MOLECULAR CHARACTERISATION OF CLOSTRIDIUM PERFRINGENS ISOLATES FROM PATIENTS AT GROOTE SCHUUR HOSPITAL

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DECLARATION

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Table of Contents

List of figures

List of tables

List of abbreviations

Amoxi-clav	Amoxicillin/clavulanic acid
AAM	Acetic acid medium
AMR	Antimicrobial resistant/resistance
BA	Blood agar
BHIB	Brain heart infusion broth
Bp	Base pair
°C	Degree Celsius
CARD	Comprehensive Antibiotic Resistance Database
CLI	Clindamycin
CLSI	Clinical and Laboratory Standards Institute
Cpa/plc	Alpha toxin
C. perfringens	*Clostridium perfringens*
CPB	Beta toxin
CPB2	Beta2 toxin
CPE	*Clostridium perfringens* enterotoxin
DNA	Deoxyribonucleic acid
dNTP	Deoxyribonucleotide triphosphate
*Erm*Q	Erythromycin resistance methylase
ELISA	Enzyme-linked immunosorbent assay
ETX	Epsilon-toxin
FBO	Food-borne outbreak
gDNA	genomic DNA
GIT	Gastrointestinal tract
HIV	Human immunodeficiency virus
Imi	Imipenem

Itx	Iota-toxin
MIC	Minimum Inhibitory Concentration
MDR	Multidrug resistant
MLS	Macrolide-lincosamide and streptogramin
MGC	Mitsubishi Gas Chemical
MgCl2	Magnesium chloride
MLST	Multi-locus sequencing type
Mtz	Metronidazole
Mxf	Moxifloxacin
NCBI	National Center for Biotechnology Information
NEC	Necrotising enterocolitis
NetB	Necrotic enteritis B-like toxin
NHLS	National health laboratory service
PCR	Polymerase chain reaction
PenG	Penicillin G
PFO	Perfringolysin O
rRNA	Ribosomal Ribonucleic Acid
Pip-taz	Piperacillin/tazobactam
RSA	Republic of South Africa
Taq	DNA polymerase from *Thermus aquaticus*
WGS	Whole Genome Sequencing

1. Abstract

Clostridium perfringens is a spore-forming bacterium isolated from various mammals and birds. In humans it causes infections ranging from non-lethal diarrhoea to tissue-destructive, lethal diseases. Infection originates from spores found in environmental reservoirs such as soil, faecal-contaminated water, and food. Infection can also be caused, when the organisms disseminate from the gastrointestinal tract (GIT) of human carriers to the rest of the body. South Africa (RSA) lacks published data on the specific strains and toxin types responsible for human clostridial infections, as only identification and drug susceptibility testing are done in routine laboratories. The main aim of this study was to characterise 19 clinical isolates of *C. perfringens* (collected between 2017- 2020) using phenotypical and molecular methods such as PCR-based toxin typing, antimicrobial susceptibility testing and whole genome sequencing. Strains were isolated from patients with invasive clostridial disease. In agreement with other studies, toxin typing confirmed that all isolates encode the Phospholipase C (*plc)* gene which encodes for the tissue-destructive alpha toxin. Of the 19 isolates tested, $^{16}/_{19}$ (84 %) belonged to Type A, i.e., these isolates only contained the alpha toxin (*cpa*) and not the other toxins used for profiling. The remaining 16% belonged to type F, thus code for both alpha and enterotoxin (*cpe*). Toxin types B, C, D, E and G, i.e., strains with PCR amplicons for the beta, epsilon, iota and NetB genes, were not found amongst this set of isolates. All isolates were susceptible to the antibiotic piperacillin (and its lactamase inhibitor partner tazobactam), the carbapenem imipenem and the fluoroquinolone moxifloxacin. Clinical resistance was observed against amoxicillin/clavulanic acid (5%) and penicillin G (10%). Overall, the least effective antibiotics were clindamycin (68%) and metronidazole (68%), with isolates exhibiting either intermediate or complete resistance. Whole genome analysis of a subset of 5 mono-and multidrug resistant (MDR) isolates did not reveal known resistance factors encoding for metronidazole resistance, but the genetic determinant for macrolide resistance (*erm*Q) was identified in one clindamycin resistant isolate. WGS also revealed that the genome of 1/5 isolates clustered with previously published human isolates originating from non-lethal gastroenteritis cases, although the enterotoxin (*cpe)* gene was not identified either via PCR-based toxin typing or whole genome analysis. The genomes of the remaining 4/5 isolates cluster with published genomes originating from the environment, birds, or unidentified animals. Multi-locus sequencing type (MSLT) reveals two isolates (GSH 46 and GSH 48) belonged to a single MLST ST274,

indicating that they were from the same origin.

2. Problem statement

Current knowledge of toxin and strain types causing human clostridial diseases in RSA is lacking. The global re-emergence of *C. perfringens* as a pathogen negatively impacting human, animal, and bird health (and thus the bioeconomy) requires appropriate epidemiological data collection, antibiotic resistance profiling and genome studies to facilitate One Health (and appropriate public health) measures (1–9). In developed countries such as USA, *Clostridium perfringens* has been reported as the second most common bacterial cause of foodborne illness, with nearly one million cases of foodborne illnesses each year (10–12), resulting in a net economic loss of US $382 million (13). While in UK it has been reported to cause ~80 000 cases of self-limiting illnesses per year (14). Therefore, there is a need to study the organism and expand our knowledge of disease- causing strains in our clinical settings, notably as RSA has a high incidence of persons with risk factors for *Clostridium* infections (for example diabetics), a large population with weakened immune system due to morbidities such as Human immunodeficiency virus (HIV), one of the highest incidences of tuberculosis (TB) requiring extended and multiple antibiotics for therapy (15–18). Previous reports have shown a strong association between TB, HIV, and diabetes (19,20). A significant portion of the South African population is also geriatric and lives in poor health and poverty in areas without proper sanitation and water (17,21,22). These factors are known risks for clostridial gastrointestinal disease. Furthermore, due to declining quality of resources such as water, the risk for gastrointestinal disease increases where access to safe drinking water is limited. Furthermore, a recent South African study identified multiple virulence factors and antibiotic resistance genes in a set of local, environmental *C. perfringens* isolates obtained from a riverbed close to a human settlement (23). Similar studies in the veterinarian and human health care settings could inform of risk factors if patient metadata and environmental reservoirs are known. Notably, genomic data can provide information regarding the relationship, disease transmission and genetic divergence between human, environmental and agricultural isolates. Toxin typing further would be valuable as a quick method, as it specifically could link isolates to industries such as poultry farming and associated practices that would be driving antibiotic resistance. However, no genetic studies are done during local surveys of anaerobic bacteria isolated from humans, therefore we have limited information regarding the genetic relatedness of the strains and toxin type of invasive disease-causing *C. perfringens*. The study described here is a first step in conducting a more in-depth analysis of recently isolated human, clinical isolates. Phenotypic

and genotypic data generated from these *C. perfringens* isolates obtained from local health care settings will advance our knowledge of strains and toxin types present in our setting.

3. Literature review

3.1. Members of the genus *Clostridium* and human disease

The genus *Clostridium* was first described in 1880 by Prazmowski (24), and includes Gram-positive, rod-shaped anaerobic bacteria that are capable of forming spores. While some spore-forming members of the *Clostridium* genus are either not human pathogens or rarely reported to cause human disease, the genus *Clostridium* contains several disease-causing species such as *C. botulinum, C. perfringens* (formerly *C. welchii*), *C. difficile* (recently reassigned to the genus *Clostridioides* (25), *C. baratti, C. septicum, C. leptum* and *C. tetani* (26).

Infection typically develops following ingestion of contaminated food or water, or contamination of tissue with spores from the soil. Clinical manifestations of infection with pathogenic species include self-limiting, non-lethal food poisoning as well as potentially lethal sequalae such as emphysematous cholecystitis, tetanus, botulism, pseudomembranous colitis, gas gangrene, bacteraemia, and septic shock, which is characterised by extensive red cell haemolysis, disseminated intravascular coagulation (DIC) and rapid death (26,27). A major contributor to disease establishment is the production of extracellular toxins that are responsible for tissue destruction and lysis of red blood cells (28–30). Indeed, members of this genus produce more toxins than any other known bacterial genus, including some that are ranked amongst the most lethal toxins described to date (26,31). In this regard, *C. perfringens*, the focus of the current project and this review, is particularly significant, producing more than 20 enzymes and toxins, the highest number identified in any single species (32,33). Unsurprisingly, *C perfringens*, is the most commonly cited etiological agent of clostridial disease in humans and is second only to members of the *Bacteroidetes* phylum as a leading cause of all anaerobe infections (34).

C. perfringens is ubiquitously present in a range of environments. While it has been isolated from diseased individuals (those with a self-limiting gastroenteritis or invasive disease) it has also been observed in the gastrointestinal tracts of humans and animals in the absence of disease, suggesting that it occasionally exists as a commensal (35–37). Furthermore, it has been isolated from soil, water (various water sources including the soil underneath water bodies), contaminated vegetables, and sewage (35–37). The organism grows over a range of temperatures (20 °C to 50°C), with an optimum

growth temperature of 45°C (38,39). This enables survival in diverse environments, including partially cooked, contaminated meat and poultry. *C. perfringens* strains that cause food poisoning are known to be heat resistant and their spores can survive common cooking procedures (40,41).

3.2. The *C. perfringens* genome

3.2.1. General features

The genus *Clostridium* originally contained a diverse group of bacteria, now divided into 20 clusters based on phylogenies constructed using ribosomal RNA sequences. Cluster I comprise the type species (*Clostridium butyricum*) and closely related species such as *C. perfringens* (42,43). Species from Cluster I are regarded as *Clostridium sensu stricto*, true representatives of spore-forming clostridial and phylogenetically distinct from other clusters (44).

C. perfringens was the first Gram-positive anaerobic bacterium for which a genetic map was elucidated (28,45). Typically, the *C. perfringens* genome size ranges from 2.8–4.1 Mb with a low GC content of 28.6% (46–49). The observed genome size variation is hypothesised to be due to extensive horizontal transfer of extrachromosomal elements such as insertion sequences and plasmids amongst *C. perfringens* isolates in the environment (46,47). Using the concept of genome openness (indicating the ability of a species to acquire new genes and identification of members of the species harbouring novel genes not yet described in the species pangenome), the *C. perfringens* openness index was higher than 51 other bacteria analysed. This suggests a greater ability of this microbe to obtain new genetic elements (49,50).

Numerous attempts to classify *C. perfringens* using various typing schemes (toxin typing, MLST *etc.*) have been published. One of the first comprehensive genome analyses used 56 published genomes and identified a pangenome of 11667 genes (49,50). This analysis revealed a plastic, diverse pangenome, comprising only 12.6% core genes while 87.4% of genes were attributed to the accessory genome (46). A more recent extensive core genome analysis of a larger dataset (using 282 genomes, from diverse environments) and MLST of 1,431 highly conserved core genes confirmed that *C. perfringens* strains clusters into 5 phylogroups I-V (47). Genome analysis also revealed the presence of putative novel toxins whose role in disease is currently unknown (1). Other interesting features of the available *C. perfringens* genomes are incomplete pathways for the biosynthesis of amino acids and tricarboxylic acid (TCA) cycle, which is expected in an obligately anaerobic microbe (48). However, an abundance of genes code for enzymes in fermentation and glycolytic pathways are

typically observed. These assist *C. perfringens* to invade the host and degrade the host tissues providing essential amino acids (48).

3.2.2. Mobile genetic elements and virulence

Notably some toxin genes and enzymes in *C. perfringens* are found exclusively on the chromosome, while others can be located on either plasmids or the chromosome (51). An example is the *cpe* gene, which encodes the gastroenteritis-causing enterotoxin (CPE) (12). Toxin-bearing plasmids frequently also carry antimicrobial resistance elements and serve as a reservoir for the spread of antimicrobial resistance (28,51,52). The presence of virulence and antimicrobial resistance encoding genes on plasmids contributes to the risk of strains acquiring these genes and functions via horizontal gene transfer (51). As a consequence of this, strains of the same toxin type are not necessarily closely related at a phylogenetic level (46).

C. perfringens plasmids range between small (2.4Kb) to very large (404 kb) in size (51). Some *C. perfringens* strains carry more than one plasmid, with some plasmids carrying more than one distinct toxin gene (47,51,53). Most *C. perfringens* toxin-carrying plasmids are closely related, suggesting a common evolutionary origin. Horizontal gene transfer is aided by mobilizable plasmids containing all elements necessary for gene transfer between *C. perfringens* isolates. These plasmids are classified in 3 groups, based on their characteristic conjugative apparatus. These groups are the Tcp, the Pcp and the pIP404 plasmids. Of these, the Tcp plasmids are unique to *C. perfringens* and contain the *tcp* locus, which contains a set of genes encoding proteins necessary for mediating highly efficient conjugative transfer of these plasmids (54,55). In the gastrointestinal tract, transfer of such plasmids from an infecting strain to *C. perfringens* already present in the normal intestinal microbiota may help to amplify and prolong an infection, (51,56)

3.2.3. Whole genome sequence to study the molecular epidemiology of *C. perfringens*

Several studies demonstrate the utility of whole genome sequence (WGS) analysis to discern the environmental sources and epidemiology of outbreak strains, enabling the design of appropriate preventative strategies. Using WGS to analyse the *C. perfringens* strains is of importance, as *C. perfringens* has been reported to be the main cause of foodborne outbreaks and other severe diseases. In the United States of America (USA), *C. perfringens* was reported to be the second cause of gastroenteritis which is related to food poisoning with approximately million cases per year (12).

