.2.3 DNA quantification

Genomic DNA of *C. difficile* isolates was quantified as described in Chapter 3, section 3.2.1.3.

4.2.2.4 Performing WGS

WGS was performed as described in Chapter 3, section 3.2.1.4.

4.2.3 *C. difficile* genome sequence analysis

A new project was generated on the Ridom Seqsphere+ software version 9.0.10 to keep the analysis from Chapter 3 and this chapter separate (Junemann et al., 2013). Genome sequence analysis was performed as described in Chapter 3, section 3.2.1.5. Paired-end reads were mapped to the *C. difficile* M120 reference genome (NC_017174.1) (He et al., 2010).

4.2.3.1 Multilocus sequence typing (MLST) target gene definition.

The sequence types of the *C. difficile* isolates were obtained as described in Chapter 3, section 3.2.1.6.

4.2.3.2 Core genome multilocus sequence typing (cgMLST) target gene definition

The differentiation of closely related strains was carried out as described in Chapter 3, section 3.2.1.7.

4.3 Results

4.3.1 WGS of *C. difficile* ST11

4.3.1.1.1 Samples included in this study

Sequence data was obtained for 186 *C. difficile* isolates from human, animal, environmental and food recovered in Ireland between 2018 and 2023. The detailed statistics for the quality of sequencing data for the abattoir samples, as provided by Novogene, are shown in Supplementary Table S1. There were no clinical details available for the any of the *C. difficile* isolates from human samples.

4.3.2 MLST

The 2 abattoir isolates were confirmed as ST11 by MLST. *In silico* MLST analysis revealed that all 186 *C. difficile* ST11 isolates had allelic conservation in the seven housekeeping genes (*adk5*, *atpA8*, *dxr5*, *glyA11*, *recA9*, *sodA11*, and *tpi8*), which is consistent with ST11 and clade 5. All isolates carried both toxin A and B genes (*tcdA*, *tcdB*) and the binary toxin genes (*cdtA*, *cdtB*).

4.3.3 SNP analysis

Next, the phylogenetic distribution of *C. difficile* ST11 strains of human, animal, environmental and food origin was explored to understand the potential for zoonotic transmission, either directly or indirectly. To determine if the sequences were genetically distinct, a minimum spanning tree (MST) was generated (Figure 4.1). This analysis indicated clustering of *C. difficile* strains of different origins, which may suggest bidirectional spread between hosts. The 186 isolates differed from 0 to 202 alleles in pairwise comparison, the mean difference was 10.23 alleles, and the median allelic difference was 6 alleles. Following the standard approach of Eyre et al. (2013), a genetic relationship was defined as ≤2 SNPs in the core genome between *C. difficile* isolates and is indicative of a plausible clonal transmission event. Clonal groups (CGs) of isolates are defined as 0-2 allelic differences, and isolates are grouped into the same cluster if they differ by ≤6 alleles. There were four clusters identified (≤6 allelic differences), with the largest cluster group (cluster 1) containing 169 genomes. In general, there was no geographic grouping of isolates, as isolates from the same farms and hospitals were

widely distributed across the MST, and there was overlap between strains from clinical and non-clinical sources.

The largest cluster, designated cluster 1 on the MST in Figure 4.1, was comprised of 169 *C. difficile* isolates of human (n=72), pig (n=54), environmental (n=38), and food (n=3) origin, and isolates were distributed independent of sampling time or location, as isolates from different farms and hospitals often clustered into the same CG. For focused analysis of this cluster, a neighbour joining (NJ) circular tree was constructed (Figure 4.2). CGs of isolates were seen throughout the tree, which includes a CG of *C. difficile* isolates of human, food (coleslaw and cottage cheese), and environmental origin differing by 0 SNPs.

Cluster 2 was comprised of 4 human *C. difficile* isolates, and 6 pig *C. difficile* isolates. The human isolates originated from different tertiary referral hospitals, which are over 200km apart: 3 from centre 1, and the fourth isolate came from centre 2. The 6 pig *C. difficile* isolates came from 2 different farms, and there was 0 SNPs between them. Clusters 3 and 4 each contained 2 isolates from the environment.

4.3.3.1 Clonal relationships among *C. difficile* isolates from diverse sources

Across the 4 phylogenetic clusters, 29 CGs were identified encompassing 135 out of 186 (72.5%) *C. difficile* isolates from diverse sources. Of these CGs, 21/29 (72.4%) were made up of isolates from the same host species, which is supportive of intraspecies transmission. The remaining 8 CGs (27.5%) were comprised of isolates of clinical and nonclinical origin, which is consistent with interspecies transmission.

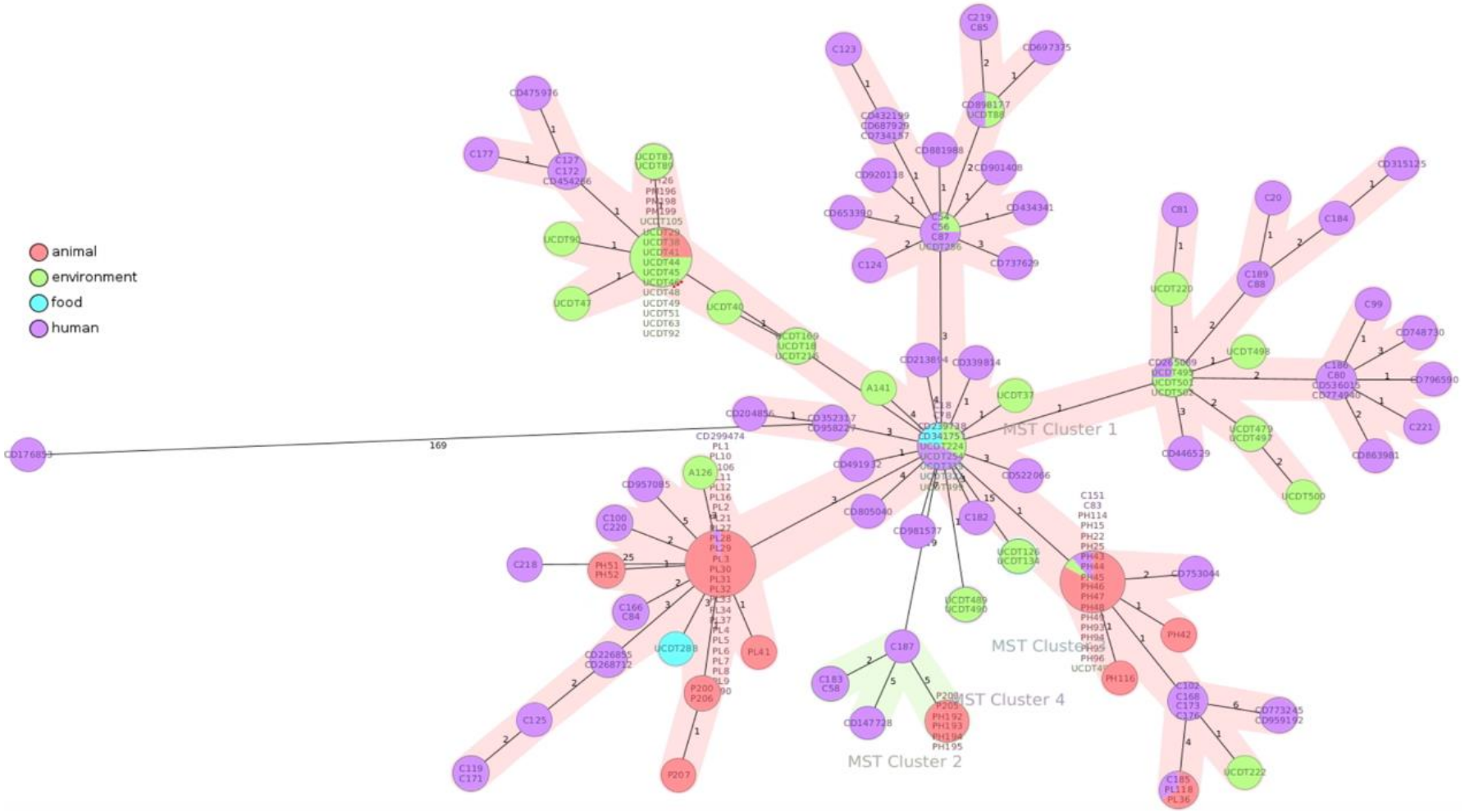


Figure 4.1 Phylogenetic tree of 186 *C. difficile* ST11 isolates from different sources.

Tree is coloured by the source of isolate (legend on left); "animal" refers to pig isolates, "human" contains isolates from two different centres, and "environment" isolates are from abattoir, water, soil, and faeces samples.

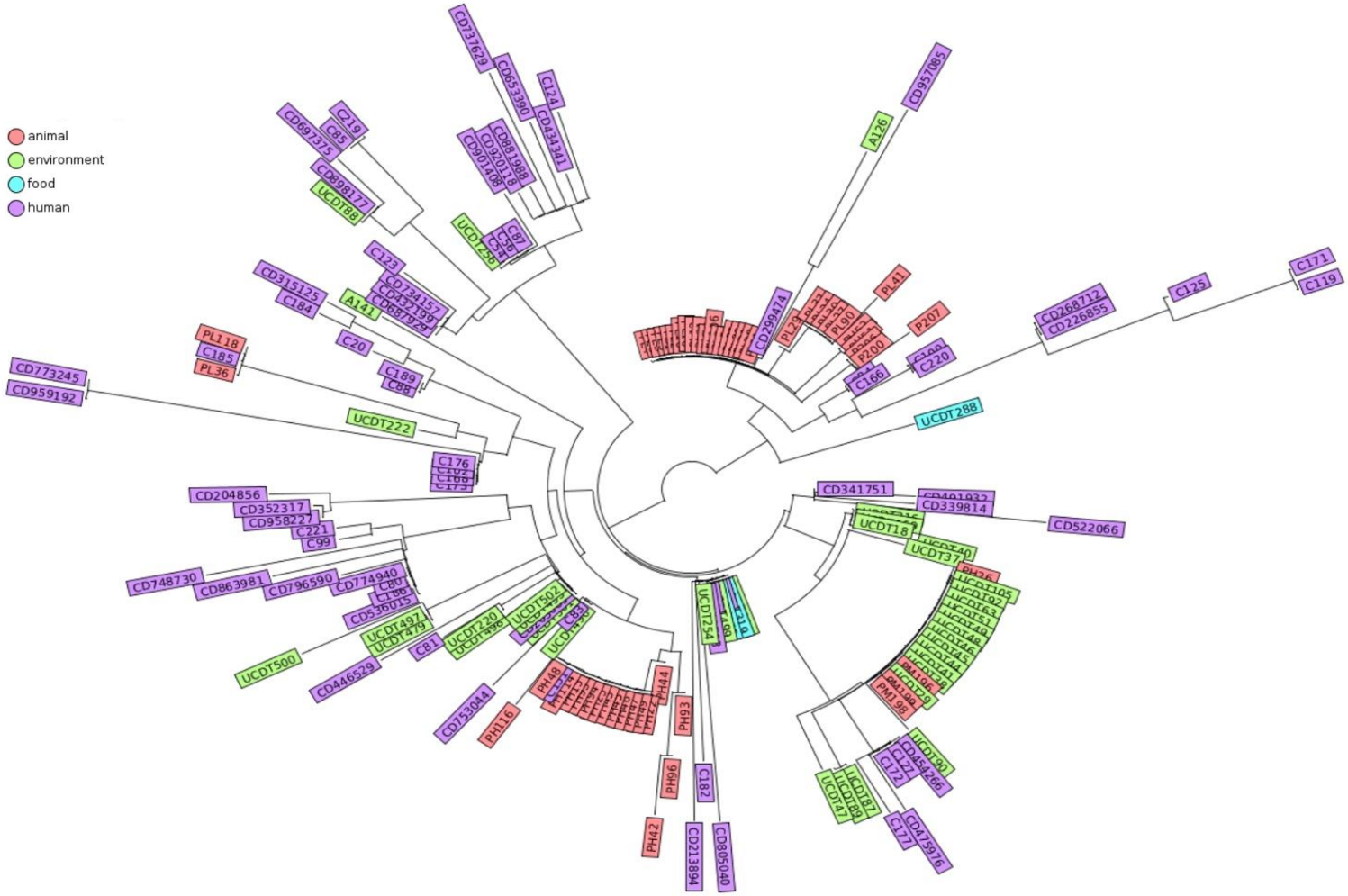


Figure 4.2 A maximum likelihood phylogenetic neighbour joining (NJ) circular tree for cluster 1 (169 *C. difficile* isolates).
Generated by the Ridom™ SeqSphere+ core genome multilocus sequencing typing (cgMLST) scheme version 2 (2147 genes) and MLST scheme (7 genes). Tree is coloured by the source of isolate (legend); "animal" refers to pig isolates, "human" contains isolates from 2 different centres, and "environment" isolates are from abattoir, water, soil, and faeces samples.

4.3.3.1.1 Clonal relationships between human and environmental isolates

There were 3 out of 8 CGs that were made up of human and environmental *C. difficile* isolates only, which are shown in Figure 4.3.

Figure 4.3(a) shows a CG that is made up of 4 *C. difficile* isolates, 3 are of human origin and 1 is of environmental origin. The 3 human isolates are from the same hospital, centre 1, and the environmental isolate originates from a sheep carcase in an abattoir. There were 0 SNP differences between the 4 isolates.

Figure 4.3(b) shows a CG that is made up of 2 *C. difficile* isolates, 1 human isolate and 1 environmental isolate. The human isolate originates from centre 2, and the environmental isolate originates from a soil sample that was taken on a sheep farm. There were 0 SNP differences between the 2 isolates.

The CG shown in Figure 4.3(c) is made up of 4 *C. difficile* isolates, 1 human isolate and 3 environmental isolates. The human isolate originates from centre 2. The environmental isolates all originate from a poultry farm, one isolate was obtained from a water sample and the other two isolates were cultured from soil samples. There were 0 SNP differences between the 4 isolates.

4.3.3.1.2 Clonal relationships between pig and environmental isolates

There was 1 out of 8 CGs that were comprised of pig and environmental *C. difficile* isolate, which is shown in Figure 4.4(a) and is comprised of 16 *C. difficile* isolates, which are 4 pig and 12 environmental isolates. The pig isolates originate from 2 different farms, 3 from a MAU farm, and 1 from a HAU farm. Out of the 12 environmental isolates, 10 originate from a cattle farm, with 5 isolates cultured from soil samples and 5 isolates cultured from water samples. The remaining 2 isolates were obtained from a faecal sample and a soil sample on a sheep farm. There were 0 SNP differences between the 16 *C. difficile* isolates.

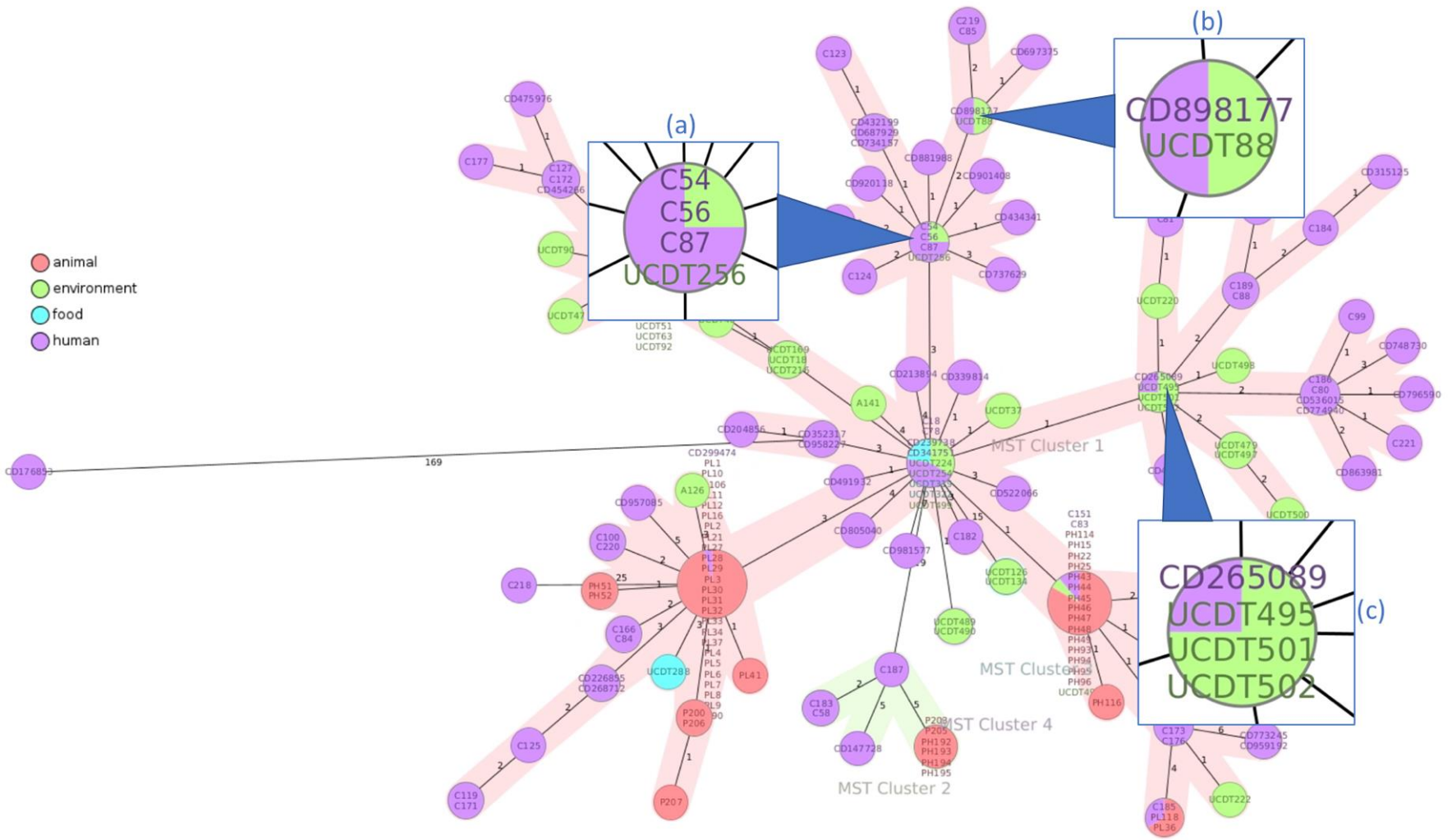


Figure 4.3 Minimum spanning tree (MST) demonstrating the clonal relationship between human and environmental *C. difficile* isolates.
Legend shown on the left, (a) CG with 3 human and 1 environmental *C. difficile* isolates, (b) CG with 1 human and 1 environmental *C. difficile* isolates, and (c) CG with 1 human and 3 environmental *C. difficile* isolates. The isolate label describes the original hospital, "C" isolates are centre 1, and "CD" are centre 2.

4.3.3.1.3 Clonal relationships between pig and human isolates

There were 2 out of 8 CGs that were comprised of pig and human *C. difficile* isolates only, which are shown Figure 4.4(b) and Figure 4.4(c). Figure 4.4(b) shows a CG that is made up of 26 *C. difficile* isolates, 25 of these isolates are of pig origin, and 1 isolate is of human origin, from centre 2. The 25 pig *C. difficile* isolates are all from pigs on LAU farms, 15 of which are from one farm, and 10 are from another farm. The human *C. difficile* isolate was cultured from centre 2. The maximum difference between the 26 *C. difficile* isolates was 1 SNP.

Figure 4.4(c) shows a CG that is made up of 3 *C. difficile* isolates, 2 from pigs and 1 from humans. The pig isolates were both cultured from a LAU farm. The human isolate was cultured from centre 1. There were 0 SNP differences between the 3 *C. difficile* isolates.

4.3.3.1.4 Clonal relationships between three different sources of isolates

There were 2 out of 8 CGs that were comprised of *C. difficile* isolates from 3 different origins, which are shown in Figure 4.5. Figure 4.5(a) shows a CG that is made up of 9 *C. difficile* isolates, which are 4 human, 3 environmental, and 2 food *C. difficile* isolates. The 4 clinical isolates originate from 2 different hospitals – 2 were cultured from centre 1, and 2 were cultured from centre 2. Out of the 3 environmental isolates, 2 were cultured from soil samples on a poultry farm. The remaining environmental isolate was cultured from a sheep carcase in an abattoir. The 2 food isolates were cultured from cottage cheese and coleslaw from supermarket retailers. The maximum difference between the 9 *C. difficile* isolates was 2 SNPs.

Figure 4.5(b) shows a CG that is made up of 18 *C. difficile* isolates, which are 15 pig, 2 human, and 1 environmental *C. difficile* isolates. The 15 pig isolates all originate from HAU farms, with 10 isolates cultured from one HAU farm, and the remaining 5 isolates cultured from another HAU farm. The human isolates were obtained from the same hospital, centre 1. The environmental sample was cultured from a soil sample from a poultry farm. The maximum difference between the 18 *C. difficile* isolates was 3 SNPs.

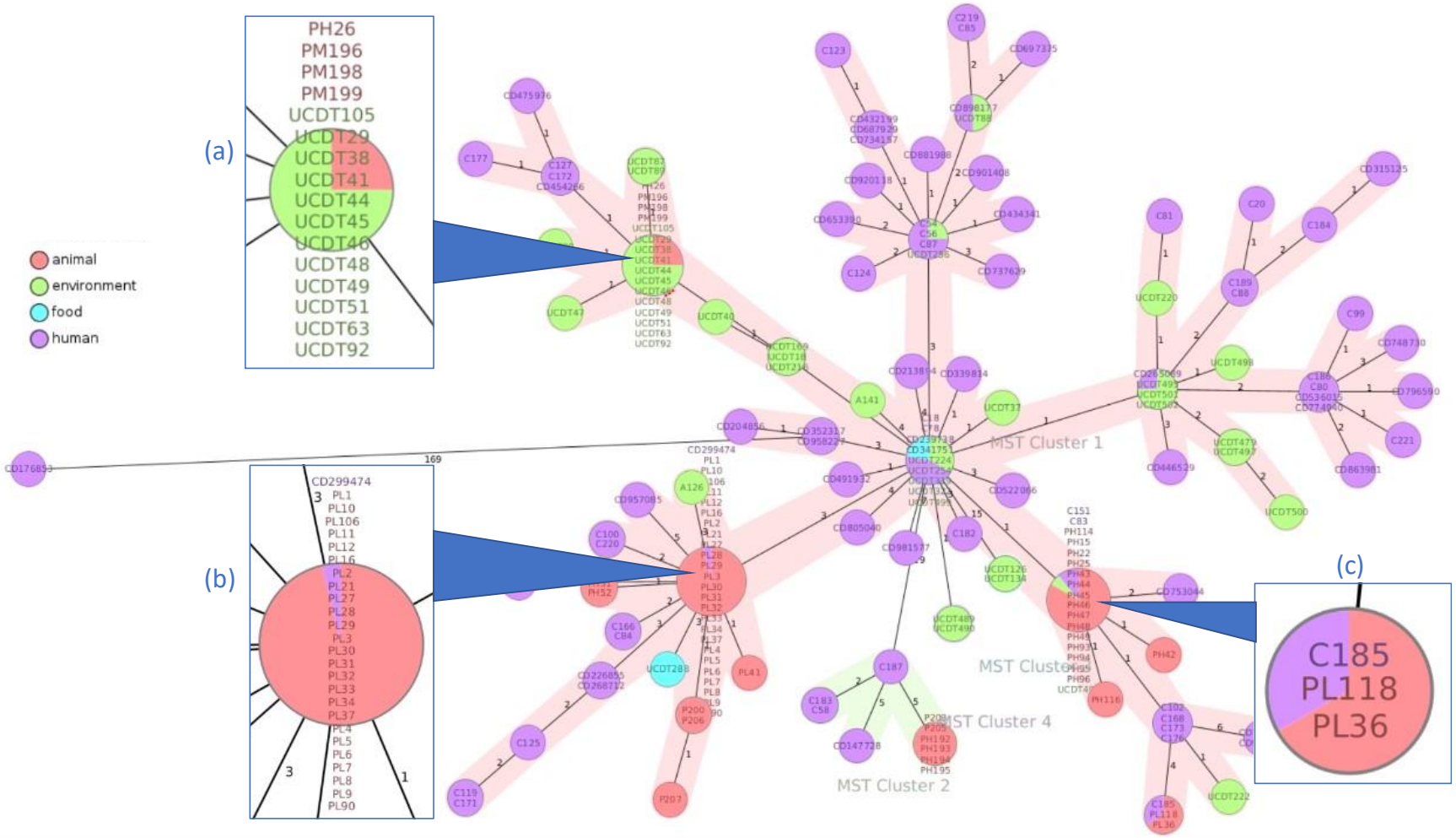


Figure 4.4 Minimum spanning tree (MST) showing the clonal relationship between pig and environmental, and pig and human *C. difficile* isolates. Legend shown on the left, (a) CG with 4 pig and 12 environmental *C. difficile* isolates, (b) CG with 1 human and 25 pig *C. difficile* isolates, and (c) CG with 1 human and 2 pig *C. difficile* isolates. The isolate label describes what type of AMU the pig farm is, PL is LAU farms, PM is MAU farms and PH is HAU farms. The isolate label also describes the hospital, "C" isolates are centre 1, and "CD" are centre 2.

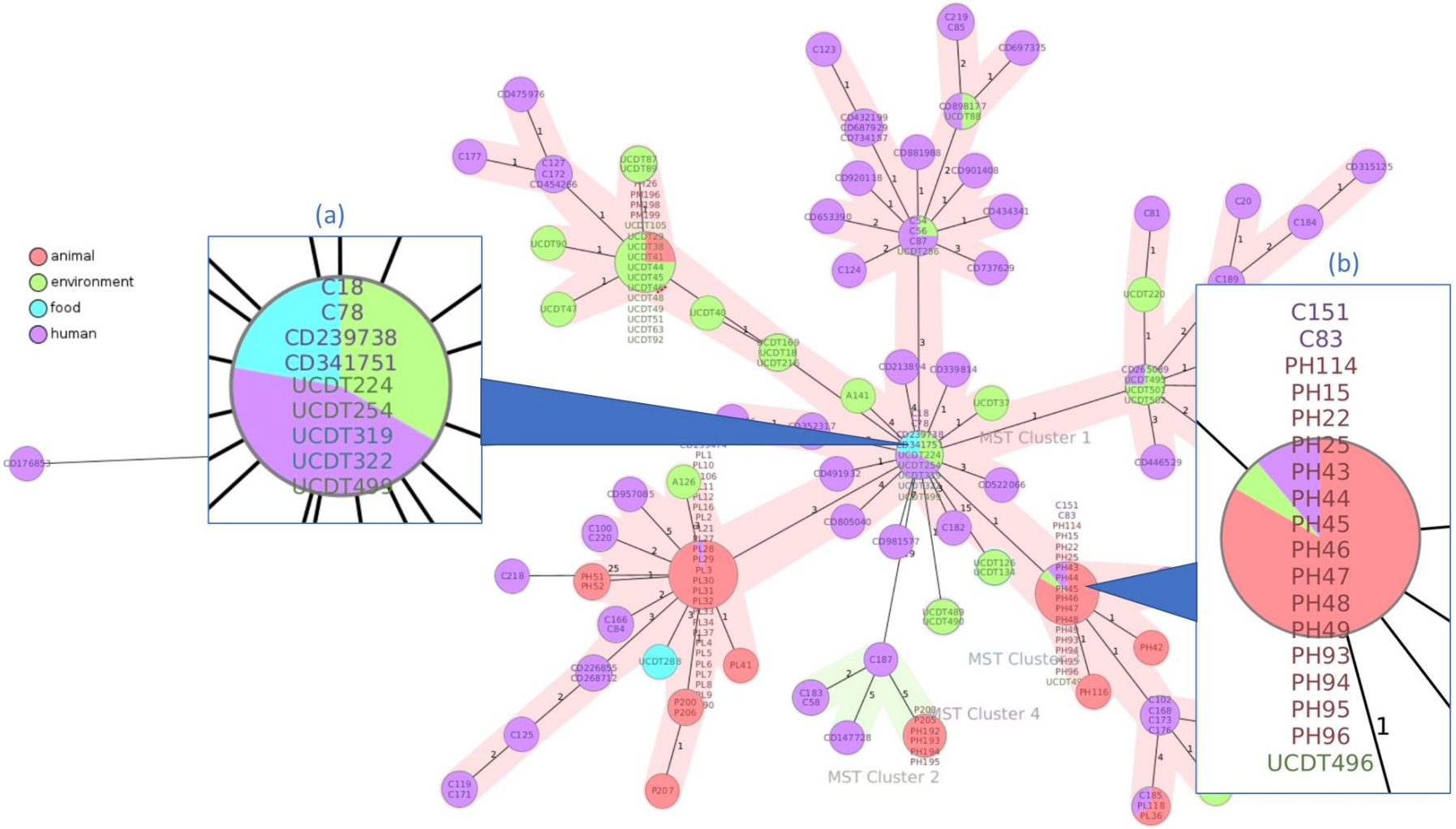


Figure 4.5 Minimum spanning tree (MST) demonstrating the clonal relationship between three diverse sources of *C. difficile* isolates.
Legend shown on the left, (a) CG with 4 human, 3 environmental, and 2 food *C. difficile* isolates, and (b) CG with 15 pig, 2 human, and 1 environmental *C. difficile* isolate. The isolate label describes what type of AMU the pig farm is, "PL" is LAU farms, "PM" is MAU farms and "PH" is HAU farms. The isolate label also describes the hospital, "C" isolates are centre 1, and "CD" are centre 2.

4.4 Discussion

RT078/ST11 is an important lineage of *C. difficile* that has established widespread reservoirs in production animals worldwide (Rodriguez et al., 2016a, Knight et al., 2019). It is also a major infection source for both HA-CDI and CA-CDI in Europe, Canada, and North America (Bauer et al., 2011b, He et al., 2013). There is increasing evidence suggesting that CDI has a substantial zoonotic component, supported by the shared ancestry and genetic overlap of ST11 strains of animal and human origin. Environmental reservoirs such as food, air, water, and exposure to animals may play an important role in human CDI (Lin et al., 2015).

Using WGS, this study investigated a total of 186 *C. difficile* ST11 isolates. Sequence data was provided by Marcos et al. (2021), who reported *C. difficile* prevalence rates of 50%, 40% and 83%, from cattle, sheep, and broiler farms, respectively, and RT078 was the predominant ribotype, having been found on all farms sampled. Clinically relevant, hypervirulent strains are prevalent in pigs, as previously highlighted in Chapter 2 and 3, but are also widely reported in other production animals, which underlines the requirement for a "One Health" approach to *C. difficile* involving veterinarians, environmental scientists, and medical professionals (Squire and Riley, 2013).

ST11, which belongs to clade 5, is a diverse evolutionary lineage of *C. difficile* (Knight et al., 2019) . This clade is divergent from the four other *C. difficile* clades within the MLST loci and possess several unique genome regions (Dingle et al., 2011). Interestingly, some of these genomic regions exhibit approximately 80% sequence similarity to the anaerobic bacteria *Streptococcus pyogenes* and *Thermoanaerobacter* spp., which is suggestive of gene transfer across large phylogenetic distances (He et al., 2010). Isolates from clade 5 have also been found to carry different sets of mobile genetic elements that encode for predicted virulence factors and antimicrobial resistance (Hargreaves et al., 2016, Elliott et al., 2014). As a result, RT078 isolates of *C. difficile* are mapped to the ST11 reference genome M120, to capture the strain relatedness and genetic variability of *C. difficile* ST11.

This analysis revealed intraspecies and interspecies transmission of *C. difficile* ST11 isolates. Genetically indistinguishable isolates (0 SNP differences) were found across the hospitals studied, and farms, that were geographically distinct, which is indicative of

long-range dissemination of *C. difficile* clones and a potential zoonotic transmission network nationally (Knight et al., 2019). In 2018, Knetsch et al. (2018) performed a WGS analysis on 247 *C. difficile* RT078 isolates from humans and animals that were collected between 1996 and 2012 from 22 different countries across four continents (North America, Europe, Australia, and Asia). Phylogenetic analysis revealed clustering of *C. difficile* strains of animal and human origin, which also supported bidirectional spread of *C. difficile* between diverse sources. The authors concluded that the lack of geographic clustering seen in their study is characteristic of repeated international transmission of *C. difficile* ST11 between humans and production animals. The findings of Knetsch et al. (2018) were further supported by a study by Knight et al. (2019), who analysed 200 ST11 genomes of *C. difficile* from diverse sources and found intraspecies and interspecies CGs (isolates differing by ≤2 SNPs). These CGs were distributed irrespective of location, and analysis revealed an internationally disseminated network of clones which is indicative of zoonotic and/or anthroponotic transmission.

The close genetic similarities of *C. difficile* isolates from human samples, animals, and the environment, and the intermingling of the isolates in clusters is indicative of transmission of *C. difficile* between animals and humans. Although the most plausible route for the spread of *C. difficile* is dissemination through the food chain, studies to investigate the prevalence of *C. difficile* contamination in retail meats have yielded variable results (Primavilla et al., 2018, Jobstl et al., 2010, Razmyar et al., 2017, Weese et al., 2010a, Limbago et al., 2012). Low contamination rates in retail food products is supported by the low carriage of *C. difficile* on carcases in slaughterhouses and meat processing facilities (Warriner et al., 2017). This does not exclude food as a potential route of transmission, but it is likely that there is another vehicle that is important in the dissemination of *C. difficile*, potentially through an environmental reservoir (Moloney et al., 2021). The presence of *C. difficile* ST11 on farms in soil and water provides evidence that human and animal by-products, such as manure or compost, could contribute to environmental contamination with *C. difficile* (Moono et al., 2016).

Foods other than meat products (which were examined in the current study) have been investigated for the presence of *C. difficile*. *C. difficile* has frequently been reported in vegetables, with rates ranging from 2.8% to 18.2% in ready-to-eat salads, to 30% in root

vegetables (Eckert et al., 2013, Tkalec et al., 2019, Lim et al., 2018). It is interesting to note that all the *C. difficile* ST11 isolates identified in the study by Marcos et al. (2023), and included in this study, were found in ready-to-eat food products. This is further backed up by the intermingling of 2 out of 3 *C. difficile* isolates from food with human and environmental *C. difficile* isolates in this study.

C. difficile ST11 was isolated from water on all farms examined by Marcos et al. (2021), and the current study has shown that some of these isolates are genetically indistinguishable from human *C. difficile* isolates. *C. difficile* has been isolated in other studies from water samples from the sea, river, lake, swimming pools and tap water (al Saif and Brazier, 1996, Zidaric et al., 2010, Janezic et al., 2016). *C. difficile* can survive the sewage treatment process, and discharged effluent can contaminate rivers and seas (Romano et al., 2012, Lim et al., 2020b). A study in Finland by Kotila et al. (2013) demonstrated that waterborne transmission of *C. difficile* is possible, after the accidental contamination of the drinking water distribution system with sewage effluent from a municipal wastewater treatment plant caused an outbreak of CDI in the community. The release of contaminated effluent into seas could have an impact on seafood, and *C. difficile* has been isolated in shellfish, with rates ranging from 3.9% to 49% in molluscs (Troiano et al., 2015, Pasquale et al., 2012). The occurrence of hypervirulent *C. difficile* strains in shellfish is of concern as shellfish is often consumed raw, or inadequately cooked.

Although *C. difficile* was recovered from food samples reported by Marcos et al. (2021), and genetic relatedness was uncovered between food and human *C. difficile* isolates, this does not directly equate to *C. difficile* being a foodborne pathogen. The source of contamination is unknown and may have occurred at any critical control point along the food chain such as at the food source, the processing facility, the food handler, or the food transporter (Kwon et al., 2016). There is no evidence of a direct link between food and CDI, and human exposure may be zoonotic, waterborne, environmental or person to person.

An important limitation to this study is the lack of patient metadata from both centres. Unfortunately, we were not permitted to collect or analyse any patient information, other than the centre of origin, due to ethics restrictions. The inclusion of this data, such

as if it was a recurrent infection, or a CA-CDI case,, or information on the patient's occupation, could help to further clarify and enhance any epidemiological links (Frentrup et al., 2020).

4.5 Conclusions

The current study corroborates the findings of Knetsch et al. (2018) and Knight et al. (2019), by identifying CGs of *C. difficile* isolates from different farms and hospitals, which further indicates that zoonotic spread of *C. difficile* may not be confined to geographical regions or local populations of humans and animals. This study highlights the necessity for whole genome sequencing of *C. difficile* to provide more detailed epidemiological information than PCR ribotyping, and to map the transmission in both hospital and the environment.

5 Antimicrobial susceptibility profiles of ST11 *Clostridioides difficile* isolates

5.1 Introduction

C. difficile appears to have a high capability to adapt to change in environmental conditions, through genetic and metabolic alterations (Spigaglia, 2016). The occurrence/recurrence of CDI is attributed to *C. difficile* resistance to antibiotics that are commonly used to treat bacterial infections (other than CDI), but it also is an important contributor to epidemiological changes and newly emergent strains of *C. difficile* (Spigaglia, 2016, Imwattana et al., 2021).

Currently, there are two antimicrobial agents, vancomycin and fidaxomicin, that are recommended for first-line treatment of CDI in Europe and North America (van Prehn et al., 2021, McDonald et al., 2018). The development of resistance to the few antibiotics that are effective in treating CDI, although rare, poses a challenge for both patients and healthcare professionals (Wickramage et al., 2021). The US Centers for Disease Control and Prevention (CDC) has highlighted *C. difficile* as an organism that poses the highest antimicrobial resistance (AMR) burden and threat (CDC, 2019). AMR is important in the dissemination and pathogenesis of *C. difficile*, as the risk of developing CDI is increased if *C. difficile* is resistant to the antibiotic administered to the host, where the *C. difficile* is not directly affected by the antibiotic usage (Dingle et al., 2019, Drudy et al., 2006).

Outbreaks of CDI are attributed to the emergence of hypervirulent strains, and a common characteristic of these endemic *C. difficile* strains is AMR (Sholeh et al., 2020). The acquisition of AMR in *C. difficile* strains has been a key driver of genetic and epidemiological diversity and with the spread of *C. difficile* as a nosocomial infection in the 1980s. This was linked to intrinsic resistance to cephalosporins (Imwattana et al., 2020). Likewise, clindamycin resistance has been associated with outbreaks of CDI due to *C. difficile* RT017 (Kuijper et al., 2001). In addition, the rapid dissemination of the hypervirulent RT027 strain was facilitated by the acquisition of increased fluoroquinolone resistance (O'Connor et al., 2009, He et al., 2013). Resistance to fluoroquinolones is attributed to nonsynonymous mutations, which are mutations that change an amino acid in a protein, in the quinolone resistance-determining regions (QRDR) of DNA gyrase subunits *gyrA* and *gyrB* and can cause increased resistance to ciprofloxacin and moxifloxacin (Mac Aogain et al., 2015a).

In Ireland, CA-CDI accounts for approximately 30% of cases nationally each year, and since CA-CDI patients do not clearly have the traditional risk factors that are associated with CDI, production animals such as pigs have been identified as a possible reservoir (Knight et al., 2019). In addition, as shown in previous chapters, genetically indistinguishable isolates of *C. difficile* between human and pigs indicates that CDI could be a zoonotic infection.

Pig farms are the highest consumer of veterinary antimicrobials in Ireland, and tetracycline is one of the most used antimicrobials used in agriculture, particularly in medicated feed (O'Neill et al., 2020). Tetracycline resistance determinants are often found in *C. difficile* genomes and are particularly common in ST11 genomes (Dingle et al., 2019). Similar to the outbreaks of CDI associated with RT017 and RT027, as discussed above, selective pressure from tetracycline use appears to be an important driver of *C. difficile* ST11 infections in humans (Sholeh et al., 2020). Macrolides and penicillins are also used in medicated feed for pigs, but they are the most common antimicrobial classes that are administered by injection (O'Neill et al., 2020). This study by O'Neill et al. (2020) was performed between 2017 and 2018, and since then these antimicrobials have been listed as the highest priority critically important antimicrobials for human medicine by the WHO (WHO, 2019). Additionally, the EU brought in legislation in 2022 restricting the use of these critically important antimicrobials for humans in the veterinary sector (EU, 2022).

Multidrug resistance (MDR) in bacterial isolates is defined as resistance to three or more antimicrobial classes (Knight et al., 2019). Studies involving production animals, meat products and vegetables have reported *C. difficile* resistance to multiple groups of antibiotics (Marcos et al., 2023, Pirs et al., 2013, Thitaram et al., 2016).

This study aims to determine the susceptibility profiles of *C. difficile* ST11 isolates cultured from pig and human samples, as detailed in Chapter 2. In addition, the genome of these isolates will be investigated to elucidate genotypic resistance.

5.2 Methods

5.2.1 Phenotypic Antimicrobial susceptibility testing (AST)

5.2.1.1 Selection of isolates for AST

With the purpose of performing a comparison between phenotypic and genotypic AMR results, 55 ST11 *C. difficile* isolates that had been subjected to WGS in Chapter 3 were selected for AST. There were 28 pig *C. difficile* isolates, and 27 human *C. difficile* isolates included in the study. The pig isolates were categorised by farm antibiotic usage: 14 isolates were from LAU farms, 1 isolate was from a MAU farm and 13 isolates were from HAU farms.

Furthermore, the 28 pig isolates were split across different farms and stages of the pig, as shown in Table 5.1. There were no ST11 isolates identified towards the older stage of the pig, as described in Chapter 2, which is why isolates were predominantly from the sow and piglet stage. At the time that isolates were being selected, 27 human *C. difficile* isolates had been sequenced, so these were all chosen for AST.

Table 5.1 The number of isolates chosen for AST from each stage of pig and farm

Stage of Pig	Farm 1	Farm 2	Farm 3	Farm 4	Farm 6	Total
Sow	3	3	3	4	1	14
Piglet	2	2	3	3	-	10
Weaner 1	1	1	-	-	-	2
Weaner 2	-	2	-	-	-	2
Finisher	-	-	-	-	-	0
Slaughter	-	-	-	-	-	0
Total	6	8	6	7	1	28

5.2.1.2 In vitro susceptibility tests using Sensititre™ plates

Susceptibility to penicillin, imipenem, cefoxitin, clindamycin, metronidazole, tetracycline, moxifloxacin, and vancomycin was determined using the ANAERO3 Sensititre™ plates (Thermo Scientific™, UK) according to the manufacturer's instructions and Pirs et al. (2013). These plates contained two-fold serial dilutions of the antibiotics (Table 5.2). *C. difficile* isolates were cultured on Columbia blood agar plates at 37°C for

48 hours in anaerobic conditions. *C. difficile* isolates were added to cation-adjusted Mueller Hinton Broth (MHB) with TES (Thermo Scientific, UK) and the turbidity was read using a DensiCHEK™ Plus Nephelometer (Biomerieux, Basingstoke, UK). Isolates were suspended in the broth to reach a turbidity standard of 0.5 McFarland. Inoculated MHB with TES was prepared within 10 minutes of removal of *C. difficile* isolates from anaerobic conditions. An aliquot of 100 µl was transferred to a tube of 11 ml of Brucella broth (Thermo Scientific™, UK) to reach a concentration of $1x10^6$ CFU/ml, and the tube was inverted 8-10 times to ensure a homogenous solution.

The inoculated Brucella broth was poured into a sterile reagent reservoir and a multichannel pipette (Pipetman Neo™ Multichannel Pipette, Gilson™) was used to add 100 µl to each well of the 96-well ANAERO3 Sensititre plates, taking care not to touch the pipette tip to the bottom of the wells. A well of broth with no antibiotic or bacteria was used as a negative control. Strains *C. difficile* NCTC 13366 (PCR RT027, fluoroquinolone resistant strain) and *Bacteroides fragilis* ATCC 25285 (vancomycin resistant) were included for internal control of the procedure. The final inoculum was inoculated into the sensititre plates within 10 minutes of preparation. The wells were covered with a perforated seal, which are supplied with the ANAERO3 sensititre plates. Plates were incubated in stacks of no larger than three plates, at 37°C for 48 hours in anaerobic conditions using the Whitley A25 anaerobic workstation in the main microbiology laboratory at St James's Hospital (SJH).

After 48 hours, the MIC breakpoints were read manually by observing *C. difficile* growth in the bottom of the well using a Thermo Scientific™ Sensititre™ Manual Viewbox and values were noted.