Analysis of a set of foodborne outbreak strains (FBOs), collected over a period of five years from cases in England, showed that >95% of the outbreak strains harboured the enterotoxin-encoding *cpe* gene (2). However, outbreaks were primarily caused by strains where the *cpe* gene was carried on one of two different plasmids (pCPF5603 or pCPF4969), with only 14% of outbreak isolates carrying *cpe* on the chromosome. In the same analysis, WGS indicated that a more closely related subset of strains associated with 9 FBOs shared a common source (district care homes). Other interesting observations include the delineation of the isolates into seven lineages, with lineage I being relatively distinct from the other lineages. Strains from the care facility could also be distinguished from isolates from cases not living in care homes. For example, isolates obtained from care homes generally encoded a larger number of toxins compared to isolates from non-care home cases. This is significant because lineages coding for more toxin genes are hypothesised to be more virulent (40). Isolates from the care-home cases, specifically from lineages II–IV, also had identical toxin profiles (mostly), including sialidase genes (*nanI*, *nanJ* and *nanH*), the haemolysin gene *pfo*, and *cpb*2 (coding for beta-2 toxin (CPB2). With the exception of tetracycline-resistance genes, acquired antimicrobial resistance genes were mostly lacking in care home isolates. Amongst the non-care home strains, lineage I was characterised by strains with the same set of toxin genes (including *cpe*) and lacking other specific toxins *(pfo, cpb2* and *nanI*), and less than 20% of these isolates encoded tetracycline-resistance genes. Most of the non-care home isolates clustering in the other six lineages also encoded toxin genes *cpe*, *nanI* and *pfo*, while about one third of these isolates also encoded *cpb*2. However, these six lineages were enriched for tetracycline resistance. A more recent publication, reporting on the analysis of 30 Finnish isolates of foodborne origin as well as 260 available genomes, further demonstrated that *cpe*-bearing strains used in the analysis were exclusively observed in phylogenetic clusters III, IV, and V (183/290 strains analysed) (57). Yet another study, using 185 *C. perfringens* published genomes from isolates of various origins (animals, humans, and birds), revealed that more than 99% of sequences analysed encoded the alpha toxin, collagenase, a sialidase encoded by *nanH*, and alpha-clostripain, whereas the beta toxin, epsilon toxin, iota toxin, and binary enterotoxins were represented in less than 5% of strains. The *netB* toxin was only observed in avian isolates. Similarly, the NetE, NetF, and NetG encoding genes were specific to canines and horses, raising the possibility that these genes are host specific. These are useful observations and could be used to trace reservoirs of disease-causing isolates (53).

3.2.4. *C. perfringens* WGS in South Africa

In RSA, genomic information for Clostridia is scarce. The only *Clostridium* genome reported from a local clinical setting is that of a recently isolated, single case of infant botulism (58). Recently the genomes of *C. perfringens* cultures from water sediment were published (23). These genomes had genomic features observed in other reported, publicly available genomes from outside of Africa (46,47,53). In addition to the paucity of genome information, little is known regarding toxin carriage and diversity in clinical *C. perfringens* strains causing disease in RSA. Toxin typing of *C. perfringens* isolates is not routinely done in local diagnostic laboratories, unless foodborne outbreaks occur, and even then, is typically limited to an immune test to discern the presence of the CPE toxin. A research report, compiled by the Centre for Enteric Diseases (National Institute of Communicable Diseases, RSA) indicated "that the scope of pathogens tested for is typically very restricted, and therefore a range of foodborne pathogens will not be detected even if they are present" (59). One recent publication reported on a small outbreak in a psychiatric care facility in Cape Town. Two affected individuals demised; however, no strain information was collected beyond detection of the presence or absence of the CPE enterotoxin detection in faecal samples (60). Nevertheless, this paper highlighted the risk of foodborne outbreaks in care facilities, also noting a concern for persons using specific medications and increased risk for clostridial disease.

In summary, various phylogenetic analysis of publicly available genomes confirms earlier observations that *C. perfringens* isolates are (mostly) host and disease specific. Genetic analyses have revealed the presence of five phylogroups (I-V), of which phylogroup I originates mostly from foodborne disease and darmbrand isolates of human origin (47,61). Phylogroups II -V contain isolates from different hosts (animals, humans, and environment), indicating potential spread and transmission between hosts. Another observation made for phylogroup I was that associated strains tended to have smaller genomes, suggesting a loss of chromosomal genes and an increase in mobile elements (47).

3.2.5. Multilocus sequence typing

Multilocus sequence typing (MLST) is a commonly used technique, which is based on gene-to-gene sequencing. It was first proposed by Maiden *et al*. (1998), and expands traditional single gene microbial genotyping (e.g., 16S rRNA) to include a panel of conserved targets (62). Internal fragments of multiple conserved housekeeping genes distributed throughout the genome are amplified and assigned an allele identity based on the specific nucleotide sequence (63). Different MLST systems have been developed for *C. perfringens* strains to ensure consistent and reproducible

characterisation of *C. perfringens* strains. In 2006, a MLST system was developed by Jost *et al*. (2006) using 132 strains from different animal species (64). Additionally, Deguchi *et al*. (2009) developed another MLST system that targets eight genes including collagenase (*colA)*, heat shock protein *(groEL)*, superoxide dismutase (*sodA*), phospholipase C *(plc)*, DNA gyrase B gene (*gyrB*), sporulation sigma factors (*sigK*), phosphofructokinase gene (*pgk*) and quinolinate synthetase (*nadA)* for *C. perfringens* strains (65). These systems have been useful in tracing the pathogens involved in outbreaks and causing infections in different geographic locations (64,65).

3.3. Virulence factors of *C. perfringens*

Virulence in *C. perfringens* is driven by a large cohort of toxins and enzymes, mostly regulated by the VirR/VirS system (48). Toxins are the major driver of virulence and are categorised as major toxins (based on lethality in mouse models) and minor toxins (not lethal). The plasmids which carry these toxin genes can be lost or transferred leading to various *C. perfringens* toxin types (66). Importantly, toxin encoding genes can be found in one or more plasmids. A subset of the major toxins is used to classify the organism into seven toxin types (1). These toxin types are associated with specific disease manifestations and are host-specific, although they are sporadically also isolated from other hosts. The toxins used to type *C. perfringens* are the alpha (encoded by *cpa* or *plc*), beta (*cpb*), epsilon *(etx*), iota (*Itx*) (66), *C. perfringens* enterotoxin (*cpe*) and (*NetB*) toxins (shown in Table 3.1 and Table 3.2) (67). A multiplex PCR-based typing scheme using specific primers to discern the presence of toxins in an isolate has been published (67,68). The toxin types have either one (only *plc*) or a combination of *plc* and other major toxins. Thus far, no isolate has been found to harbour all toxins at once. Importantly, given the high rate of horizontal gene transfer between strains, toxin typing is not a useful indicator of genetic relatedness (69,70).

Table 3.1: Mode of action and location of genes encoding major toxins

Toxin (Gene)	Location	Mode of action	Ref
Alpha (*plc*)	Chromosome/ Plasmid	Phospholipase C and sphingomyelinase	(71,72)
Beta (*cpb*)	Plasmid	Pore-forming	(73)
Epsilon (*etx*)	Plasmid	Pore-forming	(33,51,74)
Iota (*itx*)	Plasmid	ADP-ribosylation of actin	(75,76)
CPE (*cpe*)	Chromosome / Plasmid	Pore-forming, disrupts tight junctions	(77,78)
Net B (*NetB*)	Plasmid	Pore-forming	(79)

Table 3.2: Disease caused by *C. perfringens* in humans and animals. Based upon Petit *et al*. (1999) and Rood and Cole. (1991)

Toxinotype	Toxin						Disease
	Alpha	Beta	Epsilon	Iota	CPE	NetB	
A	+						Human: Gas gangrene Animal: Gas gangrene
B	+	+	+				Human: Enteritis necroticans Animal(s): dysentery, necrotizing enteritis
C	+	+					Human: Enteritis necroticans Animal(s): Necrotizing enteritis and enterotoxaemia
D	+		+				Human: Multiple sclerosis Animal: Dysentery and Enterotoxaemia
E	+			+			Animal: Enteritis
F	+				+		Human: Food poisoning, non-foodborne infection Animal: Enteritis
G	+					+	Animal: Necrotic enteritis

3.3.1. **Alpha toxin**

The chromosomally encoded alpha toxin was the first *C. perfringens* toxin to be described and widely studied (80). This toxin is encoded by the phospholipase C (*plc* or *cpa*) gene and is present in all *C. perfringens* strains (71). Its mode of action is cytolytic and haemolytic, as it plays a role in host cell membrane disorganization through its phospholipase C and sphingomyelinase activity (81). The result is massive toxin-mediated cell lysis. Lysis of red blood cells (RBCs) is an observed feature, although in some instances infected RBCs are not lysed but the haemoglobin drained, giving rise to so called 'ghost cells'. The alpha toxin is located in a conserved and stable region of the *C. perfringens* chromosome and is believed to be a key virulence factor in type A strains acting synergistically with the perfringolysin O (PFO) toxin in the establishment of gas gangrene (45,82,83). Although all *C. perfringens* strains harbour alpha toxin, expression of the toxin was found to be higher in type A strains (strains in which the alpha toxin is the only major toxin) compared to other toxin types (72). Type A toxin strains are the major cause of myonecrosis (gas gangrene) in humans and associated with a high mortality rate (84).

3.3.2. Beta toxin

The Beta toxin is a pore-forming toxin encoded on a plasmid carried by *C. perfringens* type B and C strains. It is encoded by a *cpb* gene was previously discovered to be located on a large plasmid with size between 65 and 110 kb. Other virulence-related genes like *cpe* and *tpel* may also be encoded by these *cpb*-carrying plasmids (78,85,86). CPB prototoxin of about 336 amino acids which produces a matured toxin when there is a cleavage of 27 amino acids during secretion (78,85,86).

C. perfringens type B and C strains are responsible for necrotic enteritis (mainly in animals), and human disease conditions called darmbrand and pigbel (87). The toxin creates pores in red blood cells (RBCs) and other human cell types, leading to cell lysis. In the presence of protease inhibitors, it produces necrotic lesions of the GIT (88). Trypsin inhibits the beta toxin activities in the small intestine (88). This toxin is also reported to have neurological effects. This toxin is also reported to have neurological effects. Beta toxin shares sequence similarity with *staphylococcus* toxins (85,89,90) . The plasmid that encodes this toxin can also encode other genes (85,89,90).

3.3.3. Epsilon toxin

The Epsilon toxin is a pore-forming toxin, which is also encoded on a plasmid (33,51,74). It is produced by *C. perfringens* type B and D strains and is responsible for fatal animal enterotoxaemia,

particularly in sheep. While these strains are a rare cause of human infections, recently some tentative links were made between the presence of this toxin and multiple sclerosis (91).

The epsilon toxin is secreted as an inactive protoxin and is activated by proteolytic cleavage in the GIT (92). The characteristics of the epsilon toxin, normally seen in enterotoxaemia caused by toxin types B and D, include an increase in vascular permeability of the brain, kidney, and intestine. The epsilon toxin is considered a potential bioterrorism agent and is reckoned amongst the top 3 deadliest toxins known to man after *C. botulinum* and *C. tetani* neurotoxins (93). Infections in humans are rare, but possible. Laboratories are not allowed to work with strains carrying this toxin, except with permission from the South African government (94). Novel epsilon toxins have been described recently in human isolates, although these must still be characterised (68).

3.3.4. Iota toxin

Type E isolates produce the cytotoxic and dermonecrotic iota toxin. The toxin comprises two units, encoded by two iota genes found on a plasmid (75). The two major functional units of this binary toxin comprise a binding component and an ADP-ribosylating component. Strains bearing this toxin cause haemorrhagic enteric disease in cattle (75). Limited information is available regarding this infection, as toxin type E is a rare cause, and more studies are needed.

3.3.5. *C. perfringens Enterotoxin (CPE)*

The enterotoxin mediates non-lethal food poisoning and non-foodborne related diarrhoea in humans. It was first identified in *C. perfringens* type A-producing enterotoxin strains, which later became regarded as type F strains (67). It is encoded on either the chromosome or on a plasmid and is present in approximately 5% of *C. perfringens* isolates. Chromosomally encoded *cpe* strains are more heat-resistant compared to those that harbour the *cpe* gene on a plasmid, although the reasons for this are unclear (40,95). This toxin forms spores that are able to withstand harsh conditions such as high temperature, high pressure, toxic chemicals, and radiation (96). The ability of cpe, pore-forming toxin, to bind to and necrotise human ileal and colonic epithelium in vitro is significant. It also has the ability to cause cell death, or apoptosis, via the caspase-3 pathway. Therefore, the tight junction rearrangements caused by CPE could be a potential pathogenesis mechanisms underlying food poisoning (97). Enterotoxin is different from other *C. perfringens* toxin as it is not a secreted toxin but rather it is produced during sporulation, upon lysis of the mother cell. It has been reported that enterotoxin-bearing strains, on average, have a smaller genome size than other *C. perfringens* strains

(2,47). Also, in majority of food poisoning strains characterised, the *cpe* associated with Tn*5565* is flanked by *IS*1470 mobile sequence, whereas isolates from non-food borne gastrointestinal disease carry the *cpe* gene on plasmids flanked by the IS1470-like sequence or the IS1151 sequence (98–101)

While infections with CPE-producing strains are generally not lethal, death has been recorded in the young and elderly people. In the USA, *Clostridium perfringens* has been reported as the second most common bacterial cause of foodborne illness, with nearly one million cases of foodborne illnesses each year (102), reportedly causing nearly one million cases of foodborne illnesses in the USA each (11,12). While in UK, it has been reported to cause ~80 000 cases of self-limiting illnesses per year (14). One study from England performed a phylogenic analysis of *C. perfringens* strains that were present in food, and also from human (4). They reported WGS to have a high discriminatory power when compared to other standard laboratory methods (4). As expected, 96.3% (105/109) carried the *cpe* gene either in chromosome or plasmid. Interestingly 4/109 isolates were *cpe*-negative suggesting an alternative virulence gene. 16S rDNA sequence analysis, multi-locus sequence typing (MLST), and toxin gene profiling are also used during foodborne outbreaks.

3.3.6. NetB toxin

The NetB toxin is a pore-forming cytotoxin involved in the formation of lethal lesions in the GIT. *C. perfringens* isolates from poultry are associated with the presence of the NetB toxin, where they are responsible for necrotic enteritis disease. The rise of this disease in poultry has been linked directly to the widespread use of antibiotics (103,104). NetB carrying *C. perfringens* strains are not known to affect humans (33).