5.2.1.3 Etest®

Susceptibility to rifampicin and ceftriaxone was carried out using Etest® (bioMérieux, France) as the ANAERO3 Sensititre™ plates did not contain those two antibiotics. *C. difficile* isolates were cultured on Columbia blood agar plates at 37°C for 48 hours in anaerobic conditions using the Whitley A25 anaerobic workstation in the main microbiology laboratory at St James's Hospital (SJH). Etests® were removed from the freezer at least 30 minutes before required and Fastidious Anaerobic agar (FAA) plates were dried in the incubator for 15 minutes at 15°C. *C. difficile* isolates were suspended

in saline (0.9%) to reach a turbidity standard of 1.0 McFarland, which was measured using a DensiCHEK™ Plus Nephelometer (Biomerieux, Basingstoke, UK). A sterile cotton swab was soaked in the inoculum and excess fluid was removed by rotating the swab several times against the side of the tube. The swab was lawned over the entire surface of the FAA plate (Figure 5.1).

The plates were allowed sufficient time to dry before applying the Etest® strips. The Etest® package was opened by peeling back the film, and a forceps was used to apply the Etest® strip to the inoculated agar surface. Plates were incubated in stacks of no larger than five plates, at 37°C for 48 hours in anaerobic conditions.

After 48 hours, sensitivity breakpoints were read manually. The MIC value was read where the edge of the ellipse of no *C. difficile* growth intersects the side of the Etest® strip, and values were noted. For an accurate reading, it was important to ensure that the inoculum was the appropriate density, achieving semi-confluent growth on the agar plate after 48 hours, and to ensure that there was a strict edge on the zone of inhibition.

Table 5.2 Layout of the ANAERO3 Sensititre™ 96-well plate used for AST, containing serial dilutions of antibiotics.

The plate contained 14 antimicrobial agents: penicillin (PEN), amoxicillin (AMOX), amoxicillin/clavulanic acid 2:1 ratio (AUG2), piperacillin/tazobactam 4/0.5 (P/T4), cefoxitin (FOX), imipenem (IMI), chloramphenicol (CHL), erythromycin (ERY), clindamycin (CLI), metronidazole (MRD), moxifloxacin (MXF), tetracycline (TET), vancomycin (VAN). Positive and negative controls (POS CON; NEG CON) was also included. The concentration (mg/L) of the antibiotic in the well is given underneath the antibiotic name.

	1	2	3	4	5	6	7	8	9	10	11	12
A	PEN 8	AMOX 32	AUG2 32/16	P/T4 128/4	FOX 64	IMI 8	IMI 128	ERY 128	CLI 64	MRD 32	MXF 8	TET 16
B	PEN 4	AMOX 16	AUG2 16/8	P/T4 64/4	FOX 32	IMI 4	IMI 64	ERY 64	CLI 32	MRD 16	MXF 4	TET 8
C	PEN 2	AMOX 8	AUG2 8/4	P/T4 32/4	FOX 16	IMI 2	IMI 32	ERY 32	CLI 16	MRD 8	MXF 2	TET 4
D	PEN 1	AMOX 4	AUG2 4/2	P/T4 16/4	FOX 8	IMI 1	IMI 16	ERY 16	CLLI 8	MRD 4	MXF 1	TET 2
E	PEN 0.5	AMOX 2	AUG2 2/1	PIP 128	FOX 4	IMI 0.5	CHL 16	ERY 8	CLI 4	MRD 2	MXF 0.5	VAN 8
F	PEN 0.25	AMOX 1	AUG2 1/0.5	PIP 64	FOX 2	IMI 0.25	CHL 8	ERY 4	CLI 2	MRD 16	MXF 0.25	VAN 4
G	PEN 0.12	AMOX 0.5	AUG2 0.5/0.25	PIP 32	FOX 16	IMI 0.12	CHL 4	ERY 2	CLI 16	MRD 0.5	MXF 0.12	VAN 2

H	PEN 0.06	AMOX 0.25	AUG2 0.25/ 0.12	PIP 16	FOX 0.5	IMI 0.06	CHL 2	ERY 1	CLI 0.5	POS CON	POS CON	NEG CON

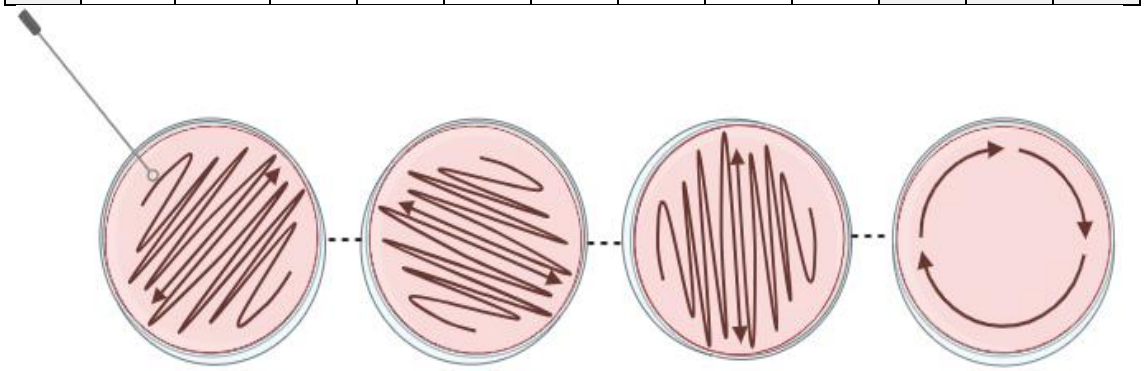


Figure 5.1 Streaking method for agar plates during Etest.
The swab was streaked over the entire surface of the agar plate. The plate was rotated approximately 60° and streaked again. This was repeated once more. Inoculation was completed by running the swab around the circumference of the agar plate.

5.2.1.4 Interpretation of Minimum Inhibitory Concentrations (MIC) and Breakpoints

The MIC was recorded as the lowest concentration of antimicrobial agent for which there was no visible growth for both methods of AST (Figure 5.2 for the ANAERO3 Sensititre™ 96-well plate and Figure 5.3 for the Etest® strips) (Baquer et al., 2021). The breakpoints (the concentration of antibiotic at which a bacteria in a patient is likely to be susceptible to treatment with an antibiotic) were interpreted following the guidelines by the European Committee on Antimicrobial Susceptibility Testing (EUCAST), or Clinical and Laboratory Standards Institute (CLSI) breakpoints, if EUCAST breakpoints were not available (EUCAST, 2024, CLSI, 2020, Mitka, 2012). If CLSI and EUCAST breakpoints were not available then MICs were considered based on the breakpoints proposed by Freeman (2021). The reported MIC breakpoints interpretive criteria for each of the antibiotics that were subject to phenotypic AST as per these guidelines are listed in Table 5.3. Isolates were categorised as susceptible (S), intermediate (I) or resistant (R), based on EUCAST recommendations (EUCAST, 2024). Isolates were categorised as having intermediate susceptibility if the MIC was equal to the recommended clinical MIC breakpoint (mg/L).

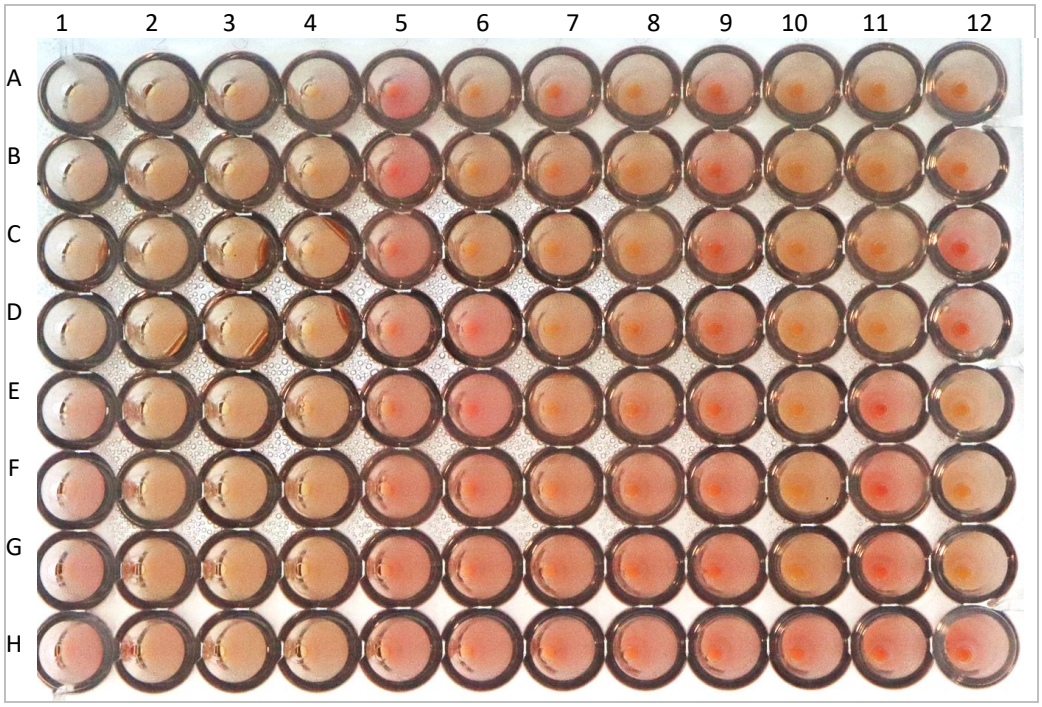


Figure 5.2 *In vitro* antibiotic susceptibility testing using the ANAERO3 Sensititre™ 96-well plate (layout of which is detailed in Table 5.2) to establish the MIC.
The lighter orange colour is indicative of no *C. difficile* growth, and the red colour indicates *C. difficile* growth, which is confirmed with growth in the bottom of the well, manually using a mirror.

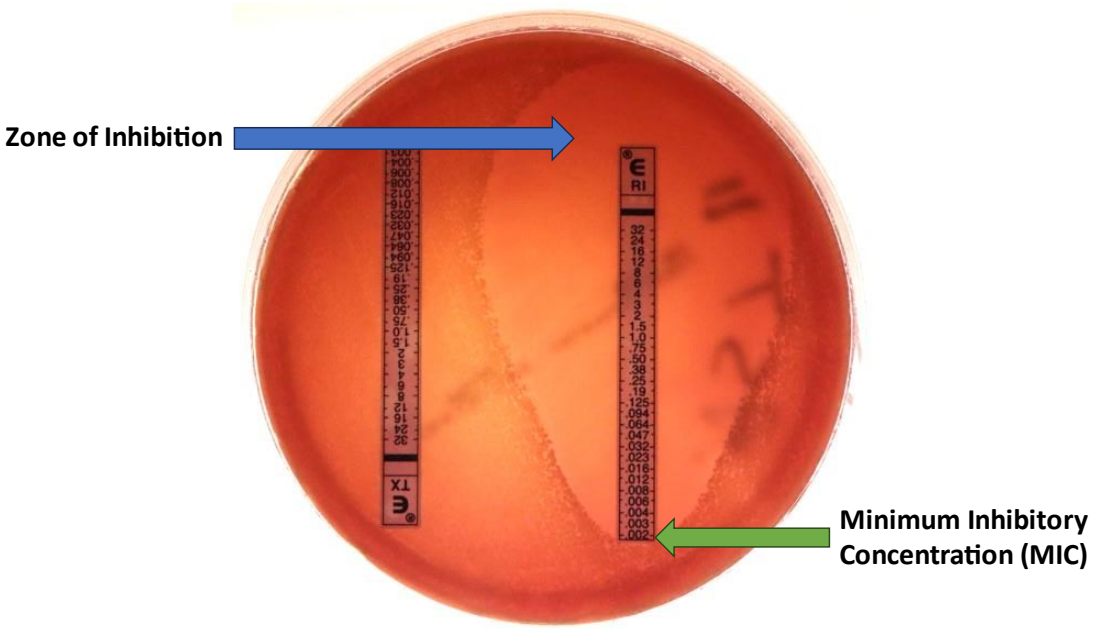


Figure 5.3 *In vitro* antibiotic susceptibility testing using the Etest® for Rifampicin and Ceftriaxone to establish the MIC.
The MIC value was read where the edge of the ellipse of no *C. difficile* growth intersects the side of the Etest® strip.

Table 5.3 Antimicrobial agents and their associated MIC breakpoint interpretive criteria. This is used to classify *C. difficile* isolates into categories as susceptible (S), intermediate (I), and resistant (R).

	MIC Breakpoint Interpretive Criteria (mg/L)		
Antimicrobial agent	**S**	**I**	**R**
Vancomycin[a]	≤2	-	>2
Metronidazole[a]	≤2	-	>2
Rifampicin[c]	≤0.004	0.008–16	≥16
Moxifloxacin[b]	≤2	4	≥8
Clindamycin[b]	≤2	4	≥8
Imipenem[c]	≤4	8	≥16
Piperacillin/Tazobactam[b]	≤32/4	64/4	≥128/4
Ceftriaxone[a]	≤16	32	≥64
Amoxicillin[c]	≤2	4	≥8
Tetracycline[b]	≤0.5	8	≥16
Erythromycin[c]	≤0.25	0.5	≥2
Chloramphenicol[c]	≤8	16	≥32
Cefoxitin[b]	-	-	≥64
Co-amoxiclav[b]	≤4/2	8/4	≥16/8

[a]Breakpoints for vancomycin, metronidazole and ceftriaxone were determined as per EUCAST V14.0 guideline (metronidazole, vancomycin) (EUCAST, 2024)

[b]Breakpoints according to the CLSI M100 ED32 guideline (imipenem, cefoxitin, clindamycin, tetracycline, moxifloxacin) were used for moxifloxacin, clindamycin, piperacillin/tazobactam 4/0.5, tetracycline, cefoxitin and co-amoxiclav (CLSI, 2020).

[c]Breakpoints as recommended by Freeman (2021) were used for rifampicin, imipenem, amoxicillin, erythromycin and chloramphenicol.

5.2.2 Genotypic antimicrobial resistance

5.2.2.1 Collection of sequence data for *C. difficile* ST11 isolates

The genomes of the 55 *C. difficile* isolates, that were selected for *in vitro* phenotypic AST testing, were screened *in silico* for acquired and intrinsic resistance determinants. The correlation between genotypic antimicrobial resistance determinants and phenotypic resistance was investigated by comparing both results for the 55 isolates.

5.2.2.2 Screening for antimicrobial resistance markers

Genome assemblies of *C. difficile* were screened for AMR markers using the National Center for Biotechnology Information (NCBI) AMRFinderPlus software tool version 3.11.2 on Ridom SeqSphere+, which finds AMR-specific genes and proteins based on the NCBI Bacterial Antimicrobial Resistance Reference Gene Database (Bio Project PRJNA313047) (Feldgarden et al., 2019).

5.3 Results

5.3.1 Antimicrobial resistance (AMR)

5.3.1.1 Phenotypic analysis

The antimicrobial susceptibility test results of 55 ST11 *C. difficile* isolates are listed in Table 5.4, and the respective MICs of all 55 ST11 isolates are shown in Figure 5.4. All the *C. difficile* isolates in this study were susceptible to metronidazole, vancomycin, piperacillin, piperacillin/tazobactam, amoxicillin, and co-amoxiclav. Susceptibility to imipenem was found for 54 *C. difficile* isolates, and 2 isolates had intermediate susceptibility. Resistance to rifampicin, moxifloxacin, tetracycline, clindamycin, and erythromycin were seen in 1/55 (2%), 37/55 (67%), 22/55 (39%), 27/55 (48%), and 32/55 (57%) of *C. difficile* isolates, respectively. All isolates were resistant to cefoxitin and ceftriaxone. *C. difficile* susceptibility to vancomycin and metronidazole was confirmed for all isolates by performing a second test using Etest strips®.

Antimicrobial susceptibility testing results, showing susceptible, intermediate, and resistant pig and human *C. difficile* isolates with breakpoints for tetracycline, erythromycin, moxifloxacin, and clindamycin, are shown in Figure 5.5. There was no difference between antibiotic usage on pig farms and the level of antibiotic resistance. Tetracycline resistance was found in 12 pig and 14 human *C. difficile* isolates. Erythromycin resistance was found in 21 pig and 10 human *C. difficile* isolates. Moxifloxacin resistance was found in 21 pig and 15 human *C. difficile* isolates. Clindamycin resistance was found in 7 pig and 15 human *C. difficile* isolates.

Phenotypic resistance to at least one antimicrobial compound in this study was noted in 47 out of 55 *C. difficile* isolates (85%). Phenotypic MDR was observed in 38 (69%) of the 55 *C. difficile* isolates studied. The most common combination of MDR was erythromycin, moxifloxacin, and tetracycline.

5.3.1.2 Genotypic analysis

SNPs and genes that were found in insertions or deletions in the genomes confer genotypic resistance, which can be a valuable tool to predict phenotypic resistance against clinically relevant antibiotics. For the 55 *C. difficile* isolates included in this study, WGS analysis revealed SNPs that are commonly associated with AMR, and which are

detailed in Supplementary Table S3. The resistance determinants that were present in the 55 genomes are detailed in Table 5.5 and Table 5.6 (Dingle et al., 2019, Wickramage et al., 2021). There were no genes encoding for vancomycin or metronidazole resistance identified by the AMRFinderPlus tool on the Seqsphere+ software in any of the genomes explored in this study.

Table 5.4 Antimicrobial susceptibility of 55 ST11 isolates tested

Isolates categorised as susceptible (S), intermediate (I) or resistant (R), based on the MIC breakpoint interpretive criteria provided in Table 5.3.

Antimicrobial Compound	Antimicrobial susceptibility		
	S % (n)	I % (n)	R % (n)
Vancomycin[a]	100% (55)	0	0
Metronidazole[a]	100% (55)	0	0
Rifampicin[c]	98% (54)	0	2% (1)
Moxifloxacin[b]	33% (18)	2% (1)	65% (36)
Clindamycin[b]	27% (15)	33% (18)	40% (22)
Imipenem[c]	100% (55)	0	0
Piperacillin/Tazobactam[b]	100% (55)	0	0
Ceftriaxone[a]	0	100% (55) *	0
Amoxicillin[c]	100% (55)	0	0
Tetracycline[b]	11% (6)	40% (22)	49% (27)
Erythromycin[c]	44% (24)	0	56% (31)
Chloramphenicol	98% (54)	2% (1) †	
Cefoxitin[b]	0	0	100% (55)
Co-amoxiclav[b]	100% (55)	0	0

Breakpoints were taken from aEUCAST (EUCAST, 2024), bCLSI (CLSI, 2020), and c Freeman (2021)

*The highest concentration for ceftriaxone on the Etest® was 32 mg/L.

†The highest concentration of chloramphenicol in the Sensititre™ plates was 16 mg/L.

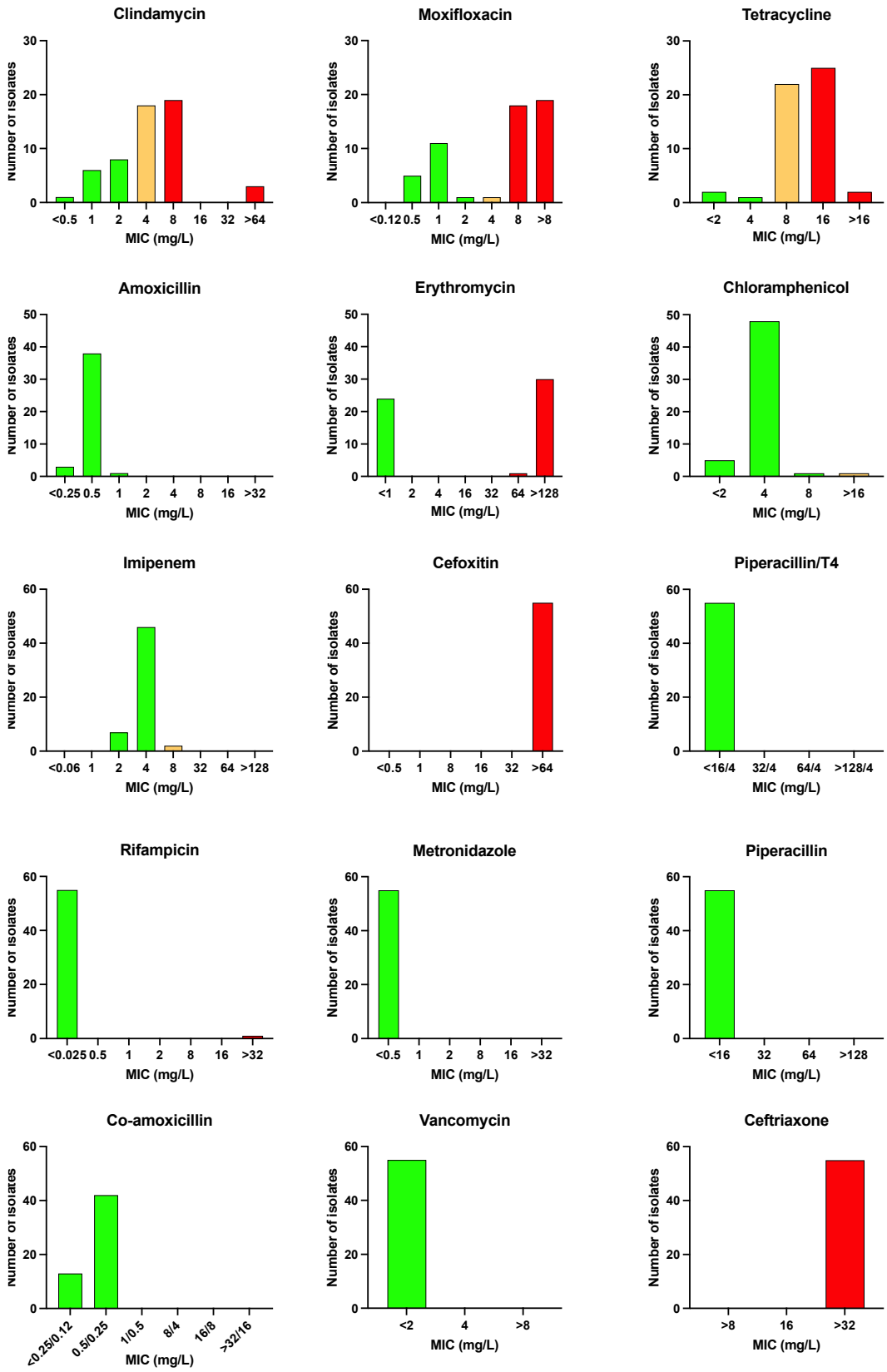


Figure 5.4 *In vitro* antimicrobial susceptibility test results showing MICs for 15 antimicrobial agents against 55 ST11 *C. difficile* isolates.

Breakpoints are as shown in Table 5.3 and are used to highlight isolates as susceptible (green bars), intermediate (yellow bars) and resistant (red bars).

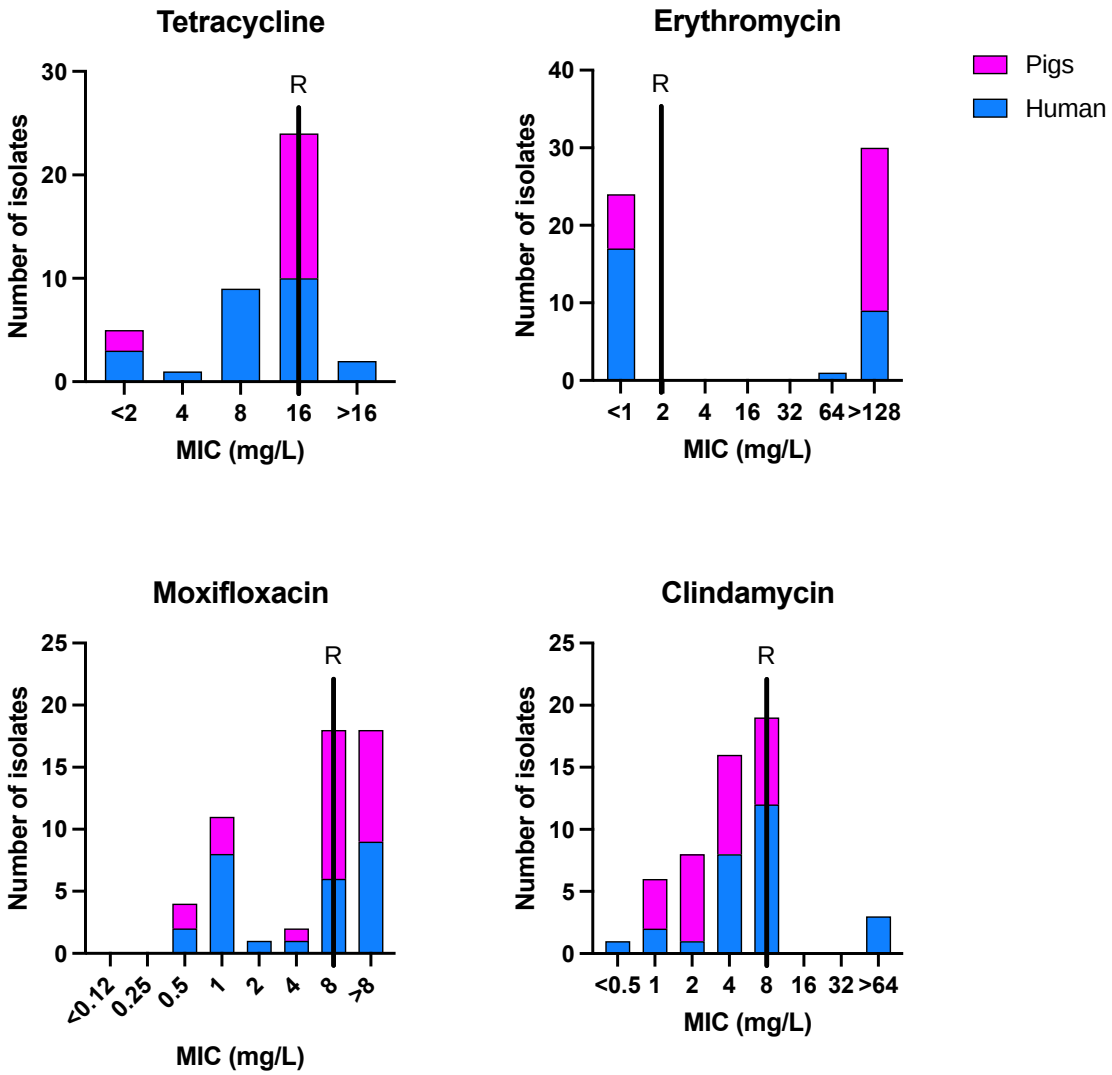


Figure 5.5 The MIC distributions of tetracycline, erythromycin, moxifloxacin, and clindamycin against 55 pig and human *C. difficile* isolates.

The MIC breakpoint of resistance is marked on the graph with an "R", therefore any isolates with an MIC higher than the breakpoint are also resistant to the antibiotic. The bars are coloured according to the source of *C. difficile* isolate, as indicated in the legend on the right – pig isolates are shown in pink and human isolates are shown in blue.

Table 5.5 AMR accessory genes and the prevalence of these genes found in 55 ST11 *C. difficile* genomes of pig and human origin.

Accessory gene	Mechanism	No. of *C. difficile* strains (%)	Predicted AMR phenotype
tetM	Ribosomal protection	52/55 (94%)	Tetracycline
tet40	Efflux pump	22/55 (40%)	Tetracycline
tet44	Ribosomal protection	3/55 (5.4%)	Tetracycline
ermB	23S rRNA methyltransferase activity	3/55 (5.4%)	MLS_B
cfrB	23S rRNA methyltransferase activity	1/55 (1.8%)	MLS_B

MLS_B = Macrolide-Lincosamide-Streptogramin B antibiotics.

Table 5.6 Amino acid substitutions that confer AMR in *C. difficile*, and the prevalence of amino acid substitutions found in 55 ST11 *C. difficile* genomes of pig and human origin.

Amino acid substitutions	Nonsynonymous substitution	No. of *C. difficile* isolates (%)	Protein modified	Predicted AMR phenotype
gyrA	Thr(82)Ile	35/55 (63%)	DNA gyrase subunit A	Fluoroquinolones
gyrB	Ser(366)Val	54/55 (98%)	DNA gyrase subunit B	Fluoroquinolones
gyrB	Ser(416)Ala	54/55 (19%)	DNA gyrase subunit B	Fluoroquinolones
gyrB	Ile(139)Arg	1/55 (1.8%)	DNA gyrase subunit B	Fluoroquinolones
rpoB	Arg(505)Lys	1/55 (1.8%)	β subunit RNA polymerase	Rifampicin

5.3.2 *C. difficile* ST11 AMR repertoire

The concordance between genotypic and phenotypic antimicrobial resistance is shown in Figure 5.6. Genotypic resistance was able to predict phenotypic resistance for tetracycline moxifloxacin, chloramphenicol, and rifampicin, but was unable to predict phenotypic resistance to erythromycin and clindamycin, which are both in the class of Macrolide-Lincosamide-Streptogramin B (MLS_B) antibiotics.

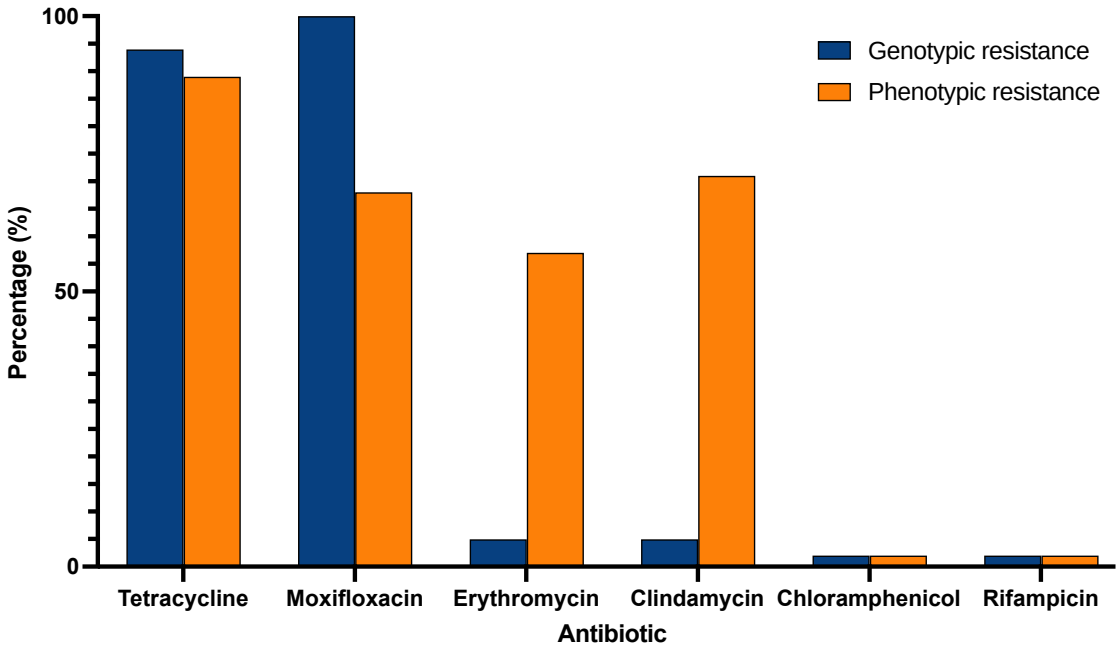


Figure 5.6 The concordance between genotypic and phenotypic resistance for 55 *C. difficile* isolates.
The genotypic resistance represents the percentage of isolates that possess genes that confer resistance to antibiotics. The phenotypic resistance represents the percentage of isolates that were resistant to antibiotics *in vitro*.

5.3.2.1 Tetracycline resistance

Susceptibility to tetracycline was measured using a EUCAST breakpoint of 16 mg/L; and 6 isolates were susceptible, 22 were intermediate and 27 isolates were resistant. One or more tetracycline resistance genes (*tetM, tet40, tet44*) were detected in 52/55 (94.5%) of the sequenced genomes. Genotypic tetracycline resistance correlated with phenotypic resistance in all strains. Tetracycline resistance gene *tetM* was detected in all 52 of these genomes, *tet40* was detected in 22 of these genomes and *tet44* was detected in 3 of these genomes.

5.3.2.2 Fluoroquinolone resistance

Using the EUCAST breakpoint of 4 mg/L for moxifloxacin; 18 isolates were susceptible, 1 was intermediate, and 36 were resistant. Mutations in genes that encode for DNA gyrase subunits (*gyrA* and *gyrB*) are commonly associated with resistance to the fluoroquinolone class of antibiotics, and all 55 (100%) of genomes possessed at least one mutation in either gyrA or gyrB. Mutations in *gyrA* were found in 35 genomes, while mutations in *gyrB* were found in all 55 genomes.

5.3.2.3 Macrolide-Lincosamide-Streptogramin B (MLS_B) resistance

The presence of *erm* genes is frequently implicated in resistance to MLS_b antibiotics (Freeman, 2021). There were 24 *C. difficile* isolates that exhibited susceptibility to erythromycin, and 31 isolates were resistant. Among the 31 isolates that were resistant to erythromycin, only 9% (3/31) carried an *erm* gene (*ermB*/*ermQ*). This indicates that *in silico* genotypic resistance for erythromycin is a poor predictor of phenotypic resistance (Ellington et al., 2017).

There were 15 *C. difficile* isolates that were susceptible to clindamycin, 18 isolates were intermediate, and 22 isolates were resistant. It was of note that out of the 22 *C. difficile* isolates that were resistant to clindamycin, 3 had highly resistant MICs (>64 mg/L), which was concordant with the 3 genomes that carried an *erm* gene.

5.3.2.4 Chloramphenicol resistance

Of the 55 isolates subjected to AST for chloramphenicol, 54 were sensitive. One isolate showed high levels of resistance (>16 mg/L). This correlated with the presence of a cfrB gene in the genome, which has also been found to play a role in conferring MLS_b antibiotic resistance (Spigaglia, 2016).

5.3.2.5 Rifampicin resistance

A mutation in the *rpoB* gene, which encodes for rifampicin resistance, was identified in 1/55 (1.8%) isolates (Figure 5.7). This *C. difficile* isolate possessed the R505K SNP (referred to as Arg(505)Lys in Table 5.6), which confers resistance to rifampicin and commonly leads to high levels of resistance (Banawas, 2018). Using the breakpoint of 0.004 mg/L for rifampicin, 54 isolates were susceptible and 1 was resistant. Genotypic

resistance showed concordance with phenotypic resistance to rifampicin *in vitro*, as the *C. difficile* isolate showed a highly resistant MIC to rifampicin (32 mg/L).

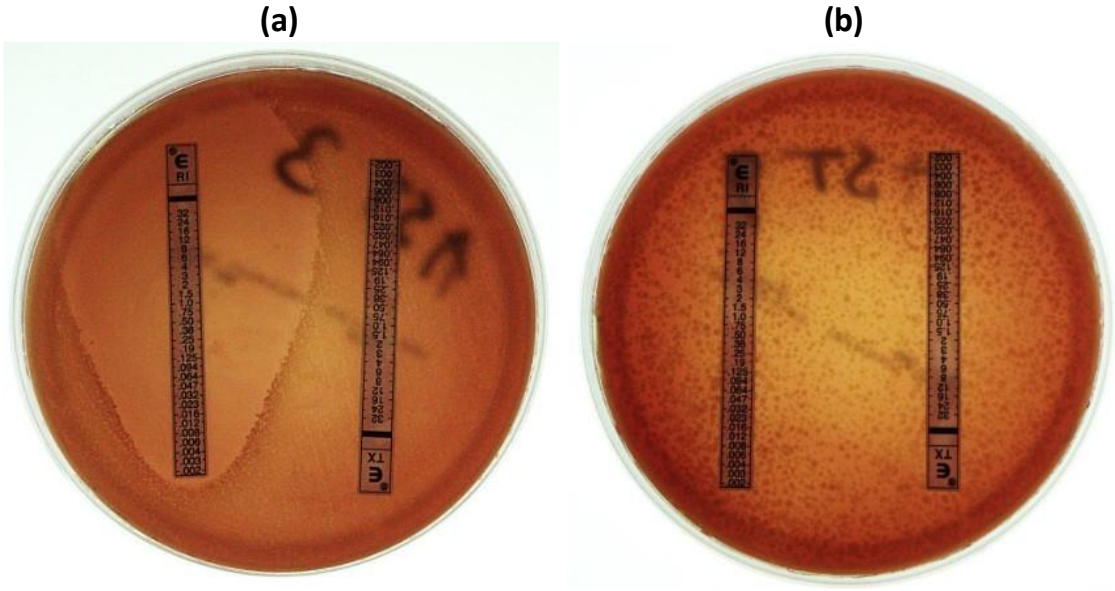


Figure 5.7 *In vitro* AST for two *C. difficile* isolate using Etest® (sample ID: AST9).
(a) Susceptibility to rifampicin (left) and resistance to ceftriaxone (right). (b) Resistance to rifampicin (left) and ceftriaxone (right).

5.4 Discussion

WGS of bacterial isolates facilitates the rapid prediction of an antimicrobial phenotype (Knight et al., 2019). In addition, WGS has a distinct advantage over conventional typing methodologies as it can locate regions on the genome that are suggestive of microbial adaptation and changes to the bacterial genome due to horizontal gene transfer and horizontal transfer of genes on mobile genetic elements (Wu et al., 2019).

C. difficile RT078/ST11 is one of the most frequently identified strains implicated in human CDI in Europe and North America (Kuijper et al., 2006, Rodriguez et al., 2016b). It is one of the most prevalent genotypes in Ireland, and was responsible for 16% of reported cases between 2021 and 2023 (HPSC, 2021, HSE-HPSC and NRL, 2023). In addition, RT078 was associated with 3 out of 51 severe cases of CDI in Ireland in 2021 (HPSC, 2021). RT078/ST11 is also the most common ribotype found in farm animals such as pigs, horses, and beef and dairy cattle (Kachrimanidou et al., 2019, Blasi et al., 2021). It was the most common ribotype found in pigs in the current study, as highlighted in Chapter 2, and in recent years has also become one of the most common ribotypes found in human CDI. Although *C. difficile* often forms part of the normal gut microbiota in both healthy pigs and humans, selective pressure from antimicrobial exposure has likely been the driving force behind *C. difficile* expansion in animals and humans (Dingle et al., 2019, Knight and Riley, 2019). This emphasises the "One Health" approach that should be taken to *C. difficile*, which recognises that human health is closely connected to animal health, and the environment (WHO, 2017).

Historically, intermediately susceptible isolates were classified as resistant, but in 2019 the interpretation of results changed to classify such isolates as having intermediate susceptibility (EUCAST). The same degree of clinical efficacy is obtained for antibiotics at susceptibility and intermediate susceptibility, but different dosing regimens are used (Kowalska-Krochmal and Dudek-Wicher, 2021). For susceptible isolates, a standard dosing regimen is maintained as normal, and therapeutic success is likely. For intermediate isolates, dosing regimens are adjusted or concentrated at the site of infection to help ensure full therapeutic success.

All the *C. difficile* isolates tested in this study, both from humans and pigs, show full susceptibility to amoxicillin, co-amoxiclav, metronidazole, and vancomycin. In general,

the use of ampicillin, amoxicillin, cephalosporins, clindamycin and fluoroquinolones in patients is associated with increased risk of developing CDI, as these broad-spectrum antibiotics disrupt the normal gut microbiota which allows for the proliferation of *C. difficile* (Miller et al., 2023). The treatment of CDI has recently changed, whereby metronidazole is no longer recommended for first-line treatment of CDI in Europe and North America, instead vancomycin is recommended as a first-line treatment. The main concern, from the viewpoint of current antibiotic therapy regimens for CDI in humans, is the emerging resistance to metronidazole and vancomycin (Pirs et al., 2013, Greentree et al., 2022). Our results provide some reassurance that the isolates included in this study were all susceptible to metronidazole and vancomycin *in vitro*, which are treatment choices for CDI. This is consistent with current literature as reports of *C. difficile* resistance to these antibiotics is still rare, however some reduced susceptibility in other ribotypes (RT027 and RT001) to vancomycin and metronidazole have been reported in a longitudinal surveillance study in Europe (Freeman et al., 2018).

Pigs are the largest consumer of veterinary antimicrobials in several countries, and this accounts for approximately 40% of total veterinary antimicrobial usage in Ireland (O'Neill et al., 2020). In the O'Neill et al. study, it was found that tetracyclines are the most used antimicrobials in Ireland by weight of active ingredient (55.81%), followed by potentiated sulphonamides (25.22%), penicillin (7.84%), and macrolides (7.84%), with 95.6% of all antimicrobial usage being classified as prophylactic. The disposal of excess pig manure generally involves spreading it on the farmer's land and neighbouring farms to reduce the need for chemical fertiliser (Nolan et al., 2012). Good quality pig manure can be used in Ireland as an organic fertiliser for grassland and tillage crops (such as barley, wheat, and oats), and is beneficial to farmers as it can significantly reduce fertiliser costs (McCutcheon and Quinn, 2020, Martin, 2007). As a result, it was not surprising to find tetracycline and erythromycin resistance in *C. difficile* isolates from pig farms. Tetracycline resistance was detected in 27/55 (48%) of *C. difficile* isolates. Previous work from our group found tetracycline resistance in 65/163 (40%) of *C. difficile* isolates from pig and humans (Mac Aogáin, unpublished data). The increase in tetracycline resistance has been noted in several studies in recent years (Knight et al., 2019). This may be because of the prolonged usage of tetracyclines both as growth

promoters and prophylactically in animals, which were used on one HAU farm in this study, as highlighted in section 2.2.2.1.1. However, there was no difference between the antimicrobial susceptibility profiles of *C. difficile* isolates on LAU and HAU farms, which is similar to what has been found by Susick et al. (2012) and Mencia-Ares et al. (2020). Therefore, acquisition of resistance may occur on all farms and is likely driven by antimicrobial selective pressure between farming facilities and the environment (Dingle et al., 2019).

Resistance to the MLS_B antibiotics, clindamycin, and erythromycin, was demonstrated in 22/55 (39%) and 32/55 (57%) of isolates, respectively. The antibiotic clindamycin is significantly associated with the highest risk of developing CDI in community settings (Brown et al., 2013). Resistance to clindamycin was observed in 22/55 (39%) of *C. difficile* isolates, which is similar to studies in Europe (Barbut et al., 2007, Spigaglia et al., 2011, Sholeh et al., 2020). Even though clindamycin is not approved for use in food-producing animals, resistance to the antibiotic has been observed in several studies on *C. difficile* strains of animal origin (Thitaram et al., 2016, Keessen et al., 2013b). Resistance may occur as a consequence of widespread MLS_B antibiotic use in animals, for example tylosin and erythromycin (Licciardi et al., 2021).

Fluoroquinolone resistance in Europe has been associated with outbreaks of CDI in hospitals. An early study on RT078 found that prior exposure to fluoroquinolone antibiotics, particularly ciprofloxacin, was particularly associated with CDI caused by this genotype (Goorhuis et al., 2008, Pirs et al., 2013). Goorhuis et al. (2008) reported high levels of moxifloxacin susceptibility (88%) in the Netherlands, however decreasing susceptibility to moxifloxacin has been reported in recent years (Krutova et al., 2020, Freeman et al., 2015). Moxifloxacin resistance was observed in 36/55 (64%) of *C. difficile* isolates in this study. This phenomenon was observed in the early 2000s with the hypervirulent *C. difficile* RT027/B1/NAP1, where this strain acquired fluroquinolone resistance and caused outbreaks in northern Europe and North America (Donskey, 2017b). Resistance to fluoroquinolones, such as moxifloxacin, could have developed as a result of extensive use of members of this drug class, especially ciprofloxacin, in hospitals across the world (Mac Aogain et al., 2015a). The World Health Organisation (WHO) classifies quinolones as the highest priority critically important antimicrobials, as

quinolones treats infections where there are limited antimicrobial therapy options available (WHO, 2019).

The cfrB gene was identified in one clinical isolate, which confers resistance to lincosamides (e.g. clindamycin), oxazolidinones (e.g. linezolid), phenicols (e.g. thiamphenicol), pleuromutilins (e.g. tiamulin), and streptogramin A antibiotics (e.g., virginiamycin M1) (Long et al., 2006, Chatedaki et al., 2019). Linezolid resistance in gram-positive bacteria is uncommon but this gene has sporadically been reported in *Clostridioides difficile* and are typically found on mobile genetic elements (Marin et al., 2015, Stojkovic et al., 2019). This is worrying as although linezolid is not used to treat CDI, linezolid treatment of patients with ventilator-associated pneumonia can protect against the development of CDI (Stojkovic et al., 2019). The detection of the *cfrB* gene also highlights the important of genotypic screening of AMR, as it can direct effective antimicrobial stewardship and infection control programmes.