3.3.7. Other toxin and enzymes

In addition to the known virulence-related gene used for toxinotyping such as alpha toxin, *C. perfringens* secrete various minor toxins and other enzymes (Table 3.3) (1,48). These enzymes play an important part in degrading the host tissues (1,48).

3.4. Disease caused by *C. perfringens*

C. perfringens causes both non-lethal and lethal disease in humans and animals (33). The ability of this bacterium to proliferate very rapidly and to produce spores that are resistant to common

household disinfectants (including hypochlorite), coupled with the potential to produce a range of deadly toxins and tissue destructing enzymes contribute to its pathogenicity and virulence (33,96).

Immune-compromised persons are at risk of developing clostridial disease, especially if they undergo GIT surgery, have solid tumours and/or require extensive antibiotic use. For example, due to diabetes-related ischaemic conditioning of parts of the body, persons living with diabetes are at risk for infections by anaerobes such as *C. perfringens*, both in the lower limbs and other body parts (16,18,105) (16,18,106).

An emerging risk has been noted for persons living chronically with HIV, the result of a rise in so-called "non-AIDS associated solid tumour malignancies" in such persons (107). Internal infections in solid tumours are typically micro-aerophilic or even anaerobic, thus providing an ideal environment for anaerobic bacteria. Indeed, there are indications that many clostridial bacteraemia could be related to spread of the organism from infected tumours (108,109). Interestingly, due to their ability to effectively lyse solid tumours, some species such as *C. novyi* are considered as potential therapeutic agents to effectively remove such tumours, albeit in a genetically modified, less lethal form that can be administered safely (110,111).

Table 3.3 Other *C. perfringens* toxins and enzymes based on modified *Kiu and Hall* (1)

Name	Gene	Location
alpha-clostripain	*cloSI*	Chromosome
Sialidase	*nanJ nanH* and *nanI*	Chromosome
Collagenase	*colA*	Chromosome
Hyaluronidase	*nagJ, nagL, nagH nagI* and *nagK*	Chromosome
Perfringolysin O	*pfoA*	Chromosome
Beta2	*cpb2*	Plasmid
Binary Enterotoxin	*becA and becB*	Plasmid
Tpel	*Tpel*	Plasmid
Lambda (mu)	*Lam*	Plasmid
Binary enterotoxin	*becA, becB*	Plasmid
Net E, Net F and Net G	*netE, netF, netG*	Plasmid

3.4.1. Disease in animals

Clostridial enteric disease is a major problem in the food and poultry industries, especially amongst new-born animals and chicks (104,112,113). Farmed animals due for human consumption, therefore,

present a risk of humans being exposed to contaminated meat. (104,112,113). Livestock are affected by clostridial necrotic enteritis, dysentery (especially lambs) and bovine enterotoxaemia. The poultry industry is impacted by necrotising enteritis, which is associated with high mortality (8,114) and is a global economic concern (8,104,113,115). Necrotising enteritis in poultry is caused by *C. perfringens* type G strains and affects mostly chickens with symptoms including wet litter and diarrhoea. One predisposing factor is the use of feed that contains high protein, which is now known to stimulate the growth of Clostridia. Co-infection with *Eimeria* spp. is another contributing factor, which due to the associated damage to the gut epithelium, facilitates the entry and proliferation of *C. perfringens* (116).

In RSA, there is limited information regarding *C. perfringens* types found in animals. A report from, Madoroba *et al*. (2010) (117), reported a predominance of type A and type D strains, which are capable of causing serious infections such as enterotoxaemia in South African animals. These data are consistent with work done by other researchers in Italy and Egypt, who reported a predominance of types A and D in sheep with suspected enterotoxaemia (118,119). In the northern part of RSA, the death of pigs suffering from haemorrhagic bowel syndrome was associated with the presence of *C. perfringens* (112).

3.4.2. Disease in humans

3.4.2.1. Lethal myonecrosis

Commonly known as gas gangrene, this anaerobic infection results from the entry of *C. perfringens* cells or spores into a wound (120,121), with vegetative cells or spores usually from the soil or the human gut. Escape from the human gut is facilitated by damage to GIT mucosa. Although in humans the incidence of this infection is very low, mortality rate is relatively high, especially if left untreated.

Myonecrosis is mostly caused by *C. perfringens* strains producing only the alpha (*plc*) toxin (82,122). Murine models have shown that mutation of the *plc* gene diminishes strain virulence, which is restored by complementation with a wild-type *plc* gene (82). Hence, the secretion of this toxin is significantly associated with the development of gas gangrene infection and the characteristic presence of gas bubbles in an infected wound. Synergism between the alpha toxin and perfringolysin (*pfo*) has been reported, aiding disease progression (82).

Different pre-clinical animal models studying gas gangrene development showed that an infectious dose of 10^9 colony forming units (or spores) is needed for an infection to be established (33,123,124). The disease is characterised by tissue destruction, resulting in decreased blood flow to the tissue,

thereby contributing to a favourable anoxic environment for the growth and proliferation of *C. perfringens*, which enhances the progression of myonecrosis (125). Once tissue destruction ensues, a significant indication of clostridial myonecrosis is the visible production of gas bubbles. If this infection is left untreated, it is fatal, hence a need for urgent diagnosis and therapy (28). Toxaemia can ultimately lead to shock, multi-organ failure and death (125).

3.4.2.2. Enteric disease caused by *C. perfringens*

Food poisoning caused by *C. perfringens* was first reported in the USA and the United Kingdom (UK) in the 1940s, where symptoms appeared after ingesting contaminated food with high numbers of the bacterium (5,126,127). Foodborne outbreaks were reported from food that is raw or not cooked properly and/or has been stored incorrectly (which allows the growth of bacterial cells) (128). The production of enterotoxin in the intestine causes fluid secretion thereby leading to diarrhoea (28). Disease symptoms typically manifest 8-12 hours after ingesting the contaminated food, presenting with acute abdominal cramps, nausea, and diarrhoea (126). *C. perfringens* enterotoxin (*cpe*) is the major virulence factor involved in this illness and functions by disrupting the tight barrier junctions in the intestinal epithelium. Foodborne infection is usually self-limiting leading to under-reporting of cases caused by this organism. In the USA, *C. perfringens* has been reported to be the second most common cause of foodborne illness with approximately one million cases reported per annum (12). Even though this illness is usually self-limiting, mortality in young and elderly patients has been seen (1).

Foodborne outbreaks caused by *C. perfringens* are a global problem. In the past few years there have been several foodborne outbreaks reported across the globe, with *C. perfringens* Type F (previously known as *C. perfringens* type A enterotoxin-producing strains) the most common cause of both foodborne and non-foodborne illnesses. Most of these outbreaks occur in group settings, such as psychiatric facilities with patients receiving psychoactive drugs, hospitals, restaurants, and care facilities for elderly people. (129,130).

A recent review from the National Institute of Communicable Diseases (NICD, South Africa) stated that there are limited studies reporting FBOs in RSA, including those caused by *C. perfringens* (131). Furthermore, incomplete reporting and inadequate sampling and laboratory procedures contribute to limited epidemiological data regarding FBOs in RSA. According to this report, from 2013-2017 there were 327 recorded outbreaks (all organisms, not just *C. perfringens*), with 11155 people involved and 49 deaths (131). From the 147 stool samples tested, 8% yielded *C. perfringens*, second in prevalence only to *Salmonella* species. From tested food samples, it was the 4th most frequent organism isolated,

after *Salmonella*, *E. coli* and *Bacillus cereus* with 3% of samples culture positive (4/132) (131). The report did not indicate specific *C. perfringens* toxin types responsible, although generally type F is the cause of FBOs. A recent report described an outbreak at a psychiatric hospital in the Western Cape Province of RSA, where two patients died due to food-poisoning associated with *C. perfringens* (60,132). Unfortunately, since no genetic typing was done, the virulence or other factors in the isolates responsible for the two deaths, which are unusual for self-limiting diarrhoea, is unknown.

Other *C. perfringens* non-foodborne illnesses include antibiotic-associated diarrhoea and sporadic diarrhoea which are usually long-lasting and more severe when compared to food poisoning cases (133).

3.4.2.3. Enteritis necroticans

C. perfringens type C strains cause fatal enteritis necroticans, called darmbrand in Germany and pigbel in New Guinea and both of which are related to the consumption of pork (134,135). This illness was firstly described in northern Germany and Denmark after World War II (136). Symptoms include abdominal cramps, bloody diarrhoea, occasional vomiting, and dehydration followed by inflammation of the small intestine (134,135,137). A major virulence factor contributing to infection is the beta toxin, with observations confirmed using animal models (138). The mortality rate for enteritis necroticans ranges between 10- 40% and the disease is mostly found in countries where there is severe malnutrition of protein origin (128,134,135,137).

Individuals with low trypsin levels, due to a protein-poor diet or pancreatic disease, or those with diets containing high concentrations of trypsin inhibitors, are at higher risk of developing enteritis necroticans (128,137). Trypsin inhibitors prevent the degradation of beta toxin. Another important factor involved in infection progression is reduced intestinal motility, resulting in increased exposure of the toxin to the intestinal walls (139). Immunisation against this toxin decreases its effect on the host and the incidence of this disease. The disease has also been described in diabetic patients (140–142). Notably diabetic patients are more prone to bacterial opportunistic infections, such as clostridial gas gangrene, due to decreased blood circulation, oxygen supply and peripheral neuropathy (143,144). The presence of tissue necrosis in hyperglycaemic environment favours the growth and proliferation of bacteria (143,144).

3.4.2.4. Necrotising enterocolitis (NEC) of neonates and preterm infants

NEC significantly affects neonates and is mostly lethal, with *C. perfringens* being one of the microbial causes (145,146). It is characterised by abdominal distension, portal venous gas and pneumoperitoneum, arterial hypotension, poor circulation, and gangrene of the colon with serious consequences (145,146). A notable risk factor for NEC is pre-term birth and low-birth weight in infants. NEC occurs in 1-5% of neonates admitted to the intensive care unit (ICU) (147,148) and is associated with significant mortality of up to 50% if not treated in time (149). Though rarely seen in full term births, occasional cases have been reported (147,148). Breastfeeding is protective compared to formula feeding, indicating the presence of protective immune factors or potentially protection offered by the breastmilk microbiota (147). Previous outbreaks have been associated with crowded nurseries where there are high incidences of gastrointestinal illnesses among carers (150).

3.4.3. Other diseases associated with *C. perfringens*

3.4.3.1. Multiple Sclerosis

The presence of epsilon toxins have been described in the brain tissue of persons suffering from multiple sclerosis (MS) (91). It is hypothesised that epsilon toxins cross the intestinal barrier and enter the circulatory system. They damage particularly kidney and brain tissue (151). In the brain they target oligodendrocytes and lead to their demyelination (152). Apart from brain tissue, the epsilon toxin has been previously isolated from human intestinal contents and stool of patients who presented with toxaemias, gas gangrene and multiple sclerosis (151,153,154). About 10% of patients suffering from multiple sclerosis have been reported to have antibodies that are specific for epsilon toxin (151). The presence of the epsilon toxin or epsilon toxins in MS is interesting and warrants investigation to discern the role of the toxin in disease manifestation.

3.5. Reservoirs of *C. perfringens*

C. perfringens ranks among the most widespread organisms found in soil, sewage, and water (35–37,155). It is also isolated from the gastrointestinal tract (GIT) and urine of some humans and animals (33), while spores have been found in tested water and air (37). The ability of this bacterium to survive for a long period in different environments is due to the production of these hardy spores, which enables it to withstand extreme conditions (156). Once vegetative cells or spores enter a suitable host, they germinate and grow rapidly if the environment is favourable (anaerobic). *C. perfringens* has the

shortest cell division time recorded for culturable bacteria, with a generation time of approximately 6-8 minutes in a suitable growth medium (28,157). Therefore, the organism has become a popular genetic model for studying the *Clostridium* genus (28).

It is important to note that *C. perfringens* can be isolated from faecal samples of both diseased and non-diseased (carriers) humans and animals, indicating that it can live as a commensal of the GIT (158). Due to its ubiquity in nature, it has been suggested as a useful indicator of faecal contamination in water, representing hardy organisms that are not sensitive to chemical disinfection of sewage (159,160). Human carriers are a potential reservoir for type A chromosomally encoded *cpe* positive isolates, and as a result they can introduce these bacteria into foods during handling (161,162). Supporting this possibility, a few faecal samples from healthy people in Finland and Japan were found to contain type A chromosomal-*cpe* isolates, confirming carriers as a potential reservoir for spreading the infection (161,162).

C. perfringens cpe positive strains are also found in soil. In these isolates the *cpe* gene is encoded on the plasmid. These strains are a potential reservoir for horizontal gene transfer to other Clostridia (129). There has been recent progress towards understanding why the type F isolates carrying a chromosomal *cpe* gene are often associated with classical food poisoning outbreaks due to *C. perfringens* (163). These strains produce a small soluble product, protein 4 (SS4), which binds to DNA (deoxyribonucleic acid). A specific variant of this SS4 protein is characterised by more effective binding to DNA and associated with an increase in clostridial resistance to heat and sodium nitrite. This could explain survival of the organism in contaminated food (40,41). More recently, a review further highlighted the role of small acid soluble proteins (SASPs), including SS4, in DNA protection from degradation and damage through binding between SASPs and bacterial DNA. These tiny proteins are only 60 to 70 amino acids in length and highly conserved amongst spore-formers such as the Clostridia (164). The SASPs are present in high concentration in spores and are tightly bound to DNA. They are only degraded and removed from DNA once the spore germination has occurred (164)

C. perfringens has been reported in the surface water systems of various countries, including RSA, where it occurs as a contaminant (23,160). This poses a risk of infection to animals and humans in rural communities where a lack of potable water necessitates the use of potentially spore-containing contaminated water systems as a source for their livestock and households (37). Indeed, a recent National Institute for Communicable Diseases (NICD) report described contaminated water samples as potential sources of contamination causing a bacterial foodborne outbreak in humans (59). Furthermore, a worrisome observation in another study done in SA, revealed that *C. perfringens* was

also found in human urine from individuals in eThekwini, indicating that carriers are shedding this microbe via their urine (17). Bearing in mind that urine is a resource to manufacture fertiliser, this is problematic as spores may survive the manufacturing process and contaminate vegetable products grown using contaminated fertiliser (17). The abovementioned reports suggest the need for improved monitoring of water sources, especially where humans are living next to rivers and other water sources and use these resources for their households. Also, where ablution and proper sewage systems are not available, citizens using the environment for urination and defecation (for example in the veld or public spaces) might also contribute to dissemination of spores in the environment.