There are concerns about fidaxomicin resistance, and, although rarely reported, resistance to fidaxomicin may emerge *in vivo* after a single course of treatment (Marchandin et al., 2023, Sholeh et al., 2020). SNPs in *rpoB* have been suggested as a possible cause for reduced fidaxomicin susceptibility, as fidaxomicin acts by inhibiting bacterial RNA synthesis (Zhanel et al., 2015). A study by Kuehne et al. (2018) found that a T3428G mutation in the *rpoB* gene of *C. difficile* was associated with reduced fitness *in vitro* and decreased virulence *in vivo*. The cited study found that fidaxomicin resistance arose in isolates that were cultured from patients with recurrences of CDI. At the time of the current study, it was not possible to investigate *C. difficile* isolates for susceptibility to fidaxomicin. However, it would be interesting to look at *C. difficile* isolates for fidaxomicin susceptibility, especially in the isolate from the current study that possesses the *rpoB* SNP.

In this study, AST was performed using broth microdilution method for expediency reasons, but this method is not recommended due to the differences in MICs in comparison to those yielded by agar dilution (Krutova et al., 2022b). Additionally, MICs can differ based on *C. difficile* RT, with higher MICs being found in epidemic RTs such as RT027 (Jon et al., 2021). Additionally, recommendations of AST differ between guidelines from EUCAST and CLSI, with different recommendations on the testing

procedure and the interpretation criteria that should be used (EUCAST, 2024, CLSI, 2020, Mitka, 2012). Many of the methods that are currently in use are adapted from other anaerobic bacteria, which may not be suitable for *C. difficile* (Jon et al., 2021). EUCAST standards are widely used in Europe, whereas CLSI breakpoints are more commonly adopted in North America, which can lead to differences in treatment decisions across these regions. Collaboration across the world is required to reach a standardised testing method, however this is challenging due to differing healthcare system structures and policies.

Another limitation of this study is the use of the NCBI AMRFinderPlus tool on the Seqsphere+ software, which investigates target regions in the core genome. *C. difficile* resistance is often carried on mobile genetic elements and plasmids, and genotypic resistance that is not carried in these regions may not be identified, for example plasmid-borne genes which are typically part of the accessory genome (Lehtinen et al., 2021). Additionally, AMRFinderPlus relies on a curated database of known resistance genes that are well characterised. Therefore, if resistance genes or mechanisms are not included in the database, or underrepresented in *C. difficile*, they may not be detected. Other tools, such as SNP-calling algorithms, may be more appropriate to detect specific mechanisms of resistance, such as mutations in NimB, or the plasmid pCD-METRO.

5.5 Conclusions

The treatment of *C. difficile* infection is challenging due to the inadequacy of treatment and, given the high rate of recurrence (8-10% in Ireland), the search for more appropriate and effective ways of treating CDI has intensified (HSE-HPSC and NRL, 2023).

The current work demonstrates the potential to use WGS as a complementary tool to phenotypic *in vitro* antimicrobial resistance testing. However, the lack of concordance between genotypic and phenotypic determinations of isolate resistance against the two MLS_B antibiotics investigated in the current study (erythromycin and clindamycin) demonstrates that the potential for WGS to be used as a predictor of antibiotic resistance, and a possible surrogate for phenotypic resistance testing, still requires further study and elucidation, as it could become an invaluable tool to combat AMR.

6 General Discussion

Chapter 6

6.1 Introduction

C. difficile causes a serious, toxin-mediated intestinal infection in humans, and continues to be the leading cause of healthcare associated diarrhoea (Depestel and Aronoff, 2013, McFarland et al., 2016). Since the emergence of *C. difficile* as an antibiotic- and healthcare-associated intestinal infection, the CDC has listed the bacterium as an urgent antibiotic resistance threat, with "urgent" being the highest level of concern to human health (CDC, 2019). *C. difficile* spores can persist in environments unfavourable to the bacterium, and cause outbreaks of infection in susceptible hosts, placing a financial and operational burden on hospitals and other healthcare facilities.

C. difficile RT078/ST11 is a prominent and emerging strain associated with high virulence and severe infections, and the possibility of a zoonotic origin, especially from pigs and calves or their respective environments, has been suggested. (Goorhuis et al., 2008, Bauer et al., 2011a). Evidence from an Australian study indicates high clonality between pig, human and environmental RT078/ST11 *C. difficile* isolate across different states, countries, and continents, raising concerns about *C. difficile* entering the food chain through contaminated foodstuffs (Knight et al., 2019). The overall aim of this project was to gain a deeper understanding into the prevalence, epidemiology, and antibiotic susceptibility of certain ribotypes of *C. difficile* that were shared by farm animals and humans. *C. difficile* was investigated in this study using a combination of PCR ribotyping, WGS and phenotypic assays, and has provided new insights into the broader *C. difficile* epidemiology in Ireland.

Historically, most CDI cases have been hospital- or other healthcare-associated, often linked to prior antibiotic therapy and assumed to have been acquired from the healthcare environment, including other patients. Recent evidence suggests a change, whereby a significant proportion of patients carry their own unique(endogenous) strain that overgrows the normal intestinal microbiota after antibiotic exposure, or other triggers such as proton pump inhibitors (PPIs). The inference is that these patients were asymptomatically colonized by *C. difficile* prior to their hospital admission.

CDI has become a “One Health” issue as *C. difficile* can also colonise or cause infections in animals (Weese, 2020). Pigs are considered a significant reservoir of *C. difficile* and have been specifically studied due to their potential role in the transmission of the bacterium to humans. (Hensgens et al., 2012).

6.2 *C. difficile* in pigs and humans in Ireland

A longitudinal study on pig farms in Ireland was conducted to determine the prevalence of *C. difficile* in pigs, and to determine on-farm shedding dynamics. Prevalence studies on *C. difficile* in farmed animals internationally, including studies in Ireland, have primarily focused on pigs (Hawken et al., 2013, Keessen et al., 2013a, Stein et al., 2017, Spigaglia et al., 2023). Toxigenic *C. difficile* has been detected in association with typhlocolitis, particularly in piglets, but has also been isolated in clinically healthy animals (McElroy et al., 2015). In this study, *C. difficile* was isolated from faecal samples from all 6 farms tested, which represented approximately 9% of the commercial pig farms in Ireland. This suggests that *C. difficile* colonisation is widespread and ubiquitous in Irish pig farms.

The prevalence of *C. difficile* is generally much higher in farmed pigs compared to other farmed animal species, though the reasons for this disparity are not fully understood. Commercial pig production units, known for their intensity, provide more opportunities for pathogen transfer, potentially influencing higher prevalence. In this study, overall, a high prevalence was observed in the first week of life (piglets; 87.3%) but this rate decreased to 23% in the eighth week of life (weaning). This prevalence is similar to what has been found in previous studies in pigs from birth to finisher stage/at slaughter, revealing a marked difference in *C. difficile* prevalence as the animal grows from birth to slaughter (Hawken et al., 2013, Susick et al., 2012). It is not fully clear why piglets display higher levels of colonisation with *C. difficile*, but previous studies have shown that vertical transmission (from sow to piglet) does not occur (Hopman et al., 2011a). Susick et al. (2012) found high levels of contamination in the piggery environment of piglets, which indicates that the environment could be a potential reservoir for *C. difficile* acquisition for piglets. Notably, the overall prevalence of *C. difficile* increases towards the finishing stage (approximately 21 weeks old) to 38%. One possible explanation for this increase could be the stress on the pigs of transfer to the finishing stage.

Previous studies on antibiotic use on pig farms as a risk factor for *C. difficile* colonisation have not conclusively established an association (Susick et al., 2012, Keessen et al., 2011). However, in this study there was a significant difference ($P < 0.0001$) in *C. difficile* prevalence between HAU farms (35.33%) and LAU farms (61.89%). This finding was unexpected, as none of the antibiotics that were administered to pigs would have been expected to inhibit *C. difficile*, but antibiotic administration could affect the morphological and functional development of the pig's intestine after the weaning stage, as discussed section 2.4. A potential reason for the difference between this study and the cited study by Susick et. al., 2012 is that pigs were sampled at four different timepoints, in comparison to our study which sampled pigs at six different time points. In addition, the cited study found a lower overall prevalence (180/1650 (11%) on commercial pig farms), which could be because of biosecurity differences on the farms, or variations in sampling approaches or isolation procedures by the investigators. The current study contributes to the understanding of the dynamics of *C. difficile* transmission in a pig farming environment, particularly on the RT078 strain.

Between 2018 and 2020, 350 human *C. difficile* isolates were obtained from faecal samples of patients with suspected or subsequently proven CDI. Most of the isolates were from inpatients but it is expected some were outpatients. This is similar to national data reported by the HPSC, whereby the most frequent ribotypes in 2020 were 078 (15%), 014 (14%), and 002 (14%) (HPSC, 2021). Of note, only circa 20% of the total CDI cases in Ireland were ribotyped between 2018 and 2020. The lack of a national reference laboratory at that time meant that microbiology laboratories referred *C. difficile* isolates abroad, e.g., to the CDRN in Leeds, UK, for ribotyping. The results of this ribotyping are not publicly available. The infrequent typing of clinical isolates makes CDI epidemiology in Ireland complex, and the extent of CDI in Ireland was unclear. However, a National Reference Laboratory for *C. difficile* was established in 2021, which will help to identify intra- and inter-hospital spread of *C. difficile* using WGS compared to other molecular tools such as PCR ribotyping which cannot provide this level of discrimination.

Notable findings in this study included a low prevalence of RT027 (<1%), which had not been anticipated prior to the study, and the dominance of RT078 in both humans and pigs (66% in pigs and 12.57% in humans). RT027 was once a dominant strain in Ireland,

and the changing epidemiology and decline of RT027 in recent years could be because of the emergence of hypervirulent strains, such as RT078 (Baldan et al., 2015). The success of an epidemic strain relies not only on its virulence, but on its transmissibility (Beceiro et al., 2013). The implementation of infection control measures and the rise in CA-CDI due to RT078, could be the reason as to why the prevalence of RT027, which is predominantly associated with HA-CDI, has declined in recent years.

6.3 Epidemiology of *C. difficile* in Ireland

Despite its advantages, WGS has limitations, including the need for sophisticated bioinformatics, quick data processing, and large data storage capabilities, making it currently costly for most diagnostic microbiology laboratories. The integration of WGS into routine diagnostic laboratories is seen as a promising step forward for real-time identification and monitoring of outbreaks, global transmission surveillance, and identification of novel pathogenic genotypes, improving the management of infectious diseases in patients and populations.

The current study employed Ridom Seqsphere^{+} as a bioinformatic tool, which is commercially available and is increasingly used by diagnostic laboratories to analyse bacterial genome sequences, including *C. difficile*. This software has developed and published a core genome MSLT, based on 2,147 core genes (Bletz et al., 2018b, Junemann et al., 2013). Seqsphere+ cgMLST allows for the alignment of *C. difficile* isolates of interest and describes the allelic differences between each isolate, which can be used to clarify if there are genomic relationships between isolates, or not. The cgMLST approach on Seqsphere+ provides rapid information to reach an epidemiological conclusion, but it has been shown to have lesser discriminatory power than wgMLST and SNP analysis (Frentrup et al., 2020, Eyre et al., 2019). The software cannot fully capture the genetic diversity of *C. difficile* as some strains with different RTs (e.g. RT078 and RT126, RT106 andRT500) have been found to be indistinguishable by Seqsphere+ cgMLST (Seth-Smith et al., 2021, Baktash et al., 2022).

There were 5 different RTs which were identified in both pig and human *C. difficile* isolates. In Chapter 3, 222 pig and human *C. difficile* isolates were successfully sequenced, focusing on RTs which were found in both sources, revealing significant

diversity between 29 different sequence types. The most common sequence types that were identified in both pig and human *C. difficile* isolates were ST11 (45%), ST8 (9%), ST2 (8%), and ST44 (4.95%).

One of the aims of this study was to investigate the epidemiology of *C. difficile* RT078 in Ireland, but more isolates were required to place this analysis within a broader context. Genome sequence data from collaborators was available for environmental and food isolates, which enriched the study's scope and findings. The hypothesis that CDI is potentially foodborne or of zoonotic origin is further supported by the recovery of similar strains of *C. difficile* from different sources. Genetically similar strains of *C. difficile*, particularly RT078, have been found in humans, production animals, and retail meat. This correlates with the findings of our study in Chapter 4, where genetically indistinguishable isolates were found between pig, human, environmental and food sources, which highlights the zoonotic potential of *C. difficile*. As *C. difficile* is a spore former, there is a possibility that pigs and humans may become colonized from the same source, which may be food or the environment. Thus, more clarity is needed as to whether pigs are the vector for *C. difficile*.

C. difficile isolates from food products that were included in this study from Marcos et al. (2021) were cultured from ready to eat food products. The presence of *C. difficile* in these food products is of serious concern to public health, as often ready to eat salads are seen as a quick “lunch on the go” option and are not washed prior to consumption. Cooking reduces the risk of human exposure to *C. difficile* and through inadvertent ingestion of spores, while ready to eat products are generally not cooked after they are purchased (Rodriguez-Palacios and Lejeune, 2011, Eckert et al., 2013). It is not clear how food products are contaminated with *C. difficile*, but it could be because of environmental contamination from soil, contamination of the food processing facility, or transmission from hands by food handlers who are carriers of *C. difficile*.

No *C. difficile* isolates were found in Irish certified pig meat products in this study. Environmental *C. difficile* that were included in this analysis from Marcos et al. (2021) were from soil and water samples from broiler, sheep, and beef farms. *C. difficile* has previously been isolated from compost (22.5%), garden soil (79%), and manure (83%) (Lim et al., 2020a, Shivaperumal et al., 2020). Unpasteurised pig manure (slurry) is often

spread on soil as organic fertiliser, as it can provide nutrients for crop growth and is a cheaper alternative to chemical fertiliser (McCutcheon and Quinn, 2020). The use of slurry poses a further risk of environmental contamination due to soil run off into rivers and streams, or contaminated irrigation water spread on land. Fertiliser is not permitted to be spread on land in Ireland between September and January, which means that it is stored in leak-proof containers during this time, which could facilitate *C. difficile* proliferation, or it is exported (Hennessy et al., 2011). As a result, the transport of contaminated soil and compost, both nationally and internationally, is a likely source for long-range dissemination of *C. difficile* isolates.

Food and live animals accounted for 7% (9.5 million) of the total imports (€141 million) to Ireland and 7% (14.8 million) of the total exports (CSO, 2022). The UK, USA and China are the top importing partners to Ireland, which could be explanation as to why the epidemiology of *C. difficile* is similar, given that RT078 has established significant reservoirs in European, North American, and Asian pigs (Knight and Riley, 2019, Candel-Perez et al., 2019). It is possible that the movement of contaminated food products and animals could contribute to the dissemination of *C. difficile* in these countries and the level of risk to the domestic and export consumer. In addition, it has been shown that the prevalence of *C. difficile* can be influenced by the stress of transportation, which could cause an increase in *C. difficile* shedding when live animals are being exported or imported (Rodriguez et al., 2016a).

An important advancement in this study in comparison to a previous study from our group by Moloney et al. (2021), is the large collection of pig *C. difficile* RT078 isolates (n=148), 60 of which were fully sequenced. Due to budget limitations, the current study was unable to sequence all the pig RT078 isolates. However, selected RT078 from all 6 farms in the study were sequenced and our analysis showed that isolates from the same farms were shared between animals in close proximity.

The incidence of CA-CDI in members of populations that were previously considered to be low risk is an ever-evolving problem as there has been a dramatic paradigm shift – *C. difficile* can no longer be viewed solely as a healthcare-associated infection. Details on the occupation of CDI patients in Ireland could provide important additional epidemiological information on *C. difficile* in Ireland. Keessen et al. (2013a) found a

carriage rate of 21% in individuals with weekly or monthly contact with pigs. This is much higher than the carriage rate of 11% in asymptomatic adults (Truong et al., 2017, Le Monnier et al., 2022). In addition, close contact with children younger than 2 years of age is associated with a higher risk of CA-CDI, which would include individuals who work in childcare as they could potentially be exposed to contaminated faeces during nappy changing (Gupta and Khanna, 2014).

6.4 Antibiotic susceptibility of *C. difficile* isolates

WGS can help with the identification and description of selective pressures on microbial pathogens, such as the global spread of *C. difficile* PCR RT027 due to excess antimicrobial prescribing. Besides detecting known genetic virulence determinants, WGS alongside phenotypic studies enables the identification and characterization of virulence genotypes. The acquisition of tetracycline AMR genes in *C. difficile* ST11 is likely driven by the intensive tetracycline use in agriculture (Dingle et al., 2019).

The use of antimicrobials on farms could lead to higher levels of antibiotic resistance on farms that use in-feed antimicrobials due to selective pressure. However, the current study found no difference between the antimicrobial susceptibility profiles of *C. difficile* isolates on LAU and HAU farms, which is similar to what has been found by Susick et al. (2012) and Mencia-Ares et al. (2020).

6.5 Limitations of this study

Combining data from this surveillance and longitudinal study with other funded research projects on the same pig farms aims to identify risk factors for CDI in animals and humans, including antimicrobial use, and opportunities for intervention. The AMURAP study collected data on the biosecurity measures that are in place, and the location of each farm. It was not possible to include that data at the time of this study, due to GDPR agreements made with the farmers, but its inclusion could provide an insight into the impact that certain biosecurity measures on farms, such as the provision of changing facilities to farm workers, and the presence of other production animals on the farms. In addition, the study in Chapter 4 found genetically indistinguishable isolates (0 SNPs) between clinical and environmental samples. Details on the proximity of the farms to local rivers, streams, and community facilities such as playgrounds could also provide a

deeper insight into the epidemiology of CDI and the relationship between animal, environmental and human *C. difficile* isolates. In addition, the diarrhoeal status of the individual pigs was not recorded, which could have provided valuable information about the health status of the pigs.

Due ethics approval restrictions, there was no linked patient metadata or epidemiological information available for any of the human *C. difficile* isolates that were included in this study. Human *C. difficile* isolates were obtained from two tertiary referral centres in Ireland, who, along with faecal samples from hospital inpatients, are also sent faecal samples from other hospitals, outpatient clinics, day ward facilities, or General Practice. In addition, it was not known what type of CDI case that the isolate was obtained from, for example if the case was a new case, a recurrent case, or a case of CA-CDI. The newly established reference lab receives patient isolates for sequencing on a weekly basis, with approximately 30% of these originating from the community (HSE-HPSC and NRL, 2023). The inclusion of this data could further contribute to our understanding of the epidemiology of CA-CDI and the dissemination of CDI throughout healthcare facilities and the community.

6.6 Future work

This study intended to build on previous studies from our group to identify epidemiological relationships between pig and human *C. difficile* isolates (Moloney et al., 2021). These were not included in this analysis as the aim of the current study was to mainly focus on isolates from between 2018 and 2020. Future work would involve an analysis on the isolates from the current study and include the isolates from the study by Moloney et al. (2021), which were isolated between 2012 and 2016, and antibiotic susceptibility profiles are available for the sequenced isolates in the study. Bioinformatic analysis could provide an insight into the change dynamic of *C. difficile* over a ten-year period and identify and monitor the longitudinal antimicrobial susceptibility of *C. difficile* isolates, similar to a study by (Freeman et al., 2020). Future work involving the inclusion of *C. difficile* isolates from the study by Moloney et al. (2021) and the isolates from the current study, as well as isolates from across Europe, could provide a deeper understanding into the epidemiology of *C. difficile* internationally.

AST in this study focused on ST11 *C. difficile* isolates, however chapter 4 identified that ST44 isolates also had genotypic relationships between pig and human *C. difficile* isolate. Future work would involve an investigation into the AST profiles of these isolates. Previous work from our group investigated alternative resistance mechanisms for fluoroquinolone resistance in *C. difficile* (Mac Aogain et al., 2015a). Genotypic fluoroquinolone resistance was reported in all 55 *C. difficile* isolates that were included in this study. Future work would involve testing these isolates against a range of fluoroquinolone antibiotics such as delafloxacin, which is a new fluoroquinolone antibiotic.

The correlation between genotypic and phenotypic antimicrobial resistance is not well defined, as discussed in Chapter 5. However, rapid detection of antimicrobial resistance is important to predict treatment outcomes to decide on optimal antimicrobial treatment for CDI, but also to limit the spread of the bacteria. Agar dilution is the gold standard for phenotypic AMR testing of *C. difficile*, however this method is labour intensive and is not feasible in routine diagnostic laboratories. Therefore, the detection of genotypic AMR markers is imperative for the surveillance and prediction of resistance patterns in the population (Brouwer et al., 2024). While it is generally linked to hospital acquired infection, the threat of AMR is now spreading throughout veterinary and environmental sectors, which highlights the importance of a One Health perspective (Muntean et al., 2022). Therefore, the use of a standardised method is imperative for constant surveillance of AMR, which could be achieved across all sectors through *in silico* analysis.

C. difficile can colonise pigs asymptomatically, and similarly, *C. difficile* is carried asymptomatically in humans. Asymptomatic carriage of *C. difficile* has been found in 11% of healthy adults, with half of these strains being non-toxigenic (Truong et al., 2017, Le Monnier et al., 2022). The surveillance of *C. difficile* carriage in workers such as farmers and childcare workers, and the inclusion of occupational data in routine surveillance, could provide an insight into the risk of CDI.

There are several routes that have been shown to be important for the acquisition of *C. difficile* in the community, as illustrated in Figure 6.1. *C. difficile* can survive the wastewater treatment process, and then can be released and further disseminated

through the environment (Chisholm et al., 2023, Xu et al., 2014, Moradigaravand et al., 2018). Therefore, strategies to control the community acquisition of *C. difficile* should also consider the need for control of the bacteria in treated wastewater. In addition, fields that have been spread with pig slurry could run off into rivers during rainy weather and cause the dissemination of *C. difficile* strains from animals through the environment. An investigation of the prevalence in wastewater and rivers could give an insight into the burden of *C. difficile* in these environments, and if bathing areas could be a risk factor for the acquisition of *C. difficile*.

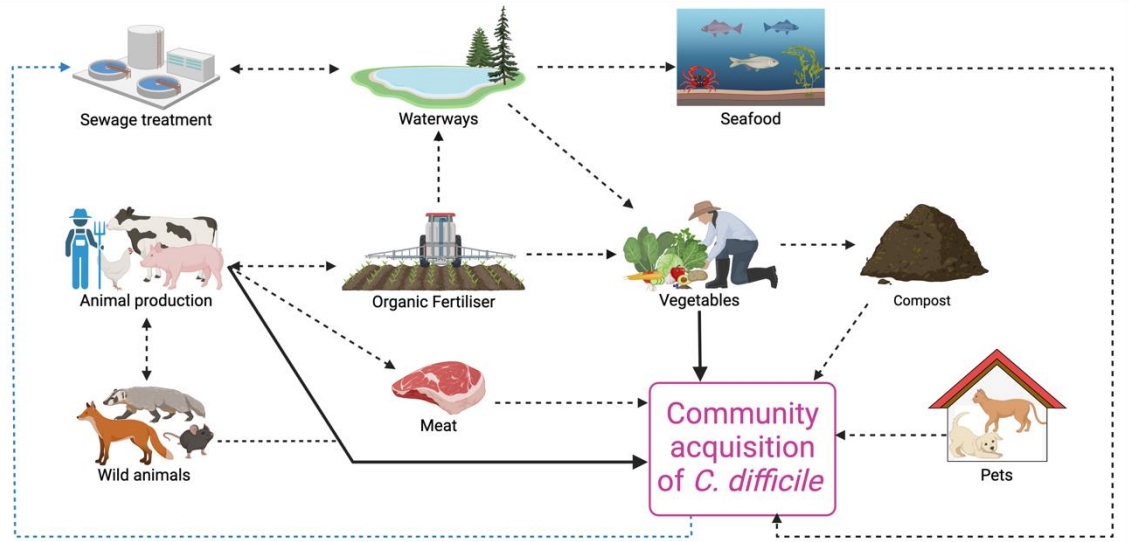


Figure 6.1 Routes that have been proposed to be important reservoirs for the community acquisition of *C. difficile*.
The solid black lines represent the evidence of close genomic relationships that we have shown in this study. The dotted lines represent the evidence that has been shown by other studies to be important. Figure adapted from (Warriner et al., 2017).

A comparison of the *C. difficile* isolates in the current study to isolates from across Europe, or the world, could provide data on the geographical distribution of *C. difficile* strains. Hypervirulent strains of *C. difficile*, in particular RT078, were found in the study by (Marcos et al., 2023) in ready to eat food lettuce products such as spinach and wild rocket. These are not commonly grown in Ireland because of the climate and are imported from countries such as Spain and Italy.

6.7 Conclusions

The findings of this study highlight genetic similarities among nosocomial, community, and veterinary infections of *C. difficile*, particularly for ST11/RT078, supporting the

concept of CDI as a “One Health” issue with interconnected transmission dynamics between human and animal populations. Genetic similarities were identified from strains from diverse sources, which supports the hypothesis of community acquisition from environmental sources. This thesis serves as a foundation for further studies on *C. difficile* epidemiology, building upon the knowledge gained from this prospective study. It emphasizes the need for a “One Health” approach to preventing CDI, considering animal, environmental and human health.

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Appendix

Supplementary Table 1 Detail statistics as provided by Novogene

Sample	Source	Library_Flowcell_Lane	Raw reads	Raw data	Effective (%)	Error (%)	Q20(%)	Q30(%)	GC (%)
P1L	Pig	EKDN220032561-1A_H5JHTDSX5_L1	5028350	1.5	99.95	0.03	96.77	90.52	30.03
P2L	Pig	EKDN220032562-1A_H5JHTDSX5_L1	7647640	1.1	99.96	0.03	96.84	90.65	29.59
P3L	Pig	EKDN220032563-1A_H5JHTDSX5_L1	9284058	1.4	99.96	0.03	96.92	90.83	29.13
P4L	Pig	EKDN220032564-1A_H5JHTDSX5_L1	7047508	1.1	99.96	0.03	96.77	90.52	29.43
P5L	Pig	EKDN220032565-1A_H5JHTDSX5_L1	7173568	1.1	99.95	0.03	96.52	90.04	29.89
P6L	Pig	EKDN220032566-1A_H5JHTDSX5_L1	6994600	1	99.95	0.03	97.13	91.29	29.66
P7L	Pig	EKDN220032567-1A_H5JHTDSX5_L1	6613984	1	99.95	0.03	97.06	91.14	29.68
P8L	Pig	EKDN220032568-1A_H5JHTDSX5_L1	6181470	2	99.95	0.03	96.96	90.93	29.59
P9L	Pig	EKDN220032569-1A_H5JHTDSX5_L1	7025568	1.1	99.95	0.03	96.66	90.28	29.37
P10L	Pig	EKDN220032570-1A_H5JHTDSX5_L1	6853442	1	99.94	0.03	96.91	90.79	29.75
P11L	Pig	EKDN220032571-1A_H5JHTDSX5_L1	6811700	1	99.95	0.03	97.04	91.09	29.98
P12L	Pig	EKDN220032572-1A_H5JHTDSX5_L1	7688158	1.2	99.95	0.03	97.07	91.17	29.67
P13L	Pig	EKDN220032573-1A_H5JHTDSX5_L1	7441848	1.1	99.95	0.03	96.79	90.57	29.1
P14L	Pig	EKDN220032574-1A_H5JHTDSX5_L1	6521460	1	99.95	0.03	97.1	91.24	28.83
P15H	Pig	EKDN220032575-1A_H5JHTDSX5_L1	6998286	1	99.95	0.03	97.13	91.26	29.06
P16L	Pig	EKDN220032576-1A_H5JHTDSX5_L1	7420314	1.1	99.96	0.03	97.07	91.13	29.27
C17	Human	EKDN220032577-1A_H5JHTDSX5_L1	6671836	1	99.95	0.03	97.16	91.35	28.71
C18	Human	EKDN220032578-1A_H5JHTDSX5_L1	8096348	1.2	99.96	0.03	96.7	90.34	28.99
C19	Human	EKDN220032579-1A_H5JHTDSX5_L1	7079144	1.1	99.93	0.03	97.31	91.72	29.1
P27L	Pig	EKDN230007732-1A_HTWTNDSX5_L4	4893488	1.7	98.4	0.03	97.84	93.37	30.21
P28L	Pig	EKDN230007733-1A_HTWTNDSX5_L4	8426200	1.3	99.08	0.03	97.45	92.44	29.82
P29L	Pig	EKDN230007734-1A_HTWMGDSX5_L1	8163418	2.1	99.25	0.03	97.81	93.22	30.12
P30L	Pig	EKDN230007735-1A_HTWTNDSX5_L4	7826708	1.2	98.73	0.03	98.08	93.9	30.09
P31L	Pig	EKDN230007736-1A_HTWTNDSX5_L4	7371118	1.1	99.26	0.03	97.71	93.02	30.05
P32L	Pig	EKDN230007737-1A_HTWTNDSX5_L4	7431768	1.1	99	0.03	98.06	93.82	30.2
P33L	Pig	EKDN230007738-1A_HTWMGDSX5_L4	7753234	2.1	99.19	0.03	97.6	92.82	30.19
P36L	Pig	EKDN230007739-1A_HTWTNDSX5_L4	7887620	1.2	99.28	0.03	97.88	93.35	29.71
P37L	Pig	EKDN230007740-1A_HTWTNDSX5_L4	9146324	1.4	99.09	0.03	97.87	93.4	30.13
P38L	Pig	EKDN230007741-1A_HTWTNDSX5_L4	8300876	1.2	98.96	0.03	97.96	93.65	29.37
P41L	Pig	EKDN230007742-1A_HTWTNDSX5_L4	7634102	1.1	98.72	0.03	97.75	93.15	30.24

Sample	Source	Library_Flowcell_Lane	Raw reads	Raw data	Effective (%)	Error (%)	Q20(%)	Q30(%)	GC (%)
P42H	Pig	EKDN230007743-1A_HTWTNDSX5_L4	8236830	1.2	99.22	0.03	97.93	93.51	29.53
P43H	Pig	EKDN230007744-1A_HTWTNDSX5_L4	3419372	1.5	99.04	0.03	97.86	93.3	29.32
P46H	Pig	EKDN230007745-1A_HTWTNDSX5_L4	9218812	1.4	99.11	0.03	97.8	93.22	29.51
P47H	Pig	EKDN230007746-1A_HTWTNDSX5_L4	6836868	1	98.93	0.03	97.92	93.5	29.84
P48H	Pig	EKDN230007747-1A_HTWTNDSX5_L4	3239344	1.1	98.8	0.03	97.94	93.5	29.48
P50H	Pig	EKDN230007748-1A_HTWTNDSX5_L4	9704606	1.5	99.1	0.03	98.01	93.73	31.09
P51H	Pig	EKDN230007749-1A_HTWTNDSX5_L4	7500542	1.1	99.22	0.03	97.92	93.52	29.98
C54	Human	EKDN230007750-1A_HTWTNDSX5_L4	8290524	1.2	99.37	0.03	97.59	92.75	29.58
C57	Human	EKDN230007751-1A_HTWTNDSX5_L4	7586796	1.1	94.11	0.03	98.06	93.92	30.79
C58	Human	EKDN230007752-1A_HTWTNDSX5_L4	8872038	1.3	99.19	0.03	98.09	93.89	29.78
C59	Human	EKDN230007753-1A_HTWTNDSX5_L4	9455308	1.4	99	0.03	97.92	93.58	30.65
C60	Human	EKDN230007754-1A_HTWTNDSX5_L4	8699476	1.3	99.02	0.03	98	93.69	29.51
C61	Human	EKDN230007755-1A_HTWTNDSX5_L4	9629542	1.4	99.29	0.03	97.94	93.51	29.2
P62L	Pig	EKDN230019210-1A_HF7WWDSX7_L4	6691392	1	99.78	0.03	97.51	92.32	29.62
P63L	Pig	EKDN230019211-1A_HF5V2DSX7_L1	6878388	1.7	99.72	0.03	97.2	91.92	29.5
P64L	Pig	EKDN230019212-1A_HF7WWDSX7_L4	9028982	1.4	99.82	0.03	97.47	92.24	29.42
P65L	Pig	EKDN230019213-1A_HF7WWDSX7_L4	8612474	1.3	99.84	0.03	97.27	91.85	29.51
P66L	Pig	EKDN230019214-1A_HF7WWDSX7_L4	8332132	1.2	99.86	0.03	97.48	92.26	30.62
P67L	Pig	EKDN230019215-1A_HF7WWDSX7_L4	7588778	1.1	99.8	0.03	97.72	92.8	29.52
P68L	Pig	EKDN230019216-1A_HF7WWDSX7_L4	8349238	1.3	99.84	0.03	97.71	92.78	29.66
P69H	Pig	EKDN230019217-1A_HF7WWDSX7_L4	7236014	1.1	99.81	0.03	97.52	92.37	29.05
P70H	Pig	EKDN230019218-1A_HF7WWDSX7_L4	7974534	1.2	99.85	0.03	97.42	92.14	29.83
P71H	Pig	EKDN230019219-1A_HF7WWDSX7_L4	9363002	1.4	99.82	0.03	97.77	92.96	29.65
P72H	Pig	EKDN230019220-1A_HF7WWDSX7_L4	10740156	1.6	99.83	0.03	97.44	92.16	29.01
P73H	Pig	EKDN230019221-1A_HF5V2DSX7_L1	8144212	2.1	99.72	0.03	97.37	92.29	29.66
C74	Human	EKDN230019222-1A_HF7WWDSX7_L4	10594386	1.6	99.83	0.03	97.55	92.43	29.44
C75	Human	EKDN230019223-1A_HF7WWDSX7_L4	8355164	1.3	99.82	0.03	97.54	92.41	29.45
C76	Human	EKDN230019224-1A_HF7WWDSX7_L4	11117966	1.7	99.85	0.03	97.58	92.46	29.21
C77	Human	EKDN230019225-1A_HF7WWDSX7_L4	9081502	1.4	99.8	0.03	97.64	92.64	29.37
C78	Human	EKDN230019226-1A_HF7WWDSX7_L4	9231946	1.4	99.84	0.03	97.61	92.53	29.61
C79	Human	EKDN230019227-1A_HF7WWDSX7_L4	7642490	1.1	99.81	0.03	97.36	92.01	29.46

Sample	Source	Library_Flowcell_Lane	Raw reads	Raw data	Effective (%)	Error (%)	Q20(%)	Q30(%)	GC (%)
C80	Human	EKDN230019228-1A_HF7WWDSX7_L4	9106052	1.4	99.86	0.03	97.46	92.2	29.75
C81	Human	EKDN230019229-1A_HF7WWDSX7_L4	6788034	1	99.81	0.03	97.8	92.97	29.32
C82	Human	EKDN230019230-1A_HF7WWDSX7_L4	9309592	1.4	99.82	0.03	97.3	91.86	29.4
C83	Human	EKDN230019231-1A_HF7WWDSX7_L4	10551138	1.6	99.82	0.03	97.5	92.3	29.13
C84	Human	EKDN230019232-1A_HF7WWDSX7_L4	10599598	1.6	99.92	0.03	97.19	91.57	29.23
C85	Human	EKDN230019233-1A_HF7WWDSX7_L4	9517404	1.4	99.93	0.03	97.62	92.47	29.17
C86	Human	EKDN230019234-1A_HF7WWDSX7_L4	8633356	1.3	99.91	0.03	97.62	92.52	28.55
C87	Human	EKDN230019235-1A_HF7WWDSX7_L4	7721464	1.2	99.93	0.03	97.4	92.01	28.62
C88	Human	EKDN230019236-1A_HF7WWDSX7_L4	8113832	1.2	99.92	0.03	97.56	92.39	28.52
P21L	Pig	EKDN230024537-1A_HFL7LDSX7_L3	13285428	2	99.79	0.03	97.57	92.7	29.05
P90L	Pig	EKDN230024542-1A_HFKLMDSX7_L2	12918098	1.9	99.84	0.03	97.73	92.75	28.72
P91L	Pig	EKDN230024543-1A_HFKLMDSX7_L2	12363194	1.9	99.74	0.03	97.75	92.75	28.25
P92L	Pig	EKDN230024544-1A_HFKLMDSX7_L2	12514160	1.9	99.82	0.03	97.82	92.93	28.14
P93H	Pig	EKDN230024545-1A_HFKLMDSX7_L2	11784722	1.8	99.72	0.03	97.87	93.11	29.07
P94H	Pig	EKDN230024546-1A_HFKLMDSX7_L2	13595126	2	99.82	0.03	97.9	93.1	28.36
P95H	Pig	EKDN230024547-1A_HFKLMDSX7_L2	11903854	1.8	99.79	0.03	97.74	92.74	28.15
P96H	Pig	EKDN230024548-1A_HFKLMDSX7_L2	13843962	2.1	99.84	0.03	97.67	92.57	28.21
C97	Human	EKDN230024549-1A_HFKLMDSX7_L2	14576110	2.2	99.79	0.03	97.81	92.76	28.47
C98	Human	EKDN230024550-1A_HFKLMDSX7_L2	11938510	1.8	99.61	0.03	98	93.4	28.17
C99	Human	EKDN230024551-1A_HFKLMDSX7_L2	13047346	2	99.55	0.03	98.05	93.51	28.18
C100	Human	EKDN230024552-1A_HFKLMDSX7_L2	13047150	2	99.82	0.03	97.84	92.98	28.67
C101	Human	EKDN230024553-1A_HFKLMDSX7_L2	14386124	2.2	99.85	0.03	98.05	93.48	28.38
C102	Human	EKDN230024554-1A_HFKLMDSX7_L2	13947660	2.1	99.72	0.03	98	93.36	28.69
P103L	Pig	EKDN230024555-1A_HFKLMDSX7_L2	13475496	2	99.82	0.03	97.95	93.2	28.95
P104H	Pig	EKDN230024556-1A_HFL7LDSX7_L3	11488620	1.7	99.76	0.03	97.38	92.32	28.5
P105L	Pig	EKDN230024557-1A_HFKLMDSX7_L2	15308490	2.3	99.87	0.03	97.72	92.74	28.35
P106L	Pig	EKDN230024558-1A_HFKLMDSX7_L2	13676418	2.1	99.81	0.03	97.43	91.98	28.52
P108L	Pig	EKDN230024560-1A_HGW5VDSX7_L3	16714602	2.5	99.87	0.03	98.04	93.98	29.25
P110L	Pig	EKDN230024562-1A_HFKLMDSX7_L2	13600106	2	99.75	0.03	97.98	93.32	27.81
P111L	Pig	EKDN230024563-1A_HFKLMDSX7_L2	15554758	2.3	99.78	0.03	97.7	92.63	28.1
P115L	Pig	EKDN230024567-1A_HFL7LDSX7_L3	14592330	2.2	99.82	0.03	97.32	92.19	28.59

Sample	Source	Library_Flowcell_Lane	Raw reads	Raw data	Effective (%)	Error (%)	Q20(%)	Q30(%)	GC (%)
C119	Human	EKDN230024571-1A_HGW5VDSX7_L3	15808538	2.4	99.66	0.02	98.14	94.29	29.52
C120	Human	EKDN230024572-1A_HGW5VDSX7_L3	14589136	2.2	99.87	0.03	98	93.89	29.05
C122	Human	EKDN230024573-1A_HGW5VDSX7_L3	16239484	2.4	99.83	0.03	97.78	93.48	29.11
C124	Human	EKDN230024575-1A_HGW5VDSX7_L3	14633000	2.2	99.8	0.02	98.13	94.22	29.54
C125	Human	EKDN230024576-1A_HGW5VDSX7_L3	13617240	2	99.86	0.03	98.03	93.98	29.54
C127	Human	EKDN230024578-1A_HGW5VDSX7_L3	12461256	1.9	99.89	0.02	98.16	94.3	29.47
C129	Human	EKDN230024580-1A_HGW5VDSX7_L3	15107874	2.3	99.88	0.02	98.08	94.12	28.39
C133	Human	EKDN230024583-1A_HGW5VDSX7_L3	17078876	2.6	99.86	0.03	97.88	93.66	28.67
C135	Human	EKDN230024585-1A_HGW5VDSX7_L3	15293352	2.3	99.86	0.03	98.05	94.06	28.93
C136	Human	EKDN230024586-1A_HFJJGDSX7_L4	12859476	1.9	99.81	0.03	97.89	93.31	28.85
C138	Human	EKDN230024587-1A_HFKLMDSX7_L2	14003352	2.1	99.73	0.03	97.87	93.06	27.98
C139	Human	EKDN230024588-1A_HFKLMDSX7_L2	15903280	2.4	99.53	0.03	97.87	93.06	28.48
C140	Human	EKDN230024589-1A_HFKLMDSX7_L2	14489128	2.2	99.79	0.03	97.95	93.27	28.55
C144	Human	EKDN230024591-1A_HFJJGDSX7_L1	13931620	2.1	99.73	0.03	97.8	93.14	28.98
P40L	Pig	EKDN230025452-1A_HGW5YDSX7_L1	16343922	2.5	99.93	0.03	98.17	93.72	29.13
C123	Human	EKDN230036860-1A_HMTYJDSX7_L4	10640814	1.6	99.89	0.03	97.92	93.62	29.82
C130	Human	EKDN230036861-1A_HMTYJDSX7_L4	23138530	3.5	99.9	0.02	98.2	94.34	29.85
C131	Human	EKDN230036862-1A_HMTYJDSX7_L4	12011764	1.8	99.86	0.03	97.95	93.68	30.22
C132	Human	EKDN230036863-1A_HMTYJDSX7_L4	9265654	1.4	99.71	0.03	97.46	92.64	30.33
C134	Human	EKDN230036864-1A_HMTYJDSX7_L4	10253856	1.5	99.91	0.03	98.03	93.9	29.78
C137	Human	EKDN230036865-1A_HMTYJDSX7_L4	9557974	1.4	99.82	0.02	98.13	94.14	30.05
C143	Human	EKDN230036867-1A_HMTYJDSX7_L4	12661488	1.9	99.89	0.03	97.87	93.49	29.53
C145	Human	EKDN230036868-1A_HMTYJDSX7_L4	13745072	2.1	99.92	0.03	97.82	93.36	29.04
C146	Human	EKDN230036869-1A_HMTYJDSX7_L4	11017440	1.7	99.88	0.03	97.97	93.74	29.74
C147	Human	EKDN230036870-1A_HMTYJDSX7_L4	12278858	1.8	99.82	0.03	98.02	93.9	31.17
C148	Human	EKDN230036871-1A_HMTYJDSX7_L4	12440674	1.9	99.89	0.03	97.76	93.23	29.39
C149	Human	EKDN230036872-1A_HMTYJDSX7_L4	9742390	1.5	99.8	0.03	97.99	93.79	29.31
C152	Human	EKDN230036873-1A_HMTYJDSX7_L4	10619350	1.6	99.9	0.03	97.79	93.31	30.44
C153	Human	EKDN230036874-1A_HMTYJDSX7_L4	11355946	1.7	99.91	0.03	97.57	92.81	30.81
C154	Human	EKDN230036875-1A_HMTYJDSX7_L4	10975412	1.6	99.9	0.03	97.84	93.45	30.51
C155	Human	EKDN230036876-1A_HMTYJDSX7_L4	10254716	1.5	99.85	0.03	97.97	93.77	29.77