3.6. Laboratory characterisation of *C. perfringens*

Rapid diagnosis of *C. perfringens* infection is a key factor contributing to a positive patient outcome (109,165,166). A retrospective study demonstrated that over a period of 10 years, early detection of the pathogen improved patient health outcomes (109). *C. perfringens* invasive infection diagnoses are based on the presence of clinical signs (gas bubbles in infected tissue are a well-recognised factor) and pathology (extensive tissue destruction, presence of so-called ghost red blood cells). Routinely, microbiological testing includes anaerobic growth on blood agar, biochemical analysis, Enzyme-linked immunosorbent assay (ELISA), and serology testing using type-specific antisera (167). Diagnostic laboratories also use automated systems such as the Vitek2 and mass spectrometers such as VITEK MS (bioMérieux, Marcy-l'Étoile, France) and Bruker MS (Billerica, Massachusetts, USA) to identify isolates based on their produced peptide profiles (168,169). Other phenotyping could also include noting the colony morphology and testing for the production of lecithinase, haemolysins, proteases, lipases (phospholipase C) and collagenase. A notable feature of the *C. perfringens* bacterium is the presence of a double zone of haemolysis on blood agar plates. Given that *C. perfringens* has a rapid growth rate, improved patient outcomes are observed if a diagnosis is made sooner as this organism rapidly disseminates in the body (109). In fact, the most effective therapy is often to remove the focal point of infection (for example an infected solid tumour) entirely (109). For self-limiting diarrhoea, faecal matter of patients with diarrhoeal symptoms suggestive of *C. perfringens*-induced diarrhoea is tested for the presence of the CPE enterotoxin (11).

PCR methods are useful in terms of sensitivity and rapid turnaround time, although they are not often used in local diagnostic settings to identify *Clostridium* spp. due to cost. However, these could identify the organism and simultaneously detect the presence of pathogen-specific toxin genes. The use of PCR and 16S rRNA sequencing has improved the characterisation and correct identification of *Clostridium* spp in clinical microbiology laboratories where they are employed (170,171).

Molecular techniques are also useful to clarify if bacteria are present in culture-negative blood cultures, where the clinical picture suggests bacterial infection, especially if ghost cells are present (172). Recently, some molecular tests such as qPCR which detects 5 serotypes (A-E) at once and a loop-mediated isothermal amplification (LAMP) in combination with a lateral-flow biosensor (LFB) which detect one of the conserved toxin gene (*cpa*) have been described, although these are not yet used in most diagnostic laboratories (for example https://www.nzytech.com/en/molecular-diagnostics/vet-fod-pharma/food-water-safety/clostridium-perfringens-qpcr-kit/ (173).

3.7. *C. perfringens* susceptibility to antimicrobials

Over the years re-emergence of antibiotics resistance in *C. perfringens* has been a concern. In certain countries antibiotics such as penicillin, bacitracin, tetracycline *etc* have been used as prophylaxis and growth promoters in animal feeds (174,175). Due to increase in the development of antimicrobial resistance and the transfer of antibiotic resistant genes from animals to humans have led to the banning of antibiotic feeds as growth promoters in the European countries (9). Tetracycline resistance has been found to reach up to 75% in poultry and human isolated samples (7,176). Furthermore, antimicrobial resistance to alternative medicine such as metronidazole and clindamycin have been observed (177–179). In Iran, 11.39% and 16.46% of *C. perfringens* from human samples, were resistant to metronidazole and clindamycin respectively (177). Surveys across the globe have confirmed the need for improved detection and susceptibility screening of anaerobic bacteria due to the emergence and spread of its resistance to multiple antimicrobial drugs. The increase in resistance has an impact on public health food and agriculture in the world and is a critical component of the 'One Health' paradigm. The latter recognises the interdependence of human, animal, poultry, crops, and environmental health (180). In RSA, published literature on antimicrobial susceptibility for anaerobic bacteria reported 100% susceptibility of *C. perfringens* strains isolated in Bloemfontein to several classes of drugs used to treat anaerobic infections (181). Decreased susceptibilities were observed for clindamycin (86%) and ciprofloxacin (99%) respectively) (181). A more recent study by Meyer *et al.* (2006) reported that 81% of *Clostridium* spp. strains isolated in Pretoria between 2003-2004 were susceptible to clindamycin (182), although *C. perfringens* was not specifically mentioned. Other than the abovementioned reports, there is a paucity of local studies on clinical *C. perfringens* isolates, with only a 2011 study reporting that *C. perfringens* isolates comprised 13% of all anaerobes obtained (183). Isolates were only resistant to clindamycin, while a *C. septicum* isolate was highly resistant to metronidazole (>256 µg/ml).

None of these studies reported genetic data on local *C. perfringens* clinical isolates, leaving a knowledge gap pertaining to strain origins and epidemiology.

Antimicrobial resistance occurs when bacteria acquire genetic changes or acquire resistance genes that allow them to survive and grow in the presence of antimicrobial drugs that would normally inhibit or kill them (184). Some studies have shown varying levels of resistance in *C. perfringens* to different antimicrobial agents (185). The following are mechanism of resistance in *C. perfringens*:

(i) Beta-lactams: *C. perfringens* can produce beta-lactamases, enzymes that can inactivate beta-lactam antibiotics such as penicillin and cephalosporins (186). The beta-lactamase, encoded by *bla* genes, are relatively common in *C. perfringens* isolates (23) Moreover, resistance to beta-lactam antibiotics can also be mediated by a decreased affinity of a single essential penicillin-binding protein (187).

(ii) Macrolides: Resistance to macrolide antibiotics, such as erythromycin, clindamycin has been reported in *C. perfringens*. Resistance to clindamycin can be caused by the presence of erythromycin resistant methylase genes (*erm*) (188). These include *erm* (B) and *erm* (Q) (188–190). Furthermore, clindamycin resistant can also be mediated by efflux pumps or modification of the target site (191).

(iii) Tetracyclines: Resistance to tetracycline antibiotics, has been observed in some *C. perfringens* isolates and this resistance can be due to the presence of tetracycline-resistance genes and or efflux pumps (192,193). The tetracycline-resistance genes include, *tetA*(P), *tetB*(P), *tetA408*(P), *tetM* and *tetQ* (194–196).

(iv) Fluoroquinolones: *C. perfringens* isolates have shown varying levels of resistance to fluoroquinolone antibiotics, such as ciprofloxacin. This resistance can be associated with mutations in the bacterial DNA gyrase or topoisomerase IV genes.

It is important to note that the prevalence of antimicrobial resistance in *C. perfringens* can vary geographically and over time. Resistance patterns can also differ among different strains and clinical isolates. The appropriate treatment for *C. perfringens* infections should be based on the susceptibility testing of the specific isolate obtained from the patient.

To effectively manage and prevent the further development of antimicrobial resistance, it is crucial to promote the appropriate and responsible use of antimicrobial agents, both in human medicine and

in veterinary practices. This includes using antibiotics only when necessary, following prescribed treatment regimens, and adhering to infection prevention and control measures.

3.8. Potential uses of *C. perfringens*

As mentioned before, *C. perfringens* are considered by some as a potential useful indicator of faecal contamination of water, industrial by-products, food, and medical products. This is primarily due to the fact that the spores of Clostridia are less sensitive to chemical treatment and adverse environments and thus could persist in water and soil (159,197). The immune-stimulating properties of the alpha toxin is also useful and, as a result, the protein is used as an adjuvant in some vaccines (198,199). Additionally, a recently developed TB vaccine using the antigenic properties of the PFO toxin showed improved immune responses in tested participants (200).

4. Rationale of study

C. perfringens is regarded as a re-emerging threat to humans and animals, affecting health and impacting economic outputs if not controlled. It is responsible for foodborne disease and clostridial myonecrosis (gas gangrene) in humans (1,128,179). Furthermore, an increase in the spread of antibiotic resistance amongst *C. perfringens* strains poses a further threat to positive patient outcomes (3,179,201). While patient outcomes can be improved if potentially lethal clostridial infections are diagnosed earlier, the need for specialised apparatus for anaerobic culture hampers routine drug susceptibility testing and typing of anaerobes such as *Clostridium* spp. As a result, strain reservoirs and genetics for anaerobic bacteria are not as well studied as for aerobes and there is no knowledge of the genotypes or toxin types of clinical strains currently causing clostridial disease in RSA. This information is critical to direct patient management and infection prevention strategies. For example, toxin typing of clinical isolates could provide valuable information about strain origins and identify infections with potential zoonotic origins. Furthermore, if drug resistant isolates of zoonotic origin are detected, it could imply that one needs to look at antimicrobial use in animals and birds reared for human consumption. Such information could inform policy guidelines and changes in farming practices that are more aligned with antibiotic stewardship principles. Clinically, improved knowledge of the organism is increasingly relevant in the sub-Saharan African setting, which has a disproportionately high burden of immune-compromised persons at risk for anaerobe infections. It is even more relevant for those with extended hospital stays, surgery, patients with intravenous lines and on antibiotic therapy, all risk factors for *C. perfringens* antibiotic associated diarrhoea and bacteremia.

With these points in mind, the current study aims to do in-depth analyses of recently isolated clinical strains from patient samples submitted for routine diagnostic analysis to a public, microbiology diagnostic laboratory in Cape Town, South Africa.

5. Aim of study

To characterise clinical isolates of *C. perfringens* using antimicrobial susceptibility phenotyping, toxin typing and whole genome sequence analysis.

6. Objectives

6.1. To confirm identity using 16S ribosomal RNA gene PCR and Sanger sequencing

6.2. To do toxin typing of *C. perfringens* isolates, using PCR to target selected toxins

6.3. To do antimicrobial susceptibility testing of *C. perfringens* isolates

6.4. To do whole genome analysis of selected clinical isolates and discern the presence of drug resistance and virulence genes.

6.5. To investigate the genomic variability and phylogenetic relatedness of the isolates

7. Methods and Materials

7.1. *C. perfringens* collection and culture

7.1.1. Ethical considerations

The protocol for this study was approved by the Ethics Committee of the University of Cape Town, HREC number 785/2019. Faculty Biosafety Committee Approval number LP/001/2020-2023.

7.1.2. Bacterial collection

A total of 19 non-duplicate *C. perfringens* isolates, recovered from clinical samples at Groote Schuur Hospital Microbiology Laboratory, National Health Laboratory service (NHLS), (Cape Town, South Africa) were analysed. Isolates were originally obtained during routine diagnostic culturing of clinical specimens from patients with suspected anaerobic infections. They were collected between November 2017 till March 2020. Isolates were pre-identified at NHLS via routine diagnostic procedures (colony, cellular morphology, and Vitek 2 ANC card ((BioMérieux, France)) (202)) as *C. perfringens* and were stored at -80^{0}C until further use.

7.1.3. Culture media

In this study, the stored, de-identified isolates were recovered and cultured on blood agar plates and in brain heart infusion (BHI, Appendix, Table 12.1) broth at 37°C for 24-48 h under anaerobic conditions using the AnaeroPack system (MGC, Japan). Blood agar, supplied by Greenpoint Media (National Health Laboratory Service [NHLS], South Africa), contained Brucella agar supplemented with 5% lysed horse blood, 5 mg/L hemin and 1 mg/L vitamin K (hereafter referred to as “BA”). Pure isolates obtained from the original stored cultures were stored at the Division of Medical Microbiology (University of Cape Town [UCT]) at −80°C and preserved in Cryobank vials containing BHI and 25% (v/v) of glycerol. Further sub-culturing was done using BHI broth.

7.2. Characterisation of isolates

7.2.1. Phenotypic characterisation.

Colonies were visually inspected for typical *C. perfringens* morphology on BA plates. Colonies presenting as large, flat colonies with irregular edges and exhibiting a double zone of haemolysis were presumptively identified as *C. perfringens* (Appendix, Figure 12.1). Observation of Gram-positive bacilli (Appendix, Figure 12.1) in Gram stains, as well as the absence of growth during aerobic incubation and finally, the presence of gas in liquid cultures were further putative confirmation. Colonies satisfying the above-mentioned criteria were selected for downstream processes (PCR identification, toxin typing, and antibiotic sensitivity testing was performed).

7.2.2. Molecular characterisation of *C. perfringens*

7.2.2.1. Extraction of genomic DNA from clinical isolates

Genomic DNA (gDNA) was isolated using commercial extraction kits (Quick-DNA fungal/bacterial miniprep kit [Zymo Research, USA]) as well as the Purelink Microbiome DNA Purification Kit (Invitrogen, USA) following the manufacturer's instructions. The quantity and quality of the extracted gDNA were determined using a Biodrop Duo spectrophotometer (www.biodrop.co.uk). The isolated gDNA was stored at -20^0 C until further use. The quality of extracted gDNA was further analysed using 1.5% (w/v) agarose gels submerged in 1x tris-acetate-EDTA (TAE) buffer (Promega, Wisconsin, USA) as previously described (203). The preparation of an agarose gels is described in Appendix 12.1.

7.2.2.2. Primers

Previously published primers (67) used to amplify fragments of the target genes (this study) were purchased from the commercial providers Inqaba Biotec (https://inqababiotec.co.za) and Integrated DNA Technologies (IDT, www.eu.idtdna.com), Table 7.1. Lyophilised primers were reconstituted in 1x tris-EDTA buffer (TE, pH8 [Sigma Aldrich]) to a final concentration of 100µM each and stored at -20°C. For PCR, primer stocks of 10 µM and 20 µM were diluted in PCR-grade water and used for PCR mixes.