Sample	Source	Library_Flowcell_Lane	Raw reads	Raw data	Effective (%)	Error (%)	Q20(%)	Q30(%)	GC (%)
C156	Human	EKDN230036877-1A_HMTYJDSX7_L4	10914238	1.6	99.88	0.03	97.71	93.12	29.32
C157	Human	EKDN230036878-1A_HMTYJDSX7_L4	10907124	1.6	99.74	0.03	98.04	93.93	30.02
C158	Human	EKDN230036879-1A_HMTYJDSX7_L4	8871694	1.3	99.86	0.03	97.82	93.36	29.71
C159	Human	EKDN230036880-1A_HMTYJDSX7_L4	8822234	1.3	99.87	0.03	97.91	93.61	30.57
C161	Human	EKDN230036882-1A_HG237DSX7_L1	10390876	1.6	99.91	0.03	97.16	91.81	29.42
C162	Human	EKDN230036883-1A_HMTYJDSX7_L4	8706640	1.3	99.9	0.03	97.76	93.21	29.56
C163	Human	EKDN230036884-1A_HMTYJDSX7_L4	9231456	1.4	99.84	0.03	97.73	93.18	29.96
C164	Human	EKDN230036885-1A_HMTYJDSX7_L4	9326868	1.4	99.89	0.03	97.75	93.21	29.79
C166	Human	EKDN230036887-1A_HMTYJDSX7_L4	11683310	1.8	99.91	0.03	97.94	93.67	30.43
C168	Human	EKDN230036888-1A_HMTYJDSX7_L4	10289504	1.5	99.89	0.03	97.81	93.35	29.68
C169	Human	EKDN230036889-1A_HMTYJDSX7_L4	8755934	1.3	99.91	0.03	97.58	92.79	29.08
C170	Human	EKDN230036890-1A_HMTYJDSX7_L4	9818758	1.5	99.9	0.03	97.9	93.54	29.9
C171	Human	EKDN230036891-1A_HMTYJDSX7_L4	8941694	1.3	99.8	0.03	97.94	93.68	30.69
C173	Human	EKDN230036893-1A_HMTYJDSX7_L4	12509126	1.9	99.89	0.03	97.87	93.51	29.64
C174	Human	EKDN230036894-1A_HMTYJDSX7_L4	11260238	1.7	99.71	0.03	97.84	93.38	29.51
C175	Human	EKDN230036895-1A_HMTYJDSX7_L4	14384140	2.2	99.81	0.03	97.89	93.55	29.37
C176	Human	EKDN230036896-1A_HMTYJDSX7_L4	10832702	1.6	99.85	0.03	97.88	93.52	30.05
C178	Human	EKDN230036897-1A_HMTYJDSX7_L4	9286410	1.4	99.91	0.03	97.89	93.54	29.27
C179	Human	EKDN230036898-1A_HMTYJDSX7_L4	8611378	1.3	99.65	0.03	97.93	93.65	29.67
C180	Human	EKDN230036899-1A_HG237DSX7_L1	10931474	1.6	99.9	0.03	97.43	92.44	29.86
C181	Human	EKDN230036900-1A_HMTYJDSX7_L4	10907792	1.6	99.83	0.03	97.95	93.69	29.69
C107	Human	EKDN230037930-1A_H7LJKDSX7_L4	9891144	1.5	99.94	0.03	97.2	91.78	28.57
C121	Human	EKDN230037931-1A_HMHHKDSX7_L1	8279312	2.2	99.92	0.03	96.8	90.9	28.68
C150	Human	EKDN230037932-1A_H7LJKDSX7_L4	6790988	1	99.95	0.03	97.14	91.65	28.68
C151	Human	EKDN230037933-1A_H7LJKDSX7_L4	8332428	1.2	99.96	0.03	96.92	91.16	28.79
C156	Human	EKDN230037934-1A_H7LJKDSX7_L4	7181204	1.1	99.95	0.03	97.14	91.62	29.24
C165	Human	EKDN230037935-1A_H7LJKDSX7_L4	6259232	0.9	99.86	0.03	97.04	91.48	29.28
C177	Human	EKDN230037936-1A_H7LJKDSX7_L4	6062692	0.9	99.91	0.03	96.96	91.31	29.05
C181	Human	EKDN230037937-1A_H7LJKDSX7_L4	6768366	1	99.94	0.03	97.16	91.73	28.92
C182	Human	EKDN230037938-1A_H7LJKDSX7_L3	7267220	1.1	99.94	0.03	96.86	91.07	28.97
C183	Human	EKDN230037939-1A_H7LJKDSX7_L3	9100818	1.4	99.96	0.03	96.85	91.03	28.83

Sample	Source	Library_Flowcell_Lane	Raw reads	Raw data	Effective (%)	Error (%)	Q20(%)	Q30(%)	GC (%)
C184	Human	EKDN230037940-1A_H7LJKDSX7_L3	8817348	1.3	99.96	0.03	97.09	91.54	28.79
C185	Human	EKDN230037941-1A_H7LJKDSX7_L3	8512112	1.3	99.95	0.03	97.13	91.64	29.06
C186	Human	EKDN230037942-1A_H7LJKDSX7_L3	13030150	2	99.94	0.03	96.99	91.37	29.04
C187	Human	EKDN230037943-1A_H7LJKDSX7_L3	13360072	2	99.94	0.03	97.02	91.42	28.28
C188	Human	EKDN230037944-1A_H7LJKDSX7_L3	11751156	1.8	99.89	0.03	97.05	91.47	29.11
C189	Human	EKDN230037945-1A_H7LJKDSX7_L3	9632920	1.4	99.96	0.03	97.02	91.39	28.85
C190	Human	EKDN230037946-1A_H7LJKDSX7_L3	10951246	1.6	99.94	0.03	97.04	91.45	28.6
C191	Human	EKDN230037947-1A_H7LJKDSX7_L3	10450100	1.6	99.95	0.03	97.02	91.39	28.22
C20	Human	EKDN230037948-1A_H7LJKDSX7_L3	11727896	1.8	99.95	0.03	97.03	91.44	28.65
C55	Human	EKDN230037949-1A_H7LJKDSX7_L3	13188636	2	99.94	0.03	96.79	90.96	28.35
PH144	Pig	EKDN230037950-1A_H7LJKDSX7_L3	10201408	1.5	99.94	0.03	96.9	91.16	28.36
PH192	Pig	EKDN230037951-1A_H7LJKDSX7_L3	9993650	1.5	99.91	0.03	97.09	91.58	28.61
PH193	Pig	EKDN230037952-1A_H7LJKDSX7_L3	12312848	1.8	99.95	0.03	97.02	91.45	29.9
PH194	Pig	EKDN230037953-1A_H7LJKDSX7_L3	11789430	1.8	99.94	0.03	96.99	91.36	28.51
PH195	Pig	EKDN230037954-1A_H7LJKDSX7_L3	10744970	1.6	99.92	0.03	96.88	91.12	28.88
PH22	Pig	EKDN230037955-1A_H7LJKDSX7_L3	11258958	1.7	99.95	0.03	96.88	91.09	28.72
PH25	Pig	EKDN230037956-1A_H7LJKDSX7_L3	11812720	1.8	99.95	0.03	97.2	91.8	28.57
PH26	Pig	EKDN230037957-1A_H7LJKDSX7_L3	7962412	1.2	99.87	0.03	97.28	91.91	28.81
PL109	Pig	EKDN230037958-1A_HMHHKDSX7_L1	9131662	1.4	99.95	0.03	96.84	90.97	28.97
PL112	Pig	EKDN230037959-1A_HMHHKDSX7_L1	9087494	1.4	99.94	0.03	96.83	90.97	28.76
PL113	Pig	EKDN230037960-1A_HMHHKDSX7_L1	9266984	1.4	99.92	0.03	97.07	91.5	28.55
PL118	Pig	EKDN230037961-1A_HMHHKDSX7_L1	7326546	1.1	99.94	0.03	96.88	91.06	28.62
PM196	Pig	EKDN230037962-1A_HMHHKDSX7_L1	7731758	1.2	99.91	0.03	96.95	91.21	28.46
PM197	Pig	EKDN230037963-1A_HMHHKDSX7_L1	8731150	1.3	99.94	0.03	96.85	90.94	28.42
C172	Human	ZKDN230026938-1A_HMF2TDSX7_L3	7166626	1.1	99.96	0.03	98	93.55	28.75
C209	Human	ZKDN230026939-1A_HMF2TDSX7_L3	8188998	1.2	99.93	0.03	98.15	93.89	29.41
C210	Human	ZKDN230026940-1A_HMF2TDSX7_L3	8195834	1.2	99.94	0.02	98.21	94.07	29.54
C211	Human	ZKDN230026941-1A_HMF2TDSX7_L3	9705796	1.5	99.96	0.02	98.23	94.12	28.77
C212	Human	ZKDN230026942-1A_HMF2TDSX7_L3	8839354	1.3	99.96	0.03	98.14	93.86	28.72
C213	Human	ZKDN230026943-1A_HMF2TDSX7_L3	8788382	1.3	99.95	0.03	98.05	93.64	28.51
C214	Human	ZKDN230026944-1A_HMF2TDSX7_L3	8816776	1.3	99.94	0.03	98.18	93.97	29.26

Sample	Source	Library_Flowcell_Lane	Raw reads	Raw data	Effective (%)	Error (%)	Q20(%)	Q30(%)	GC (%)
C215	Human	ZKDN230026945-1A_HMF2TDSX7_L3	11816720	1.8	99.95	0.02	98.23	94.11	28.69
C216	Human	ZKDN230026946-1A_HMF2TDSX7_L3	7842902	1.2	99.96	0.03	98.07	93.71	28.9
C217	Human	ZKDN230026947-1A_HMF2TDSX7_L3	8323878	1.2	99.95	0.03	98.03	93.58	28.49
C218	Human	ZKDN230026948-1A_HMHC2DSX7_L2	7035398	1.1	99.95	0.03	97.78	92.94	28.99
C219	Human	ZKDN230026950-1A_HMHC2DSX7_L2	7178710	1.1	99.97	0.03	98	93.48	28.84
C220	Human	ZKDN230026951-1A_HMHC2DSX7_L3	6857492	1	99.94	0.03	97.79	93.18	29.34
C221	Human	ZKDN230026952-1A_HMHC2DSX7_L3	7873362	1.2	99.94	0.03	97.76	93.11	28.8
C223	Human	ZKDN230026953-1A_HMHC2DSX7_L3	8345470	1.3	99.94	0.03	97.74	93.06	28.64
C225	Human	ZKDN230026954-1A_HMHC2DSX7_L3	7051676	1.1	99.92	0.03	97.72	92.96	28.52
C226	Human	ZKDN230026955-1A_HMHC2DSX7_L3	6900246	1	99.92	0.03	97.74	93.07	28.93
C227	Human	ZKDN230026956-1A_HMHC2DSX7_L3	6542142	1	99.92	0.03	97.63	92.85	28.82
C228	Human	ZKDN230026957-1A_HMHC2DSX7_L3	7962366	1.2	99.91	0.03	97.77	93.13	29
C229	Human	ZKDN230026958-1A_HMHC2DSX7_L3	6629618	1	99.91	0.03	97.77	93.17	29.09
C230	Human	ZKDN230026959-1A_HMHC2DSX7_L3	8530436	1.3	99.93	0.03	97.68	92.91	28.64
C231	Human	ZKDN230026960-1A_HMHC2DSX7_L3	9360786	1.4	99.92	0.03	97.7	92.98	29.24
C56	Human	ZKDN230026961-1A_HMHC2DSX7_L3	7632176	1.1	99.93	0.03	97.53	92.56	28.89
P200	Pig	ZKDN230026963-1A_HMHC2DSX7_L3	9217968	1.4	99.93	0.03	97.63	92.8	29.04
P202	Pig	ZKDN230026965-1A_HMHC2DSX7_L3	8516144	1.3	99.94	0.03	97.73	93.05	29.55
P203	Pig	ZKDN230026966-1A_HMHC2DSX7_L3	9727920	1.5	99.93	0.03	97.56	92.67	29.26
P204	Pig	ZKDN230026967-1A_HMHC2DSX7_L3	6660844	1	99.93	0.03	97.65	92.85	29
P205	Pig	ZKDN230026968-1A_HMHC2DSX7_L3	8937644	1.3	99.93	0.03	97.55	92.59	29.53
P206	Pig	ZKDN230026969-1A_HMHC2DSX7_L3	7617298	1.1	99.94	0.03	97.81	93.2	29.15
P207	Pig	ZKDN230026970-1A_HMHC2DSX7_L4	7242008	1.1	99.93	0.03	97.78	93.18	29.37
PH114	Pig	ZKDN230026971-1A_HMHC2DSX7_L4	7947146	1.2	99.94	0.03	97.72	93.02	28.86
PH116	Pig	ZKDN230026972-1A_HMHC2DSX7_L4	7473868	1.1	99.94	0.03	97.73	93.05	28.87
PH45	Pig	ZKDN230026973-1A_HMHC2DSX7_L4	7444656	1.1	99.92	0.03	97.6	92.71	28.85
PH49	Pig	ZKDN230026974-1A_HMHC2DSX7_L4	8812994	1.3	99.93	0.03	97.75	93.1	29
PH52	Pig	ZKDN230026975-1A_HMHC2DSX7_L4	9407644	1.4	99.93	0.03	97.85	93.29	29.47
PL117	Pig	ZKDN230026976-1A_HMHC2DSX7_L4	8304774	1.2	99.91	0.03	97.94	93.59	28.93
PL233	Pig	ZKDN230026977-1A_HMHC2DSX7_L4	7808336	1.2	99.9	0.03	98.05	93.85	28.93
PL235	Pig	ZKDN230026978-1A_HMHC2DSX7_L4	7014906	1.1	99.89	0.03	97.9	93.48	29.02

Sample	Source	Library_Flowcell_Lane	Raw reads	Raw data	Effective (%)	Error (%)	Q20(%)	Q30(%)	GC (%)
PL236	Pig	ZKDN230026979-1A_HMHC2DSX7_L4	8709566	1.3	99.92	0.03	97.89	93.47	28.5
PL237	Pig	ZKDN230026980-1A_HMHC2DSX7_L4	7247722	1.1	99.91	0.03	97.76	93.14	28.53
PL238	Pig	ZKDN230026981-1A_HMHC2DSX7_L4	5870084	0.9	99.9	0.03	97.9	93.51	29.02
PL239	Pig	ZKDN230026982-1A_HMHC2DSX7_L4	7653734	1.1	99.93	0.03	97.83	93.33	28.98
PL34	Pig	ZKDN230026984-1A_HMHC2DSX7_L4	8452868	1.3	99.92	0.03	97.86	93.43	29.28
PL35	Pig	ZKDN230026985-1A_HMHC2DSX7_L4	6370650	1	99.91	0.03	97.65	92.89	28.92
PL39	Pig	ZKDN230026986-1A_HMHC2DSX7_L4	8212058	1.2	99.88	0.03	97.67	92.96	28.94
PM198	Pig	ZKDN230026987-1A_HMHC2DSX7_L4	6813130	1	99.93	0.03	97.91	93.47	28.5
PM199	Pig	ZKDN230026988-1A_HMHC2DSX7_L4	6631308	1	99.92	0.03	98.01	93.74	30.03

Supplementary Table 2 Sequence analysis results for 222 *C. difficile* isolates in this study

Obtained from Ridom Seqsphere+, detailing the sample ID (C: human, PL: LAU pig, PM: MAU pig, PH: HAU pig), ST, collection date, the country of isolation, where the bacterium was isolated, the host, and the allelic profiles of the 7 housekeeping genes

Sample ID	ST	Collection Date	Country of Isolation	City of Isolation	Host	ST	adk	atpA	dxr	glyA	recA	sodA	tpi
C131	1	2019	Ireland	Dublin	Human	2	1	1	1	10	1	3	5
C225	10	2018	Ireland	Dublin	Human	1	2	1	2	1	1	3	1
C55	10	2019	Ireland	Dublin	Human	1	2	1	2	1	1	3	1
C150	10	2019	Ireland	Dublin	Human	1	2	1	2	1	1	3	1
C152	10	2020	Ireland	Dublin	Human	1	2	1	2	1	1	3	1
A142	101	2023	Ireland	ABATTOIR 1		1	1	2	2	1	1	23	1
PH71	101	2020	Ireland	FARM 4	Porcine	1	1	2	2	1	1	23	1
PL108	107	2020	Ireland	FARM 2	Porcine	1	4	1	6	1	3	1	1
PL68	107	2020	Ireland	FARM 1	Porcine	1	4	1	6	1	3	1	1
PL65	107	2020	Ireland	FARM 1	Porcine	1	4	1	6	1	3	1	1
PL103	107	2020	Ireland	FARM 2	Porcine	1	4	1	6	1	3	1	1
C127	11	2020	Ireland	Dublin	Human	5	5	8	5	11	9	11	8
C87	11	2020	Ireland	Dublin	Human	5	5	8	5	11	9	11	8
C18	11	2019	Ireland	Dublin	Human	5	5	8	5	11	9	11	8
C99	11	2019	Ireland	Dublin	Human	5	5	8	5	11	9	11	8
PM207	11	2019	Ireland	FARM 8	Porcine	5	5	8	5	11	9	11	8
C182	11	2020	Ireland	Dublin	Human	5	5	8	5	11	9	11	8
C102	11	2020	Ireland	Dublin	Human	5	5	8	5	11	9	11	8
C187	11	2019	Ireland	Dublin	Human	5	5	8	5	11	9	11	8
C100	11	2018	Ireland	Dublin	Human	5	5	8	5	11	9	11	8
C183	11	2019	Ireland	Dublin	Human	5	5	8	5	11	9	11	8
C189	11	2019	Ireland	Dublin	Human	5	5	8	5	11	9	11	8
PH194	11	2019	Ireland	FARM 5	Porcine	5	5	8	5	11	9	11	8
PM200	11	2019	Ireland	FARM 8	Porcine	5	5	8	5	11	9	11	8
PH116	11	2019	Ireland	FARM 3	Porcine	5	5	8	5	11	9	11	8
PL11	11	2019	Ireland	FARM 1	Porcine	5	5	8	5	11	9	11	8
PH48	11	2019	Ireland	FARM 3	Porcine	5	5	8	5	11	9	11	8

Sample ID	ST	Collection Date	Country of Isolation	City of Isolation	Host	ST	adk	atpA	dxr	glyA	recA	sodA	tpi
PH49	11	2019	Ireland	FARM 4	Porcine	5	5	8	5	11	9	11	8
PH51	11	2019	Ireland	FARM 4	Porcine	5	5	8	5	11	9	11	8
PL27	11	2019	Ireland	FARM 2	Porcine	5	5	8	5	11	9	11	8
PL28	11	2019	Ireland	FARM 2	Porcine	5	5	8	5	11	9	11	8
PL29	11	2019	Ireland	FARM 2	Porcine	5	5	8	5	11	9	11	8
PL3	11	2019	Ireland	FARM 1	Porcine	5	5	8	5	11	9	11	8
PL6	11	2019	Ireland	FARM 1	Porcine	5	5	8	5	11	9	11	8
PL30	11	2019	Ireland	FARM 2	Porcine	5	5	8	5	11	9	11	8
PL31	11	2019	Ireland	FARM 2	Porcine	5	5	8	5	11	9	11	8
PL32	11	2019	Ireland	FARM 2	Porcine	5	5	8	5	11	9	11	8
PL41	11	2019	Ireland	FARM 2	Porcine	5	5	8	5	11	9	11	8
PL33	11	2019	Ireland	FARM 2	Porcine	5	5	8	5	11	9	11	8
PM198	11	2019	Ireland	FARM 6	Porcine	5	5	8	5	11	9	11	8
PL12	11	2019	Ireland	FARM 1	Porcine	5	5	8	5	11	9	11	8
PH193	11	2019	Ireland	FARM 5	Porcine	5	5	8	5	11	9	11	8
PM206	11	2019	Ireland	FARM 8	Porcine	5	5	8	5	11	9	11	8
C166	11	2018	Ireland	Dublin	Human	5	5	8	5	11	9	11	8
C80	11	2019	Ireland	Dublin	Human	5	5	8	5	11	9	11	8
C84	11	2018	Ireland	Dublin	Human	5	5	8	5	11	9	11	8
A141	11	2023	Ireland	ABATTOIR 3		5	5	8	5	11	9	11	8
PH114	11	2019	Ireland	FARM 3	Porcine	5	5	8	5	11	9	11	8
C172	11	2019	Ireland	Dublin	Human	5	5	8	5	11	9	11	8
C88	11	2020	Ireland	Dublin	Human	5	5	8	5	11	9	11	8
PH192	11	2019	Ireland	FARM 5	Porcine	5	5	8	5	11	9	11	8
PL118	11	2019	Ireland	FARM 1	Porcine	5	5	8	5	11	9	11	8
C78	11	2019	Ireland	Dublin	Human	5	5	8	5	11	9	11	8
PL90	11	2019	Ireland	FARM 2	Porcine	5	5	8	5	11	9	11	8
PL34	11	2019	Ireland	FARM 2	Porcine	5	5	8	5	11	9	11	8
PL4	11	2019	Ireland	FARM 1	Porcine	5	5	8	5	11	9	11	8
PL36	11	2019	Ireland	FARM 2	Porcine	5	5	8	5	11	9	11	8
PL37	11	2019	Ireland	FARM 2	Porcine	5	5	8	5	11	9	11	8

Sample ID	ST	Collection Date	Country of Isolation	City of Isolation	Host	ST	adk	atpA	dxr	glyA	recA	sodA	tpi
C119	11	2020	Ireland	Dublin	Human	5	5	8	5	11	9	11	8
C54	11	2019	Ireland	Dublin	Human	5	5	8	5	11	9	11	8
PL106	11	2019	Ireland	FARM 1	Porcine	5	5	8	5	11	9	11	8
PL1	11	2019	Ireland	FARM 1	Porcine	5	5	8	5	11	9	11	8
C20	11	2019	Ireland	Dublin	Human	5	5	8	5	11	9	11	8
PL2	11	2019	Ireland	FARM 1	Porcine	5	5	8	5	11	9	11	8
PH95	11	2019	Ireland	FARM 3	Porcine	5	5	8	5	11	9	11	8
PH93	11	2019	Ireland	FARM 3	Porcine	5	5	8	5	11	9	11	8
C176	11	2019	Ireland	Dublin	Human	5	5	8	5	11	9	11	8
A126	11	2023	Ireland	ABATTOIR 1		5	5	8	5	11	9	11	8
C83	11	2019	Ireland	Dublin	Human	5	5	8	5	11	9	11	8
PL16	11	2019	Ireland	FARM 1	Porcine	5	5	8	5	11	9	11	8
PL21	11	2019	Ireland	FARM 1	Porcine	5	5	8	5	11	9	11	8
C58	11	2019	Ireland	Dublin	Human	5	5	8	5	11	9	11	8
PL8	11	2019	Ireland	FARM 1	Porcine	5	5	8	5	11	9	11	8
C56	11	2019	Ireland	Dublin	Human	5	5	8	5	11	9	11	8
PH52	11	2019	Ireland	FARM 3	Porcine	5	5	8	5	11	9	11	8
C81	11	2019	Ireland	Dublin	Human	5	5	8	5	11	9	11	8
PL9	11	2019	Ireland	FARM 1	Porcine	5	5	8	5	11	9	11	8
C168	11	2019	Ireland	Dublin	Human	5	5	8	5	11	9	11	8
PL10	11	2019	Ireland	FARM 1	Porcine	5	5	8	5	11	9	11	8
C218	11	2018	Ireland	Dublin	Human	5	5	8	5	11	9	11	8
C219	11	2018	Ireland	Dublin	Human	5	5	8	5	11	9	11	8
C220	11	2018	Ireland	Dublin	Human	5	5	8	5	11	9	11	8
C221	11	2019	Ireland	Dublin	Human	5	5	8	5	11	9	11	8
PL7	11	2019	Ireland	FARM 1	Porcine	5	5	8	5	11	9	11	8
PH195	11	2019	Ireland	FARM 5	Porcine	5	5	8	5	11	9	11	8
PH96	11	2019	Ireland	FARM 3	Porcine	5	5	8	5	11	9	11	8
PH203	11	2019	Ireland	FARM 5	Porcine	5	5	8	5	11	9	11	8
PH205	11	2019	Ireland	FARM 5	Porcine	5	5	8	5	11	9	11	8
PH94	11	2019	Ireland	FARM 4	Porcine	5	5	8	5	11	9	11	8

Sample ID	ST	Collection Date	Country of Isolation	City of Isolation	Host	ST	adk	atpA	dxr	glyA	recA	sodA	tpi
PH22	11	2019	Ireland	FARM 4	Porcine	5	5	8	5	11	9	11	8
PH25	11	2019	Ireland	FARM 4	Porcine	5	5	8	5	11	9	11	8
PH26	11	2019	Ireland	FARM 4	Porcine	5	5	8	5	11	9	11	8
PH42	11	2019	Ireland	FARM 3	Porcine	5	5	8	5	11	9	11	8
PH43	11	2019	Ireland	FARM 3	Porcine	5	5	8	5	11	9	11	8
PH44	11	2019	Ireland	FARM 3	Porcine	5	5	8	5	11	9	11	8
PH45	11	2019	Ireland	FARM 3	Porcine	5	5	8	5	11	9	11	8
PH46	11	2019	Ireland	FARM 3	Porcine	5	5	8	5	11	9	11	8
PH47	11	2019	Ireland	FARM 4	Porcine	5	5	8	5	11	9	11	8
PM196	11	2019	Ireland	FARM 6	Porcine	5	5	8	5	11	9	11	8
PM199	11	2019	Ireland	FARM 6	Porcine	5	5	8	5	11	9	11	8
C125	11	2020	Ireland	Dublin	Human	5	5	8	5	11	9	11	8
C85	11	2018	Ireland	Dublin	Human	5	5	8	5	11	9	11	8
C124	11	2020	Ireland	Dublin	Human	5	5	8	5	11	9	11	8
C186	11	2019	Ireland	Dublin	Human	5	5	8	5	11	9	11	8
PL5	11	2019	Ireland	FARM 1	Porcine	5	5	8	5	11	9	11	8
C184	11	2019	Ireland	Dublin	Human	5	5	8	5	11	9	11	8
C185	11	2019	Ireland	Dublin	Human	5	5	8	5	11	9	11	8
C123	11	2020	Ireland	Dublin	Human	5	5	8	5	11	9	11	8
C177	11	2019	Ireland	Dublin	Human	5	5	8	5	11	9	11	8
C151	11	2018	Ireland	Dublin	Human	5	5	8	5	11	9	11	8
C173	11	2019	Ireland	Dublin	Human	5	5	8	5	11	9	11	8
C171	11	2019	Ireland	Dublin	Human	5	5	8	5	11	9	11	8
PH15	11	2019	Ireland	FARM 3	Porcine	5	5	8	5	11	9	11	8
A128	12	2023	Ireland	ABATTOIR 3		1	1	1	6	4	3	5	1
PL233	13	2019	Ireland	FARM 1	Porcine	1	1	1	6	1	5	3	1
PL235	13	2019	Ireland	FARM 1	Porcine	1	1	1	6	1	5	3	1
C74	133	2018	Ireland	Dublin	Human	1	1	1	7	1	1	3	3
C122	14	2019	Ireland	Dublin	Human	1	1	1	2	1	5	5	3
C120	14	2020	Ireland	Dublin	Human	1	1	1	2	1	5	5	3
C98	14	2018	Ireland	Dublin	Human	1	1	1	2	1	5	5	3

Sample ID	ST	Collection Date	Country of Isolation	City of Isolation	Host	ST	adk	atpA	dxr	glyA	recA	sodA	tpi
C188	14	2019	Ireland	Dublin	Human	1	1	1	2	1	5	5	3
C215	14	2019	Ireland	Dublin	Human	1	1	1	2	1	5	5	3
PL110	16	2020	Ireland	FARM 2	Porcine	1	1	1	2	6	1	3	1
PL109	16	2020	Ireland	FARM 2	Porcine	1	1	1	2	6	1	3	1
PL237	16	2019	Ireland	FARM 2	Porcine	1	1	1	2	6	1	3	1
PL14	16	2020	Ireland	FARM 1	Porcine	1	1	1	2	6	1	3	1
PH70	16	2020	Ireland	FARM 4	Porcine	1	1	1	2	6	1	3	1
PL111	16	2020	Ireland	FARM 2	Porcine	1	1	1	2	6	1	3	1
C153	16	2019	Ireland	Dublin	Human	1	1	1	2	6	1	3	1
C154	16	2019	Ireland	Dublin	Human	1	1	1	2	6	1	3	1
PH69	160	2020	Ireland	FARM 4	Porcine	1	2	5	2	6	1	3	1
C61	17	2019	Ireland	Dublin	Human	1	1	1	2	1	1	5	3
PH204	17	2019	Ireland	FARM 5	Porcine	1	1	1	2	1	1	5	3
C212	18	2018	Ireland	Dublin	Human	1	1	1	2	5	1	3	1
C79	2	2019	Ireland	Dublin	Human	1	1	1	2	1	5	3	1
C132	2	2019	Ireland	Dublin	Human	1	1	1	2	1	5	3	1
C169	2	2019	Ireland	Dublin	Human	1	1	1	2	1	5	3	1
C209	2	2019	Ireland	Dublin	Human	1	1	1	2	1	5	3	1
C136	2	2019	Ireland	Dublin	Human	1	1	1	2	1	5	3	1
C77	2	2019	Ireland	Dublin	Human	1	1	1	2	1	5	3	1
C19	2	2019	Ireland	Dublin	Human	1	1	1	2	1	5	3	1
C159	2	2019	Ireland	Dublin	Human	1	1	1	2	1	5	3	1
C163	2	2019	Ireland	Dublin	Human	1	1	1	2	1	5	3	1
C164	2	2019	Ireland	Dublin	Human	1	1	1	2	1	5	3	1
C165	2	2018	Ireland	Dublin	Human	1	1	1	2	1	5	3	1
C214	2	2019	Ireland	Dublin	Human	1	1	1	2	1	5	3	1
C75	2	2018	Ireland	Dublin	Human	1	1	1	2	1	5	3	1
C228	2	2020	Ireland	Dublin	Human	1	1	1	2	1	5	3	1
C229	2	2020	Ireland	Dublin	Human	1	1	1	2	1	5	3	1
C231	2	2020	Ireland	Dublin	Human	1	1	1	2	1	5	3	1
C210	2	2019	Ireland	Dublin	Human	1	1	1	2	1	5	3	1

Sample ID	ST	Collection Date	Country of Isolation	City of Isolation	Host	ST	adk	atpA	dxr	glyA	recA	sodA	tpi
C139	2	2020	Ireland	Dublin	Human	1	1	1	2	1	5	3	1
C134	3	2019	Ireland	Dublin	Human	1	1	1	2	1	1	1	1
C190	3	2019	Ireland	Dublin	Human	1	1	1	2	1	1	1	1
C86	3	2020	Ireland	Dublin	Human	1	1	1	2	1	1	1	1
C143	3	2020	Ireland	Dublin	Human	1	1	1	2	1	1	1	1
C59	35	2019	Ireland	Dublin	Human	1	2	5	8	1	1	3	6
C57	35	2019	Ireland	Dublin	Human	1	2	5	8	1	1	3	6
C181	36	2019	Ireland	Dublin	Human	1	2	1	2	3	1	5	1
C121	36	2018	Ireland	Dublin	Human	1	2	1	2	3	1	5	1
C180	36	2018	Ireland	Dublin	Human	1	2	1	2	3	1	5	1
C170	42	2019	Ireland	Dublin	Human	1	1	1	2	1	1	7	1
PL236	44	2019	Ireland	FARM 1	Porcine	1	2	5	2	1	1	3	1
PL239	44	2019	Ireland	FARM 1	Porcine	1	2	5	2	1	1	3	1
PL63	44	2020	Ireland	FARM 1	Porcine	1	2	5	2	1	1	3	1
C107	44	2020	Ireland	Dublin	Human	1	2	5	2	1	1	3	1
PH104	44	2020	Ireland	FARM 3	Porcine	1	2	5	2	1	1	3	1
PL105	44	2020	Ireland	FARM 1	Porcine	1	2	5	2	1	1	3	1
C135	44	2018	Ireland	Dublin	Human	1	2	5	2	1	1	3	1
PL13	44	2019	Ireland	FARM 1	Porcine	1	2	5	2	1	1	3	1
C216	44	2019	Ireland	Dublin	Human	1	2	5	2	1	1	3	1
C223	44	2019	Ireland	Dublin	Human	1	2	5	2	1	1	3	1
C226	44	2019	Ireland	Dublin	Human	1	2	5	2	1	1	3	1
PL67	45	2020	Ireland	FARM 1	Porcine	1	4	1	6	1	1	5	1
PL66	45	2020	Ireland	FARM 1	Porcine	1	4	1	6	1	1	5	1
PL64	45	2020	Ireland	FARM 1	Porcine	1	4	1	6	1	1	5	1
PL40	45	2020	Ireland	FARM 1	Porcine	1	4	1	6	1	1	5	1
PH202	46	2019	Ireland	FARM 5	Porcine	1	4	1	6	1	1	10	1
PH73	46	2020	Ireland	FARM 4	Porcine	1	4	1	6	1	1	10	1
C82	49	2019	Ireland	Dublin	Human	1	1	1	2	1	5	3	3
C162	49	2019	Ireland	Dublin	Human	1	1	1	2	1	5	3	3
C213	49	2019	Ireland	Dublin	Human	1	1	1	2	1	5	3	3

Sample ID	ST	Collection Date	Country of Isolation	City of Isolation	Host	ST	adk	atpA	dxr	glyA	recA	sodA	tpi
C227	49	2020	Ireland	Dublin	Human	1	1	1	2	1	5	3	3
C140	49	2019	Ireland	Dublin	Human	1	1	1	2	1	5	3	3
C146	53	2020	Ireland	Dublin	Human	1	1	2	2	1	1	5	1
C157	53	2020	Ireland	Dublin	Human	1	1	2	2	1	1	5	1
C137	56	2019	Ireland	Dublin	Human	1	1	3	6	3	1	5	1
C155	56	2020	Ireland	Dublin	Human	1	1	3	6	3	1	5	1
PL117	571	2019	Ireland	FARM 2	Porcine	1	2	1	7	5	1	5	1
C161	58	2020	Ireland	Dublin	Human	1	1	5	7	1	1	13	1
C179	58	2019	Ireland	Dublin	Human	1	1	5	7	1	1	13	1
C147	58	2020	Ireland	Dublin	Human	1	1	5	7	1	1	13	1
C211	6	2020	Ireland	Dublin	Human	1	2	1	6	1	1	5	1
C138	6	2019	Ireland	Dublin	Human	1	2	1	6	1	1	5	1
C97	6	2018	Ireland	Dublin	Human	1	2	1	6	1	1	5	1
PL62	7	2020	Ireland	FARM 1	Porcine	1	1	1	7	1	1	5	1
PH72	7	2020	Ireland	FARM 4	Porcine	1	1	1	7	1	1	5	1
PL92	75	2019	Ireland	FARM 2	Porcine	1	7	3	13	1	10	1	1
PL112	75	2020	Ireland	FARM 2	Porcine	1	7	3	13	1	10	1	1
PL238	75	2019	Ireland	FARM 2	Porcine	1	7	3	13	1	10	1	1
PL113	75	2020	Ireland	FARM 2	Porcine	1	7	3	13	1	10	1	1
PL35	75	2019	Ireland	FARM 2	Porcine	1	7	3	13	1	10	1	1
PL38	75	2019	Ireland	FARM 2	Porcine	1	7	3	13	1	10	1	1
PL115	75	2019	Ireland	FARM 2	Porcine	1	7	3	13	1	10	1	1
PL91	75	2019	Ireland	FARM 2	Porcine	1	7	3	13	1	10	1	1
PL39	75	2019	Ireland	FARM 2	Porcine	1	7	3	13	1	10	1	1
C101	8	2020	Ireland	Dublin	Human	1	1	1	2	6	1	5	1
C178	8	2019	Ireland	Dublin	Human	1	1	1	2	6	1	5	1
C133	8	2019	Ireland	Dublin	Human	1	1	1	2	6	1	5	1
C191	8	2020	Ireland	Dublin	Human	1	1	1	2	6	1	5	1
PM197	8	2019	Ireland	FARM 6	Porcine	1	1	1	2	6	1	5	1
C174	8	2019	Ireland	Dublin	Human	1	1	1	2	6	1	5	1
C76	8	2018	Ireland	Dublin	Human	1	1	1	2	6	1	5	1

Sample ID	ST	Collection Date	Country of Isolation	City of Isolation	Host	ST	adk	atpA	dxr	glyA	recA	sodA	tpi
C129	8	2018	Ireland	Dublin	Human	1	1	1	2	6	1	5	1
C60	8	2019	Ireland	Dublin	Human	1	1	1	2	6	1	5	1
C17	8	2019	Ireland	Dublin	Human	1	1	1	2	6	1	5	1
C217	8	2020	Ireland	Dublin	Human	1	1	1	2	6	1	5	1
C230	8	2020	Ireland	Dublin	Human	1	1	1	2	6	1	5	1
C130	8	2019	Ireland	Dublin	Human	1	1	1	2	6	1	5	1
C144	8	2019	Ireland	Dublin	Human	1	1	1	2	6	1	5	1
C145	8	2019	Ireland	Dublin	Human	1	1	1	2	6	1	5	1
C148	8	2019	Ireland	Dublin	Human	1	1	1	2	6	1	5	1
C149	8	2020	Ireland	Dublin	Human	1	1	1	2	6	1	5	1
C175	8	2019	Ireland	Dublin	Human	1	1	1	2	6	1	5	1
C156	8	2020	Ireland	Dublin	Human	1	1	1	2	6	1	5	1
C158	8	2019	Ireland	Dublin	Human	1	1	1	2	6	1	5	1

Supplementary Table 3 Genotypic resistance profiles of 55 *C. difficile* ST11 isolates

Obtained from Ridom® Seqsphere⁺. Amino acid substitutions for Quinolone resistance and Rifampicin resistance are detailed.

Sample ID	Collection Date	Country of Isolation	City of Isolation	Host	Source Subtype	MLS_B	Quinolone	Rifampicin	Tetracycline
PH44	2019	Ireland	FARM 3	Porcine	animal		gyrB_S366V / gyrB_S416A		tet(M)
PH93	2019	Ireland	FARM 3	Porcine	animal		gyrB_S366V / gyrB_S416A		tet(M)
PL118	2019	Ireland	FARM 1	Porcine	animal		gyrA_T82I / gyrB_S366V / gyrB_S416A		tet(M)
C84	2018	Ireland	Dublin	Human	human		gyrB_S366V / gyrB_S416A		tet(40) / tet(M)
C99	2019	Ireland	Dublin	Human	human		gyrB_S366V / gyrB_S416A		tet(M)
PH45	2019	Ireland	FARM 3	Porcine	animal		gyrB_S366V / gyrB_S416A		tet(M)
PL29	2019	Ireland	FARM 2	Porcine	animal		gyrA_T82I / gyrB_S366V / gyrB_S416A		tet(40) / tet(M)
PH192	2019	Ireland	FARM 5	Porcine	animal		gyrA_T82I / gyrB_S366V / gyrB_S416A		tet(M)
PL106	2019	Ireland	FARM 1	Porcine	animal		gyrA_T82I / gyrB_S366V / gyrB_S416A		tet(40) / tet(M)
PH52	2019	Ireland	FARM 3	Porcine	animal		gyrA_T82I / gyrB_S366V / gyrB_S416A		tet(40) / tet(M)
PH116	2019	Ireland	FARM 3	Porcine	animal		gyrB_S366V / gyrB_S416A		tet(M)
PL1	2019	Ireland	FARM 1	Porcine	animal		gyrA_T82I / gyrB_S366V / gyrB_S416A		tet(40) / tet(M)
PL34	2019	Ireland	FARM 2	Porcine	animal		gyrA_T82I / gyrB_S366V / gyrB_S416A		tet(40) / tet(M)
C172	2019	Ireland	Dublin	Human	human	erm(B)	gyrA_T82I / gyrB_S366V / gyrB_S416A		tet(M)
C151	2018	Ireland	Dublin	Human	human		gyrB_S366V / gyrB_S416A		tet(M)
PH49	2019	Ireland	FARM 4	Porcine	animal		gyrB_S366V / gyrB_S416A		tet(M)
PL21	2019	Ireland	FARM 1	Porcine	animal		gyrA_T82I / gyrB_S366V / gyrB_S416A		tet(40) / tet(M)
C220	2018	Ireland	Dublin	Human	human		gyrA_T82I / gyrB_S366V / gyrB_S416A		tet(40) / tet(M)
C168	2019	Ireland	Dublin	Human	human		gyrA_T82I / gyrB_S366V / gyrB_S416A		tet(M)
PH193	2019	Ireland	FARM 5	Porcine	animal		gyrA_T82I / gyrB_S366V / gyrB_S416A		tet(M)
C173	2019	Ireland	Dublin	Human	human		gyrA_T82I / gyrB_S366V / gyrB_S416A		tet(M)
C176	2019	Ireland	Dublin	Human	human		gyrA_T82I / gyrB_S366V / gyrB_S416A		tet(M)
PL31	2019	Ireland	FARM 2	Porcine	animal		gyrA_T82I / gyrB_S366V / gyrB_S416A		tet(40) / tet(M)
PL5	2019	Ireland	FARM 1	Porcine	animal		gyrA_T82I / gyrB_S366V / gyrB_S416A		tet(40) / tet(M)
C209	2019	Ireland	Dublin	Human	human		gyrB_I139R		
PL30	2019	Ireland	FARM 2	Porcine	animal		gyrA_T82I / gyrB_S366V / gyrB_S416A		tet(40) / tet(M)
PM196	2019	Ireland	FARM 6	Porcine	animal		gyrA_T82I / gyrB_S366V / gyrB_S416A		

Sample ID	Collection Date	Country of Isolation	City of Isolation	Host	Source Subtype	MLS_B	Quinolone	Rifampicin	Tetracycline
PL28	2019	Ireland	FARM 2	Porcine	animal		gyrA_T82I / gyrB_S366V / gyrB_S416A		tet(40) / tet(M)
PH114	2019	Ireland	FARM 3	Porcine	animal		gyrB_S366V / gyrB_S416A		tet(M)
PH51	2019	Ireland	FARM 4	Porcine	animal		gyrA_T82I / gyrB_S366V / gyrB_S416A		tet(40) / tet(M)
PL12	2019	Ireland	FARM 1	Porcine	animal		gyrA_T82I / gyrB_S366V / gyrB_S416A		tet(40) / tet(M)
PH25	2019	Ireland	FARM 4	Porcine	animal		gyrB_S366V / gyrB_S416A		tet(M)
PH26	2019	Ireland	FARM 4	Porcine	animal		gyrA_T82I / gyrB_S366V / gyrB_S416A		
PL90	2019	Ireland	FARM 2	Porcine	animal		gyrA_T82I / gyrB_S366V / gyrB_S416A		tet(40) / tet(M)
C56	2019	Ireland	Dublin	Human	human		gyrB_S366V / gyrB_S416A		tet(M)
PH22	2019	Ireland	FARM 4	Porcine	animal		gyrB_S366V / gyrB_S416A		tet(M)
C119	2020	Ireland	Dublin	Human	human		gyrA_T82I / gyrB_S366V / gyrB_S416A		tet(40) / tet(M)
PL27	2019	Ireland	FARM 2	Porcine	animal		gyrA_T82I / gyrB_S366V / gyrB_S416A		tet(40) / tet(M)
C100	2018	Ireland	Dublin	Human	human		gyrA_T82I / gyrB_S366V / gyrB_S416A		tet(40) / tet(M)
C88	2020	Ireland	Dublin	Human	human		gyrA_T82I / gyrB_S366V / gyrB_S416A		tet(M)
C87	2020	Ireland	Dublin	Human	human		gyrB_S366V / gyrB_S416A		tet(M)
C166	2018	Ireland	Dublin	Human	human		gyrB_S366V / gyrB_S416A		tet(40) / tet(M)
PL41	2019	Ireland	FARM 2	Porcine	animal		gyrA_T82I / gyrB_S366V / gyrB_S416A		tet(40) / tet(M)
C124	2020	Ireland	Dublin	Human	human	cfr(B) / erm(Q)	gyrB_S366V / gyrB_S416A	rpoB_R505K	tet(M)
C85	2018	Ireland	Dublin	Human	human		gyrA_T82I / gyrB_S366V / gyrB_S416A		tet(44) / tet(M)
C83	2019	Ireland	Dublin	Human	human		gyrB_S366V / gyrB_S416A		tet(M)
C125	2020	Ireland	Dublin	Human	human		gyrA_T82I / gyrB_S366V / gyrB_S416A		tet(40) / tet(M)
C102	2020	Ireland	Dublin	Human	human		gyrA_T82I / gyrB_S366V / gyrB_S416A		tet(M)
C78	2019	Ireland	Dublin	Human	human		gyrB_S366V / gyrB_S416A		tet(M)
C219	2018	Ireland	Dublin	Human	human		gyrA_T82I / gyrB_S366V / gyrB_S416A		tet(44) / tet(M)
C127	2020	Ireland	Dublin	Human	human	erm(B)	gyrA_T82I / gyrB_S366V / gyrB_S416A		tet(M)
C171	2019	Ireland	Dublin	Human	human		gyrA_T82I / gyrB_S366V / gyrB_S416A		tet(40) / tet(M)
C80	2019	Ireland	Dublin	Human	human		gyrB_S366V / gyrB_S416A		tet(M)
C81	2019	Ireland	Dublin	Human	human		gyrB_S366V / gyrB_S416A		tet(M)
C123	2020	Ireland	Dublin	Human	human		gyrA_T82I / gyrB_S366V / gyrB_S416A		tet(44) / tet(M)