Table 7.1: Primer sets used in this study

Toxin	Forward (5'-3')	Reverse (5'-3')	Size (bp)	Ref*
Alpha (*plc*)	GCTAATGTTACTGCCGTTGA	CCTCTGATACATCGTGTAAG	324	(204)
Beta (*cpb*)	GCGAATATGCTGAATCATCTA	GCAGGAACATTAGTATATCTTC	196	(204)
Epsilon (*etx*)	GCGGTGATATCCATCTATTC	CCACTTACTTGTCCTACTAAC	656	(204)
Iota *(iap*)	ACTACTCTCAGACAAGACAG	CTTTCCTTCTATTACTATACG	446	(204)
Enterotoxin (*cpe*)	GGAGATGGTTGGATATTAGG	GGACCAGCAGTTGTAGATA	233	(204)
NetB	CTTCTAGTGATACCGCTTCAC	CGTTATATTCACTTGTTGACGAAAG	738	(67)
16S rRNA primers	AGAGTTTGATCCTGGCTCAG[1]	AAGGAGGTGATCCAGCCGCA[2]	1400	(205)

[1]16S rRNA primer 27F, [2] rRNA primer 1541R (205), bp; base pair, Ref; Reference

7.2.2.3. Polymerase chain reaction (PCR) amplification

All PCR reactions for bacterial identification (16S rRNA), alpha toxin and other toxins were performed in a final volume of 25µl using a thermal cycler (Applied Biosystems). PCR amplicons were separated by gel electrophoresis in 1.5% (w/v) agarose gels as described in Appendix 12.2. Unless stated otherwise, PCRs were done using the Promega GoTaq PCR reagents (Promega, Wisconsin, USA) (Table 7.2). The PCR cycling conditions were as follows: An initial denaturation step of 95ºC for 10 min, followed by 35 cycles with a denaturation step at 94ºC for 30 sec, annealing at 55ºC for 30 seconds and extension at 72ºC for 45 seconds (90 min for 16S rRNA PCR total time). A final extension at 72ºC for 5 minutes, followed by a holding step at 12ºC completed the PCR.

Table 7.2: PCR reagent compositions

PCR component	1 x reaction (µl)	Concentration of reagents
Nuclease-free water	13.75	-
5X Green Go Taq buffer	5	1 X
$MgCl_2$ (25mM)	2	2 mM
dNTPs (10mM)	0.5	0.2 mM
Primers (Forward) (10µM)	0.5	0.2 µM
Primers (Reverse) (10µM)	0.5	0.2 µM
Taq Polymerase (5 U/ µl)	1.25	0.25U/µl
Template DNA*	1.5	
Total	25 µl	

*DNA concentrations were not standardised, but all PCRs contained less than 100ng of template

7.2.2.4. PCR to identify *C. perfringens* alpha toxin

Although isolates were identified as *C. perfringens* during routine diagnostic evaluations, the identities of colonies were further confirmed via a single-plex PCR to detect the presence of the *plc* gene. This gene is unique to *C. perfringens* and present in all isolates described thus far in the literature (71). An amplicon with a length of 324bp was regarded as confirmation of identity as *C. perfringens*.

7.2.2.5. Bacterial identification using 16S rRNA gene PCR and Sanger sequencing

Genetic confirmation of identity was done by amplification and sequencing of the 16S rRNA gene from isolated organisms. Reaction mixtures were as described in Table 7.2, with PCR cycling program conditions as described in the section 7.2.2.3, using universal primers 27F and 1541R (205,206) in Table 7.1. The 25µL uniplex PCR to detect toxins contained 1.5 µL of purified gDNA (>100 ng), 5 µL of 5× PCR buffer, 2 mM of MgCl2, 0.2 mM of dNTPs, 0.25U/µl of Taq polymerase and 0.2µ M of primers. To confirm the identity, 16S PCR products were analysed via Sanger dideoxy sequencing at Inqaba biotech (https://inqababiotec.co.za/), with sequencing in one direction using the 27F primer (207,208). The resulting sequences were compared to the National Center for Biotechnology Information (NCBI) nucleotide database using the Basic Local Alignment Search Tool (BLAST 2.11.0+**)** (209,210), to confirm the species identity of the isolates.

7.2.2.6. Uniplex PCR to identify *C. perfringens* toxin individually

Toxin gene primers were first evaluated using single-plex PCR reactions and previously published primers (67). The protocol was modified due to suit the reagents to generate the *C. perfringens cpb, etx, iap* and NetB toxin gene fragments. Briefly, the 25µL PCR contained 1.5 µL of purified gDNA (>100 ng), 5 µL of 5× PCR buffer, 2 mM of MgCl2, 0.2 mM of dNTPs, 0.25U/µl of Taq polymerase and 0.2µ M of primers. Positive PCRs generating amplicons of 196 bp (*cpb*), 656 bp (*etx*), 446 bp (*iap*), 738 bp (*netB*) respectively were regarded as presumptive confirmation that tested strains harboured the relevant toxins. However, due to local regulatory restrictions that prohibited the purchase of specific *C. perfringens* strains without the necessary permits, no positive control strains or DNA were available for these initial PCRs. Therefore, generated amplicons were Sanger sequenced and compared to the NCBI nucleotide database using the BLAST algorithm (209,210). Strains with confirmed toxin carriage were used as positive controls in subsequent PCRs.

7.2.2.7. Multiplex PCR to determine toxin typing of *C. perfringens* isolates

To identify the toxin genes simultaneously, a previously described multiplex PCR (67) with slight changes was carried out to detect the major *C. perfringens* toxin genes: *cpa* (alpha), *cpb* (beta), *etx* (epsilon), *iap* (iota), *cpe* (enterotoxin) and *netB* (NetB) of *C. perfringens* (67,204). The PCR mixtures were prepared in a total volume of 25 µl which included 1.5 µL of purified gDNA (>100 ng), 12 µL of Promega GoTaq G2 Hotstart 2xMaster Mix, and a 2.5µL cocktail of 2µM primers as described in Table 7.3 (67,204). Primers corresponding to each toxin are listed in Table 7.1 and the conditions described in section 7.2.2.3.

Table 7.3: PCR components for multiplex PCR to detect the *C. perfringens* toxins.

PCR component (Stock concentration)	1 x reaction (µl)
Nuclease-free water	8.5
Promega GoTaq G2 Hotstart 2xMaster Mix	12.5
Primers[#] (20µM)	2.5
Template DNA*	1.5
Total	25 µl

*Template DNA all less than 100ng per reaction. #: The concentration of each primer was 0.4µM and were mixed before added to PCR.

7.3. Antibiotic susceptibility testing

The antimicrobial susceptibilities of 19 *C. perfringens* isolates were determined using the Epsilometer test (E-test) method and interpreted following the guidelines of the Clinical Laboratory Standards Institute, CLSI (211,212) with additional consideration of EUCAST guidelines where necessary (213). Seven antimicrobials, namely, amoxicillin/clavulanic acid, piperacillin/tazobactam, penicillin G, imipenem, clindamycin, metronidazole, and moxifloxacin were tested (Table 7.4). E-test strips were obtained from Liofilchem (Roseto degli Abruzzi, Italy). Isolates were prepared as described by the manufacturer (http://www.liofilchem.net/login.area.mic/technical_sheets/MTS14.pdf) and testing done on Brucella agar containing 5% laked sheep blood, 5 µg/mL hemin, and 1 µg/mL Vitamin, as recommended for drug susceptibility testing of anaerobe bacteria (213). Bacterial inoculums were prepared using McFarland standards and nephelometry (Densicheck instrument, BioMérieux, France) and bacterial lawns prepared on AAM plates using sterile cotton tipped swabs. MIC test strips were applied with sterile forceps, whereafter plates were incubated anaerobically for 48 hours (212). *B. fragilis* ATCC 25285 was used as a quality control strain, requiring that its MIC results be within the range as described by the CLSI guidelines. Based on the MIC values, the isolates were classified either as susceptible, intermediate, or resistant (Table 7.4) (213).

Table 7.4: Antimicrobials tested and recommended interpretation.

Antibiotics	Antibiotic conc. range on E-test strip (ug/ml)	CLSI guideline breakpoints		
		S	**I**	**R**
Amoxicillin/Clavulanic Acid	0.016-256	≤4	8/4	≥16
Piperacillin/Tazobactam	0.016-256	≤16/4	16/8	≥32/16
Penicillin G	0.016-256	≤0.5	1	≥2
Imipenem	0.002-3	≤4	8	≥16
Clindamycin	0.016-256	≤2	4	≥8
Metronidazole	0.016-256	≤8	16	≥32
Moxifloxacin	0.002-32	≤2	4	≥8

7.4. Whole genome sequencing

7.4.1. Selection of *C. perfringens* strains for whole Genome Sequencing

A selection of five *C. perfringens* isolates that were phenotypically resistant to metronidazole were subjected to whole genome sequencing (Table 7.5). These isolates were from the liver, brain, and unknown sites.

The selected isolates (GSH 21, GSH 40, GSH 46, GHS 48 and GSH 60) were subjected to whole genome sequencing using a commercial service provider (Inqaba Biotec, Pretoria). Their service includes Illumina library preparation (214), fragment analysis on the Bio-Analyser and sequencing of generated libraries using an Illumina NextSeq instrument to generate paired end 300bp (2X150 base) reads (214). The resulting reads were checked for quality using FastQC v0.11.9 (215) and trimmed using Trimmomatic v3.0 (216). *De novo* assembly was done using SPAdes v3.9.1 (217) and the resulting assemblies analysed using Quast (V.5.0.2) (218) to determine the genome size, G+C content and N_{50} metric. Gene prediction and annotation were conducted using the Rapid Annotation Subsystem Technology (RAST) server (219–221). Virulence factors (including toxins) were detected using abricate (version 1.0.1) https://github.com/tseemann/abricate and screening against the Virulence Factor database (VFDB) (222). ResFinder (Centre for Genomic Epidemiology) (223).

Comprehensive Antibiotic Resistance Database (CARD), RAST and NCBI were also used to determine the antimicrobial virulence factors present in the isolates (224–226).

Table 7.5: Summary of isolates subjected to WGS

Isolate	PCR toxin type	DST*	Origin
GSH 21	A	Mtz-R, Cld-I	Site not identified
GSH 40	A	Cld-R, Mtz-I,	Liver
GSH 46	A	Mtz-R	Brain
GSH 48	A	Aug-R, PenG-R Mtz-R, Cld-R	Site not identified
GSH 60	A	MtzR, Cld-I	Site not identified

*Abbreviations: DST, Drug sensitivity testing; Mtz, metronidazole; Cld, Clindamycin; PenG, Penicillin G; Aug, Amoxicillin/clavulanic acid; R, resistance.

7.4.2. Multilocus sequence typing

Multilocus sequencing typing (MLST) was performed *in silico* on the five strains for which whole genome sequence data were available using eight housekeeping genes (*colA, groEL, soda, plc, gyrB, sigK, pgk* and *nadA)* (65) and comparison to the PubMLST database (227,228), last updated on 2022-07-04. A wrapper script (https://github.com/tseemann/mlst) was used to automate the extraction of each allele from the assembled contigs.

7.4.3. Identification of core genome SNPs

To investigate the phylogenetic relationship of *C. perfringens* strains, a core-genome alignment was performed to determine the single nucleotide polymorphisms (SNPs) within all genomes. A total of 186 genomes were included in the analysis, including the five genomes sequenced in this study and 181 genomes downloaded from the NCBI RefSeq database. Where data were available, strain origin was noted, including isolates from the environment, diseased and healthy hosts, different types of clinical manifestations (gas gangrene [human], food poisoning [human], necrotic enteritis [poultry], enterotoxaemia [sheep], and haemorrhagic enteritis [dogs and foals]). Single nucleotide polymorphisms (SNPs) were identified using kSNP (V2) (229). Maximum likelihood phylogeny analysis on the resulting alignment was done using RAxML (8.2.12) (230), using the GTRCAT approximation for nucleotide substitution (selected based on a ModelTest-NG analysis (231). Bootstrap values in the final tree are the result of 500 rapid bootstrap inferences.

8. Results

8.1. Site of infection and sample types

A total of 19 clinical *C. perfringens* isolates were included in the study (Table 8.1). All isolates used in the study were stored cultures obtained from patient samples previously submitted for routine diagnostic Microbiology. The most common source of isolation was blood (20%), followed by swab (16%) and brain (16%). Some of the sample sites were not indicated or information was not available. No other metadata were available and, since isolates were deidentified for this study, this could not be followed up by a retrospective review of patient folders.

Table 8.1: *C. perfringens* clinical isolates obtained via routine diagnostic testing.

Isolates	Sample type	Bodily site	PCR Lane
GSH 6	Brain Abscess	Brain	1
GSH 16	Blood culture	Blood	2
GSH 19	Tissue	Tissue	3
GSH 21	Swab	Not indicated*	4
GSH 22	Blood culture	Blood	5
GSH 23	Swab	Not indicated*	6
GSH 26	Swab	Not indicated*	7
GSH 28	Brain Abscess	Brain	8
GSH 24	Blood culture	Blood	9
GSH 36	Swab	Left tibia	10
GSH 40	Fluid/aspirate	Liver	11
GSH 41	Tissue	Left foot	12
GSH 42	Wound	Wound	13
GSH 46	Brain abscess	Brain	14
GSH 47	Not indicated*	Not indicated*	15
GSH 48	Not indicated*	Not indicated*	16
GSH 60	Not indicated*	Not indicated*	17
GSH 66	Blood culture	Blood	Lane 1, data not shown
GSH 75	Not indicated	Not indicated	Lane 2, data not shown

*Not indicated: The sample site was not indicated

8.2. Identification of *C. perfringens* and detection of Alpha toxin

Isolates in this study were identified by a combination of phenotypic characteristics, Gram staining and sequencing of specific DNA targets (16S rRNA and alpha toxin genes).

C. perfringens colonies exhibited the characteristic double zone of haemolysis on blood agar, and were mostly umbonate in appearance, i.e., irregularly shaped (mostly) flat colonies that are slightly elevated in the centre (Appendix, Figure 12.1). Microscopically, Gram-positive rod-shaped cells were observed (Appendix, Figure 12.1). Further PCR confirmation showed that an internal *plc* amplicon with the expected product size of 324bp was present for all putative *C. perfringens* colonies, verifying the presence of the ubiquitous *C. perfringens* alpha toxin in each of the 19 isolates (Figure 8.1). The positive control was absent at the time experiment was done; therefore, the identity of bands was confirmed using Sanger sequencing and sequence comparison using the BLAST algorithm.

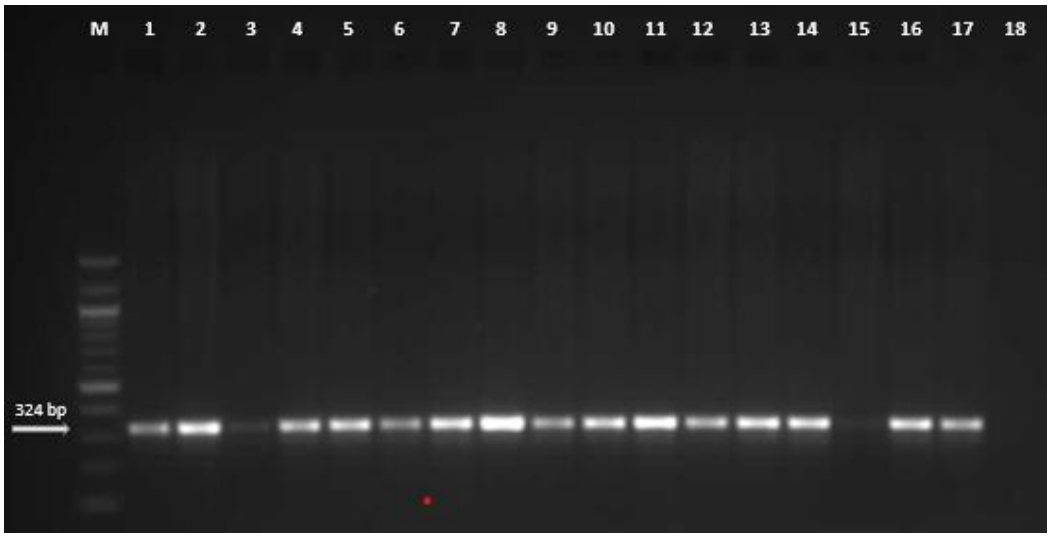

Figure 8.1: Agarose gel electrophoresis showing amplification of a 324 bp fragment of the *C. perfringens* alpha-toxin gene. Lane M: 100bp DNA ladder (NEB Biolab, UK) lane 1-17 plc gene lane 18 no template control (water). Lane 15 contained a faint *plc* band. No positive control was used for this PCR, instead amplicon identities (including lane 15) were confirmed with Sanger sequencing and comparison with known, published *plc* sequences.

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8.3. Identification using 16S rRNA PCR and Sanger sequence analysis

Amplification of the full-length 16S rRNA gene from all isolates yielded the expected product size of 1400bp (gel electrophoresis data not shown). All isolates, (except GSH 48, not subjected to 16S rRNA) exhibited at least 98% identity to published sequences of *C. perfringens* (Table 8.2). GSH 48

was not subjected to Sanger sequencing, but its phenotypic appearance, presence of the *plc* gene and WGS in the last experiment, confirmed its identity (section 8.7).

8.4. Detection of other major toxin genes using singleplex PCR

In order to screen isolates for additional major toxin genes, all confirmed *C. perfringens* isolates were checked using singleplex PCRs, followed by Sanger sequencing of the resulting amplicons. Among 19 analysed isolates, 42% (8/19) of isolates yielded an amplicon for the *cpb* PCR (beta toxin, product size of 196bp) (Figure 8.2). However, upon sequencing of the putative *cpb* products, it was found that the amplicon represented non-specific product. The enterotoxin gene (*cpe*), with amplicon size of 234bp, was detected in 3 isolates (Figure 8.3 (singleplex PCR) and Table 8.2 (multiplex PCR)). The remaining major toxin genes (epsilon, iota and NetB) were not detected in any isolates. However, in the absence of positive control strains we cannot exclude the possibility that the lack of products in these reactions was due to reaction failure, rather than the strains not harbouring the genes.

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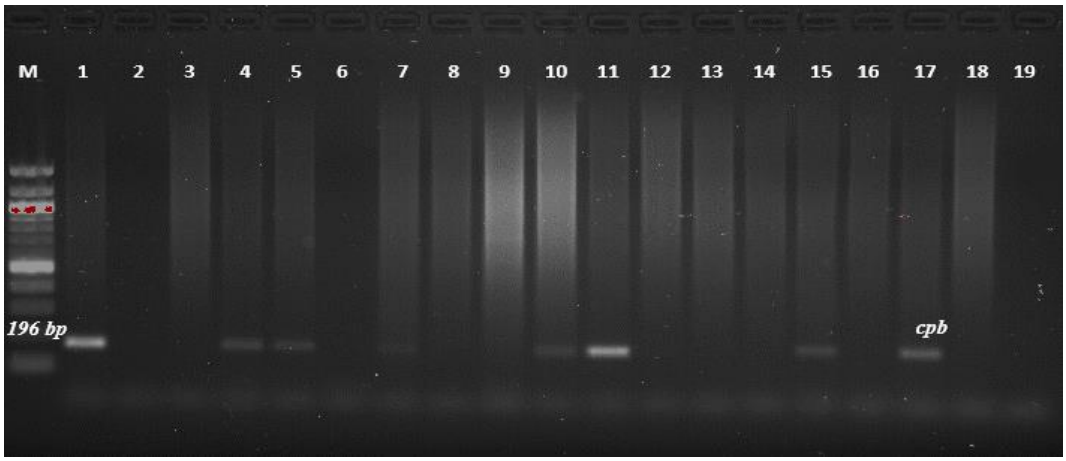

Figure 8.2 Agarose gel electrophoresis showing amplification of a 196 bp fragment of the *C. perfringens* beta toxin (*cpb*) gene. Lane M: 100bp DNA ladder (NEB Biolab, UK), lanes 1-18 *cpb* gene, lane 19. No template control (water). Lanes 19, empty lane. Amplicons were sequenced to confirm ID. GSH40 (lane 11) and GSH60 (lane 17) were further subjected to WGS.

Table 8.2: *C. perfringens* identity confirmation and toxin gene confirmation.

Isolate	Toxin genes multiplex PCR	Toxin typing	Sequence similarity to closest 16S rRNA match in the RefSeq database (%)
GSH 6	*plc*	A	99.69
GSH 16	*plc*	A	99.37
GSH 19	*plc*, *cpe*	F	99.58
GSH 21	*plc*	A	99.36
GSH 22	*plc*	A	99.16
GSH 23	*plc*, *cpe*	F	99.24
GSH 26	*plc*	A	99.16
GSH 28	*plc*, *cpe*	F	99.37
GSH 24	*plc*	A	99.26
GSH 36	*plc*	A	98.65
GSH 40	*plc*	A	99.72
GSH 41	*plc*	A	99.18
GSH 42	*plc*	A	98.49
GSH 46	*plc*	A	99.68
GSH 47	*plc*	A	99.7
GSH 48	*plc*	A	ND*
GSH 60	*plc*	A	99.37
GSH 66	*plc*	A	99.15
GSH 75	*plc*	A	99.25

*ND: Not done, due to poor sample quality and technical error. The sample was confirmed with the presences of *plc* gene which is present in all *C. perfringen*s. Furthermore, GSH 48 was subjected to.

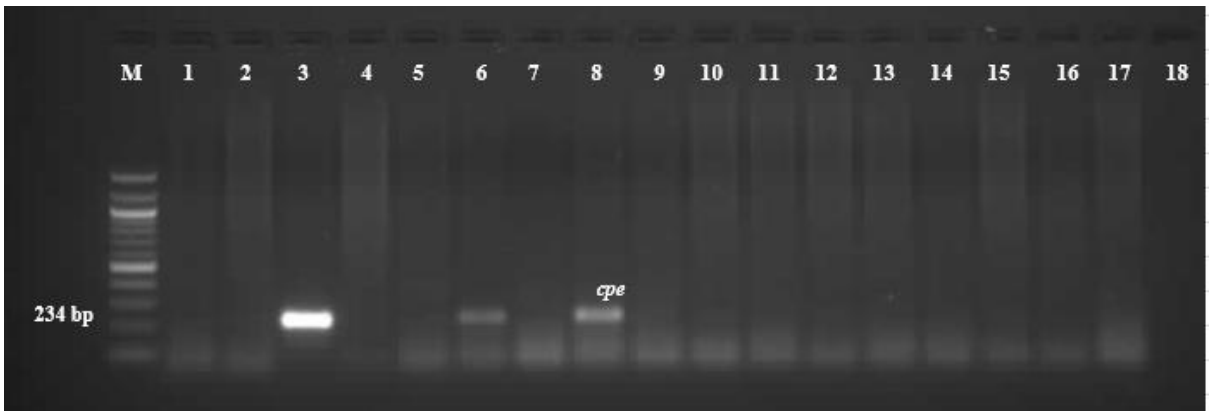

Figure 8.3: Agarose gel electrophoresis showing amplification of a 234 bp fragment of the *C. perfringens* enterotoxin (*cpe*) gene, with single plex PCR. Lane M: 100bp DNA ladder (NEB Biolab, UK), lanes 1-17 *cpe* gene, lane 18 no template control (water). Amplicons were sequenced to confirm their identity.

8.5. *C. perfringens* clinical isolates toxin typing using multiplex PCR

A previously previously described multiplex PCR method was used to simultaneously detect the toxin types of *C. perfringens* isolates (56). In this study, six sets of previously described primers (Table 7.1) were used to produce amplicons of a specific size corresponding to the various targets simultaneously. Based on the major toxin genes produced and classification using the recently revised typing scheme (56), all isolates examined in this study were identified as either type A or type F. Eighty-four percent (16/19) of these isolates belong to Type A (meaning only alpha (*plc*) gene amplified), Type A was thus found to be the most prevalent. The remaining 3 isolates (3/19) belonged to type F, meaning these were positive for both alpha (*plc*) and enterotoxin (*cpe*) genes. The multiplex PCR was found to be discordant with the results from the uniplex as 42% (8/19) of isolates yielded amplicons that were of similar size with beta toxin band. However, Sanger sequencing showed it was not this toxin, but non-specific amplification. Type B, C, D, E and G (which contains beta, epsilon, iota and NetB genes respectively) were not found in any of the genotyped isolates, as seen by a lack of PCR amplification with relevant primer sets. The lack of a positive control, however, makes it difficult to determine whether the PCR failed or whether these genes were indeed not present.

Discerning the reported site of infections (blood or tissue or a swab from infected site), Type A isolates were isolated from various infected sites including brain, blood, tibia, liver, foot, and an unspecified wound. Type F isolates were obtained from an unidentified tissue site, a swab with no provided information on the site of infection, as well as infected brain tissue.

8.6. Phenotypic drug susceptibility profile

A total of 19 *C. perfringens* isolates were phenotypically tested for susceptibility to seven antibiotics using the E-test method (Figure 8.4, Table 8.3). The susceptible, intermediate, and resistant breakpoints for each antimicrobial were interpreted according to CLSI recommendations (2020) (Table 7.4). The *B. fragilis* ATCC 25285 strain was used as a quality control strain in this study and was within acceptable range when tested against all antimicrobials.

All *C. perfringens* isolates from different origins were susceptible to piperacillin/tazobactam, imipenem and moxifloxacin.

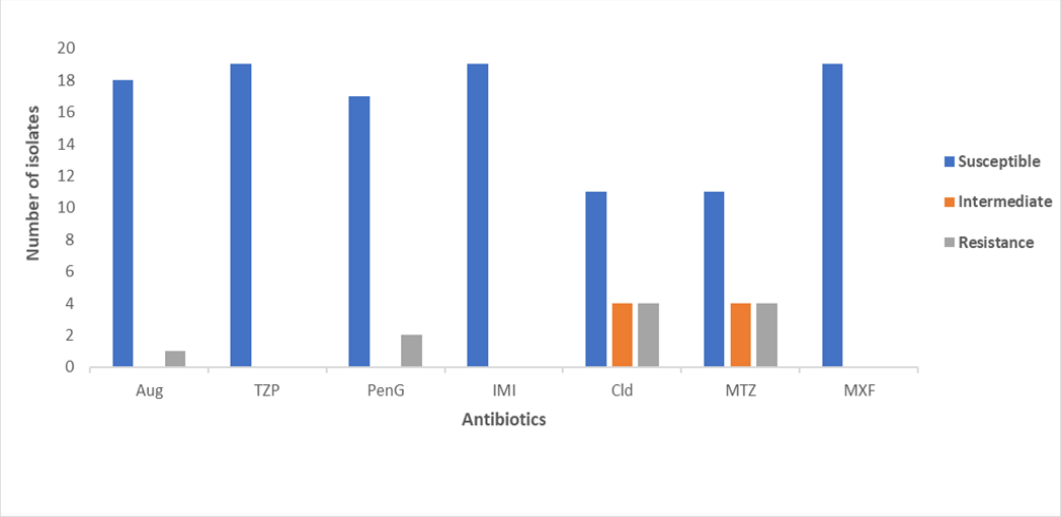

Figure 8.4: *In vitro* antimicrobial susceptibility testing of *C. perfringens* clinical isolates. Data were interpreted according to CLSI guidelines. Please note that some isolates exhibited resistance to more than one antibiotic.

For amoxicillin/clavulanic acid and penicillin G, susceptibility was observed in 95% (18/19) and 89% (17/19) of isolates, respectively. In contrast, 21 % (4/19) of isolates were resistant to clindamycin and metronidazole, respectively. An additional 21% (4/19) of isolates exhibited intermediate resistance metronidazole and clindamycin. Overall, 42% (8/19) of isolates were susceptible to all tested antibiotics. Monoresistance (to one antibiotic) was observed in 11% (2/19) of the isolates, while the remaining drug resistant isolates 21% (4/19) (GSH6, GSH24, GSH40 and GSH48) exhibited multidrug (MDR) (resistance to more than one drug) in either metronidazole, clindamycin, and / or beta lactam.

In terms of site of infection, clindamycin resistant isolates were from brain abscess (GSH 6), blood culture (GSH 24), fluid/aspirate (liver, GSH 40) and unknown origin. Metronidazole resistant isolates

were obtained from brain abscesses, blood cultures and from swabs (unidentified site of infection, GSH 48). The MDR isolates were from blood culture (GSH 24), liver (GSH liver), and swab (unidentified site of infection, GSH 48).

Table 8.3 : Susceptibility profile of isolates tested.

Isolates	Mtz			Cld			PenG			Aug			Tzp			Mxf			Imi		
	S	**I**	**R**	**S**	**I**	**R**	**S**	**I**	**R**	**S**	**I**	**R**	**S**	**I**	**R**	**S**	**I**	**R**	**S**	**I**	**R**
GSH 6		**24**				**>24**	0,023		**>24**	0,016			0,016			0,38			0,064		
GSH 16	4			0,094			0,047			0,023			0,032			0,125			0,094		
GSH 19	8			0,094			0,032			0,016			0,023			0,19			0,064		
GSH 21			**>256**		4		0,38			0,19			0,19			0,38			0,19		
GSH 22	4				4		0,047			0,047			0,064			0,25			0,125		
GSH 23	6			1			0,064			0,047			0,19			0,38			0,094		
GSH 26	3			2			0,064			0,032			0,047			0,5			0,064		
GSH 28	4			0,19			0,064			0,094			0,064			0,19			…0,25		
GSH 24			**>256**			**>256**			**>256**	0,75			0,5			0,032			0,125		
GSH 36	2			0,19			0,125			0,094			0,125			0,38			0,094		
GSH 40		16				**24**	0,032		**24**	0,016			0,023			0,38			0,094		
GSH 41	2				3		0,38			0,125			0,25			0,38			0,25		
GSH 42		16		2			0,047			0,032			0,032			0,25			0,064		
GSH 46			**>256**	1,5			0,094			0,064			0,125			0,38			0,094		
GSH 47	3			1			0,047			0,047			0,047			0,25			0,094		
GSH 48			**>256**			**>256**			**>256**			**>256**	1			0,25			0,25		
GSH 60		24		1			0,094			0,032			0,047			0,38			0,125		
GSH 66	6			0,064			0,047			0,023			0,016			0,19			0,125		
GSH 75	2				6		0,032			0,032			0,064			0,25			0,094		

*Abbreviations: S; susceptible, I; intermediate, R; resistant, Mtz; metronidazole, Cld; clindamycin, PenG; penicillin G, Aug; amoxicillin/clavulanic acid, Tzp; Piperacillin/tazobactam, Mxf; Moxifloxacin, Imi; Imipenem. CLSI guidelines breakpoint (213): Refer to Table 7.4.

8.7. Whole genome sequencing

Whole genome sequencing, carried out for five isolates, GSH 21, GSH 40, GSH 46, GHS 48 and GSH 60, selected based on their antimicrobial susceptibility patterns and sequencing budget for the project. All isolates exhibited phenotypic intermediate or full resistance to either metronidazole and/ or clindamycin. GSH 40 and GSH 60 were phenotypic intermediate resistant to metronidazole while the rest of isolates (GSH 21, GSH 46 and GSH 48) showed phenotypic resistant to metronidazole (Table 8.3 and Table 8.4). For the study reported here, the genomes were scrutinised only for previously reported virulence factors, drug resistance markers and the phylogenetic relationship with published *C. perfringens* genomes available on National Center for Biotechnology Information (NCBI, http://www.ncbi.nlm.nih.gov/)**.** More in-depth analysis will be done in future studies, which will include WGS data of other isolates in this study.

Table 8.4 Summary of isolates subjected to WGS.

GSH	PCR toxin type	DST*	Origin
GSH-21	A	Mtz-R, Cld-I	Site not identified
GSH- 40	A	Cld-R, Mtz-I,	Liver
GSH-46	A	Mtz-R	Brain
GSH-48	A	Aug-R, PenG-R Mtz-R, Cld-R	Site not identified
GSH-60	A	MtzI	Site not identified

*Abbreviations: DST; drug sensitivity testing, Mtz; metronidazole, Cld; clindamycin, PenG; penicillin G, Aug; amoxicillin/clavulanic acid, R; resistance. I, intermediate level of drug susceptibility, R, clinical resistance.

8.7.1. General genomic features

Assembled genomes ranged in size between 3.28-3.46 Mbp (Table 8.5), which falls within the genome size range for published *C. perfringens* genomes (2.9-4.1 Mbp) (46,47). The G+C content ranged from 27.9-28.24%, in keeping with the expected low G+C content of *C. perfringens* strains (46,47). Genome fragmentation was assessed using the N50 metric (50%

or more of the genomic sequence data is present on contigs of at least this length) and ranged between 0.16-1.9 Mbp.

Table 8.5: General genomic features of sequenced *C. perfringens* isolates.

Assembly	LP_ GSH21	LP_GSH40	LP_GSH46	LP_GSH48	LP_GSH60
Number of contigs	25	55	103	52	57
Total length (bp)	3283994	3328517	3372470	3342032	3468219
GC (%)	28.13	27.97	28.24	28.18	27.96
N50 (bp)	1908625	162457	255658	668943	350954
Number of CDS*	2947	2955	3088	3026	3117
Number rRNA	6	6	10	11	6
Number tRNA	78	75	76	74	76

*CDS; Coding sequence

8.7.2. Virulence factors

Toxin and virulence factor genes were identified by screening assemblies against the VFDB, and the results are reported in Table 8.6 (222). In addition to the major toxin genes used for toxinotyping (*plc*), the five genomes showed the presence of several additional toxins and virulence factors, including alpha-clostripain (*closI*), sialidase (*nanJ*) and (*nanH*), collagenase (*colA*), hyaluronidase (*nagJ*, *nagL*, *nagH*), which were present in all 5 isolates. Additionally, perfringolysin O (*pfoA*) (absent in GSH 40, liver), sialidase (*nanI*) (absent in GSH 40) and hyaluronidase (*nagI, nagK*) (absent in GSH 60) were detected in all but one of the isolates mentioned. Three out of five (60%) isolates GSH 21 (unidentified), GSH 46 (brain), GSH 48 (unidentified) harboured putative beta2 toxin genes, all of which matched the atypical, non-frameshifted form of the toxin gene (accession number AY609161) described by Jost *et al.* (2005) (232). In all cases, virulence gene hits showed good coverage (≥92.95%) and high nucleotide identity (>92%) to the genes in the VFDB database.

Table 8.6: Toxin and virulence factor genes identified in the sequenced genomes (222).

Virulence factors	Accession number[#]	GSH 21	GSH 40	GSH 46	GSH 48	GSH 60
		Gene coverage % and (Nucleotide identity%)				
Alpha-toxin: phospholipase C (*plc*)	NP_560952	100 (99.25)	100 (99.17)	100 (99.25)	100 (99.25)	100 (98.63)
Theta-toxin: Perfringolysin O (*pfoA*)	NP_561079	100 (98.74)	ND*	100 (98.87)	100 (98.78)	92.95 (88.83)
Alpha-clostripain (*cloSI*)	NP_561762	100 (97.33)	100 (98.16)	100 (97.08)	100 (97.08)	100 (95.68)
Sialidase (*nanJ*)	NP_561469	100 (99.97)	100 (97.02)	100 (98.07)	100 (98.07)	99.97 (98.13)
Sialidase (*nanI*)	NP_561641	100 (98.37)	ND*	100 (98.80)	99.97 (98.80)	100 (98.13)
Sialidase (*nanH*)	YP_695432	100 (96.69)	100 (96.59)	100 (96.26)	100 (96.26)	99.39 (96.50)
kappa-toxin: collagenase (*colA*)	NP_561089	100 (98.04)	100 (97.95)	100 (99.34)	100 (99.34)	100 (97.65)
Mu-toxin: hyaluronidase (*nagI*)	NP_561797	100 (98.61)	100 (99.05)	100 (98.61)	100 (98.610	ND*
Mu-toxin: hyaluronidase (*nagJ*)	NP_562150	100 (98.80)	100 (98.14)	100 (100)	100 (100)	100 (97.70)
Mu-toxin: hyaluronidase (*nagK*)	NP_562195	100 (99.08)	100 (98.10)	100 (97.92)	100 (97.92)	ND*
Mu-toxin hyaluronidase (*nagL*)	NP_562439	100 (99.88)	100 (96.84)	100 (100)	100 (100)	100 (95.69)
Mu-toxin hyaluronidase (*nagH*)	NP_561107	100 (98.79)	100 (98.59)	100 (98.75)	100 (98.75)	100 (97.36)
Beta2 toxin (*cpb2*)		100 (100)	ND*	100 (100)	100 (100)	ND*

*ND: Not detected. #, the accession number for the gene in the VFDB with the closest match. Sequence identity indicated as a percentage in the bracket.

The toxin types obtained via PCR-based toxin typing correlated with the whole genome sequence data of all five isolates (Table 8.7). However, the typing scheme did not include a PCR for the beta2 toxin, which was identified in the genomes of some of the sequenced isolates. This toxin is not part of the standard PCR-based toxin typing scheme.

Table 8.7: Correlation between multiplex PCR toxin typing with WGS.

Isolate	Multiplex PCR – Toxin gene	Whole genome sequencing type	Agreement
GSH 21	A	A	100%
GSH 40	A	A	100%
GSH 46	A	A	100%
GSH 48	A	A	100%
GSH 60	A	A	100%

8.7.3. Antimicrobial resistance determinants in sequenced genomes

The data from the five genomes showed the presence of different putative antimicrobial resistant (AMR) genes with high identity (>90%) to genes present in the ResFinder (224) (223), ARG-ANNOT (233) and CARD (234) databases (Table 8.8). Tetracycline-resistance was the most common antibiotic resistance trait with all of the isolates carrying at least 1 putative tetracycline-resistance gene. Interestingly, in three isolates only a *tetB*(P) gene was detected, whereas in the remaining 2 isolates, both *tetA*(P) and *tetB*(P) genes were present. However, in each case the *tetB*(P) gene was located at the end of a contig, while a fragment of the *tetA*(P) gene was detected at the end of another contig, raising the possibility that poor assembly in the region may have precluded identification of the latter gene (7). The remaining two isolates harbored both *tetA*(P) and *tetB*(P) genes. A putative *ermQ* gene, which encodes Macrolide-Lincosamide-Streptogramin (MLS) resistance, was detected in a single clindamycin resistant isolate (GSH40). Putative ATP-binding cassette transporters (ABC) transporters, which may be involved in antibiotic efflux were also detected in all the isolates. Finally, all isolates also included homologues of the *C. perfringens* SM101 *mprF1* gene (CPR_1564), which may play a role in resistance to cationic antimicrobial peptides (e.g., daptomycin).

Table 8.8: Distribution of antibiotic resistance genes and transporters in *C. perfringens.*

	***tetA*(P)**	***tetB*(P)**	***ermQ***	**Efflux ABC transporter**	***mprF***
	Coverage% and (Nucleotide identity%)				
GSH 21	100 (89.52)	100 (99.75)	ND	100 (100)	100 (95.61)
GSH 40	ND	100 (99.75)	100 (100)	100 (100)	100 (95.09)
GSH 46	ND	100 (99.75)	ND	100 (100)	100 (97.54)
GSH 48	ND	100 (99.75)	ND	100 (100)	100 (97.54)
GSH 60	100 (100)	100 (99.69)	ND	100 (100)	100 (93.74)

9. *ND: Not detected. (); Sequence identity indicated as a percentage in the bracket. *erm* (Q), erythromycin resistance methylase. MprF, Multi-peptide resistance factor.

9.1.1. Multi-locus sequencing typing

To investigate the genetic diversity of the five clinical isolates (GSH 21, GSH 40, GSH 46, GHS 48 and GSH 60), *in silico* MLST was carried out using the *C. perfringens* PubMLST database (228). Based on the analysis, 3/5 (60%) of the isolates were novel MLST subtype (STs) (Table 8.9). GSH 21 was closely related to two previously reported STs (ST143 and ST144) with a single allele difference in each case. GSH 40 was more distantly related to two STs (ST339 and ST401), both having 3 allele differences and furthermore appeared to harbour a novel version of the *nadA* allele. GSH 60 was distantly related to ST40 with six allele differences and putative novel versions of the *colA*, *gyrB* and *nadA* alleles. In contrast, isolates GSH 46 and GSH 48 had the same subtype, ST274, suggesting that they are closely related.

Table 8.9: *C. perfringens* multi-locus typing sequence

Isolate	MLST subtype	Closest ST(s)	*colA*[*†]	*groEL*	*sodA*	*plc*	*gyrB*	*sigK*	*pgk*	*nadA*
GSH21	Novel	ST143, ST144 (1 allele different)	19	5	79	5	1	2	3	1
GSH40	Novel	ST339, ST401 (3 alleles different)	1	66	89	117	25	12	4	~96 (7)
GSH46	274	NA	61	6	1	7	1	5	4	1
GSH48	274	NA	61	6	1	7	1	5	4	1
GSH60	Novel	ST40 (6 alleles different)	~62 (6)	5	130	146	~50 (1)	11	8	~122 (1)

[*] In each case, alleles were assigned based on matches to the *C. perfringens* typing scheme hosted on PubMLST

[†] Where there were nucleotide differences between the closest reference allele and the isolate sequence, the total number of SNPs is given in brackets.

9.1.2. Phylogenetic relatedness to isolates with publicly available genomes.

A maximum-likelihood (ML) tree was generated based on core genome alignments of the five GSH strains, and a hundred and seventy-eight (178) publicly available genomes including three recently published environmental genomes from RSA. The phylogenetic analysis grouped the *C. perfringens* strains into 5 phylogenetic clades (I-V), Figure 8.5. Clade I was the largest clade consisting of 53 *C. perfringens* strains from different hosts: animals, food, human, and environmental. Clade II, with 27 isolates, contained strains from avian, human, food, and environmental sources, with the majority from human foodborne outbreaks or food samples. Clade III (n = 17 isolates) primarily contained ruminant, human, and avian isolates. Clade IV was the smallest clade and included 12 ruminant, avian, rodent and food isolates. Clade V (n = 36 isolates) was primarily represented by isolates of canine and equine origin.

Three isolates from this study (GSH21, GSH46, GSH48) clustered within clade I. GSH 21 was closely related to an environmental isolate, SC4-C24, which was originally isolated from a river system in South Africa (23). GSH 46 and GSH 48 clustered with isolates from clinical

and foodborne outbreaks (type F strains responsible for non-lethal diarrhoea) and were identical to one another (n = 0 core genome single nucleotide polymorphisms [SNPs]). GSH 40 aligned with clade II isolates of human origin, while GSH 60 clustered within clade V, most closely related to a type D strain of ruminant origin. However, for both GSH 40 and GSH 60, the relatively long branch lengths suggest significant divergence between these isolates and their closest relatives in the phylogeny.

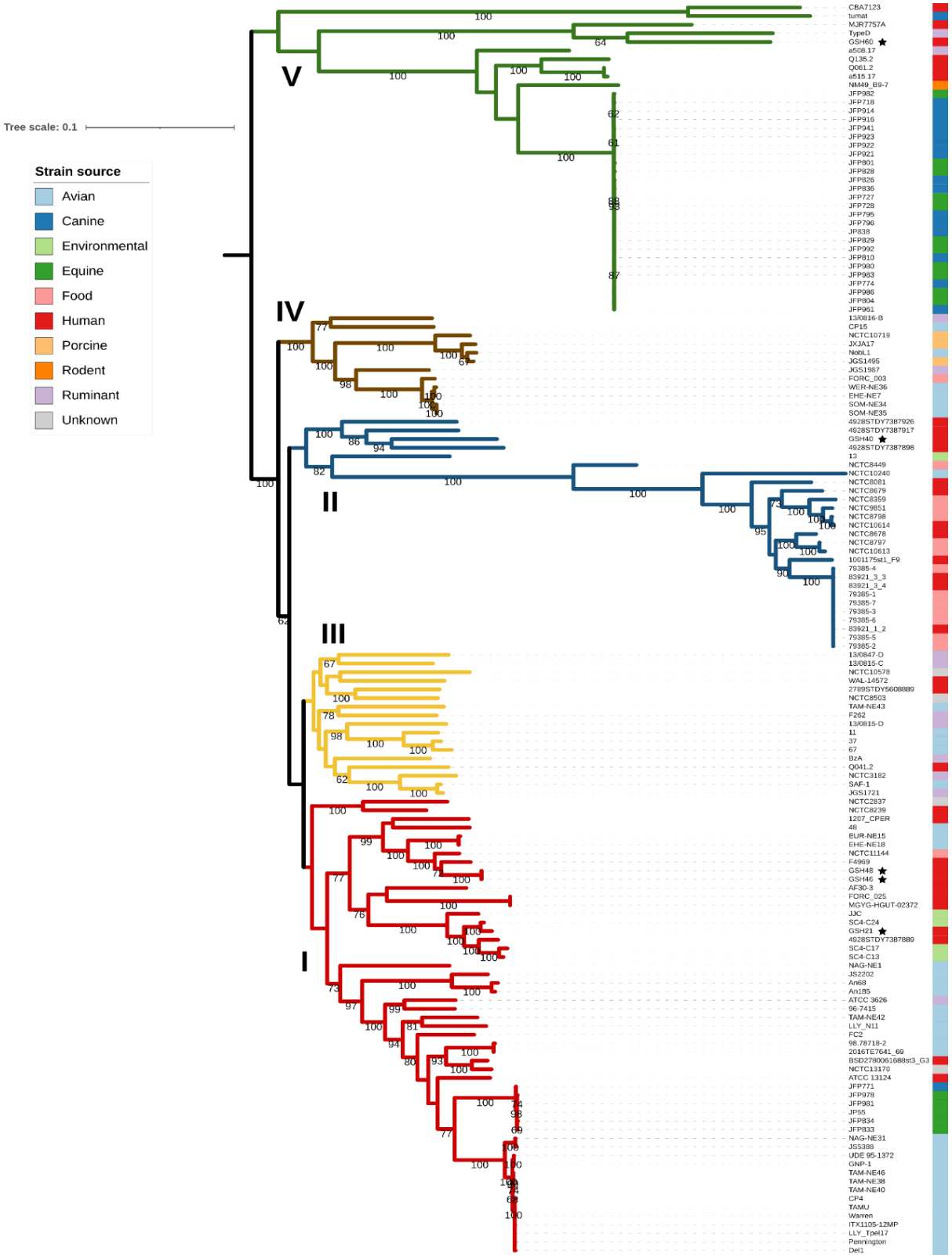

Figure 8.5: Phylogenetic relationship of *C. perfringens* strains. Hundred and eighty-six (186) genomes (including 5 genomes sequenced in this study (indicated by black stars) and rest downloaded (from publicly available databases). The *C. perfringens* strains were grouped into 5 phylogenetic clades (I-VI).

10. Discussion

C. perfringens is one of the more prevalent anaerobic bacteria causing anaerobic infections in both humans and animals (32,33). However, in RSA, genetic data regarding human clinical isolates are scarce, with only a single, published, case study reporting a blood culture isolate obtained from a diabetic patient, and identified as *C. perfringens* via sequencing of the 16S rRNA gene. From other environments, three genomes from environmental isolates have been sequenced in recent years (23). No published literature regarding genome sequences from local studies on *C. perfringens* from animals, food, and birds were available at the time of this study, while numerous studies reports on this microbe from ostrich, sheep, street vendors, yet only 1 study on genomic data from the environment (23,112,113,117,235,236). Most studies conducted previously in clinical isolates, only focused on antibiotic susceptibility testing and not toxinotyping (182,183,237). One recent clinical study reported on *C. perfringens* from a foodborne outbreak in a psychiatric hospital, with 2 deaths. Yet only alpha toxin profiling was done (60,132).

For this study, 19 *C. perfringens* isolates obtained from Groote Schuur clinical samples were phenotypically and genotypically characterised. These isolates were identified via routine diagnostics and using Vitek2 ANC cards (BioMerieux, France). The morphology of selected isolates was consistent with typical *C. perfringens* characteristics, i.e., Gram-positive rod-shaped cells and double zone haemolysis on blood agar. All the clinical isolates were also positive for the *plc* gene, which agrees with previously published data indicating that the alpha toxin encoding gene (*plc*) is present in all *C. perfringens* strains (45).

For this study, we aimed to investigate the molecular characteristics of clinical *C. perfringens*, thus analyses of clinical information and patient metadata were not within the scope of this study. Nevertheless, sample type information was available for some isolates. Isolates were cultured from brain abscesses, blood cultures, swabs, tissue, fluid/aspirate from the liver, tissue from left foot and an unspecified infected wound swab. This is consistent with what is reported in the published literature, i.e., infected soft tissues, blood cultures, infected limbs *etc*. Published works from decades ago reported on *C. perfringens* isolated from blood cultures of patients presenting with bacteremia (238) and infected tissues presenting with gas bubbles (239–241). The presence of *C. perfringens* in the blood often is the result of transient bacteraemia by Clostridia seeded into the blood by an unknown bodily infectious focus

following trauma, surgery-related intestine leakage or cancer (notably infected solid tumours) (108,242,243). Also, important to note are the comorbidities that increase the risk for clostridial infections, notably diabetes-related ischaemia, and chronic wounds of the lower limbs (a risk factor for clostridial gas gangrene) (244). Cancer, diabetes, and other immunosuppressive conditions are the common underlying disease for *C. perfringens* bacteraemia or sepsis (243). Unfortunately, the clinical data associated with our samples were not available for this study.

To discern the toxin types responsible for disease, a multiplex PCR with previously described primers was employed, attempting to classify the 19 isolates into the seven known toxin types A-G (67). The majority of our *C. perfringens* isolates were classified as type A, where only the alpha toxin gene was detected (16 out 19 isolates). This agrees with previous reports describing type A isolates as the predominant toxin type isolated from patients (33,66,245). Besides gastrointestinal diseases, toxin type A strains are also implicated in extraintestinal infections such as gas gangrene (myonecrosis) (26,246). In some cases, gas gangrene arises because of surgery, when tissues have been contaminated with intestinal tract content. However, the presence of Type A in the human GIT in the absence of disease has been noted, and it forms part of the normal intestinal microbiota in carriers (33,247).

The remaining 3 out of 19 isolates were classified as toxin type F, i.e., positive for both the *plc* and *cpe* genes. While this study examined isolates from non-diarrhoeal samples, the literature indicates that strains belonging to this toxin type are responsible for 5-20% of foodborne disease and non-foodborne gastrointestinal diseases such as antibiotic-associated diarrhoea. In foodborne outbreaks, the enterotoxin (CPE) is usually the most identified (50,158,161). Limited information is available regarding type F isolates causing invasive disease. In the present study, the *cpe*-positive isolates (confirmed with PCR and Sanger sequencing) were derived from an infected tissue, a superficial swab, and a brain abscess. It could be hypothesised that these isolates disseminated from the GIT of the individuals, but in the absence of clinical data, this cannot be confirmed. Although associated with non-lethal foodborne outbreaks, enterotoxin-bearing strains have been associated, albeit in rare reports, with lethal disease (248). An interesting observation is that enterotoxin-bearing strains, on average, have a smaller genome size than other *C. perfringens* strains (47,249). Also noteworthy is that *cpe*-bearing isolates from cases of food poisoning have the gene located on the chromosome, which is flanked by IS1470 mobile sequence, whereas isolates from non-food borne gastrointestinal disease carry the *cpe* gene on plasmids flanked by the IS1470-like sequence or the IS1151 sequence (130,245). Future analyses are necessary to determine the location of the *cpe* gene in

the isolates from the current study. This can be done using PCR that will target different loci where the *cpe* gene has been previously reported (130,250,251). In this way, the PCR will distinguish isolates that carry the chromosomal *cpe* locus, a plasmid *cpe* locus flanked by the IS1470-like sequence or the IS1151 sequence as per Miyamoto *et al.* (2004) (130).

Based on the PCR data in this study, the preliminary conclusion is that toxin types B, C, D, E, and G were not isolated. However, the lack of positive control DNA means that the possibility of PCR-failure, leading to false negatives, cannot be ruled out. For example, it is possible that sequence variability in the gene regions may have affected primer binding. Though this is unlikely, it should be noted that these well-established primers were designed using genome data from non-African strains. However, reported data from other geographical regions support the broad range applicability of these primers to detect toxin types from diverse locations. Alternative approaches, such as whole genome sequencing are needed to confirm the lack of additional toxin genes in the isolate set from this study.

It is noteworthy that 42% (8/19) isolates were positive for a potential *cpb* amplicon via the single-plex PCR (produced amplicon of expected size), but no amplicons were observed for the multiplex PCR. Sanger sequencing revealed a different DNA sequence that is not *cpb*. Two of the isolates, GSH40 (lane 11) and GSH60 (lane 17), that yielded false-positive bands for *cpb* were also subjected to WGS which confirmed the absence of *cpb* gene, further suggesting that the PCR amplicons for *cpb* were false positives. The beta toxin has been shown to cause severe illness with high mortality rate in both humans and animals (252). It is known to be produced by *C. perfringens* type B and C strains that are responsible for necrotic enteritis (animals), darmbrand and pigbel in humans (87). These diseases are mostly characterized by the loss of intestinal mucous leading to inflammation of the intestinal wall and bleeding into the lumen resulting in bloody stool (253). No published information on human infection by toxin type C is available for RSA. On the rest of the African continent, case reports of patients with pigbel/necrotic enteritis have been published for patients in Nigeria, Uganda, and Ghana (254–256). No confirmation of the toxin type for these cases has been done though. As mentioned, contaminated food as a source needs to be investigated. Notably, type C strains are more resistant to heat inactivation, thus ingestion via contaminated, improperly cooked food is problematic (257) and the agricultural meat industry needs improved quality control (258). Improved antibiotic stewardship practiced in meat producers should be encouraged to prevent development of reservoirs of drug resistant bacteria and spread of genes via plasmids. As noted, *C. perfringens* has a very plastic genome and plasmids are widely shared between strains. The

cpb gene was previously found to be carried on a large plasmid ranging from ~65 to 110kb. These *cpb*-carrying-plasmids may also encode other genes involved in virulence such as *cpe* and *tpel* (78,85,86).

Worldwide there is an increase of antimicrobial resistance amongst anaerobic bacteria (259–262). This is of great concern for public health as it limits options for treatment, thereby increasing treatment costs and mortality (263). Several studies have shown that the antimicrobial resistance profiles of anaerobic bacteria differ per country and that they are likely linked to the preferred antimicrobial regimes used in each region. Appropriate antimicrobial therapy with early diagnosis and treatment have been shown to have good clinical outcomes, but regular use of clindamycin and metronidazole are associated with higher numbers of resistant cases (166,264). Given that the last antimicrobial survey on anaerobic bacteria in RSA was published in 2011 (183), we were interested in updating the analysis. Here we report antimicrobial susceptibility testing of 19 *C. perfringens* clinical isolates using seven antimicrobial drugs that are prescribed for treating anaerobic infections in RSA. The results suggested good activity of amoxicillin/clavulanic acid and imipenem against *C. perfringens* isolates (susceptibility of 95% and 100% respectively). A previously published study in 1998, also examining isolates from a clinical setting in Cape Town, reported 100% susceptibility to amoxicillin-clavulanate (237). The results from the current study thus represent a 5% decrease in susceptibility over 24 years. However, the aforementioned study used a different method of testing (agar dilution technique), and a different growth medium (Wilkins–Chalgren agar vs sheep blood agar), which may have affected the classification of strains (265,266). Susceptibility to amoxicillin/clavulanic acid and imipenem is typically what is reported in other geographical regions. For example, a South-Eastern Hungarian study reported excellent activity of amoxicillin/clavulanic acid and imipenem (179), while a study in Costa Rica reported 100% susceptibility of *C. perfringens* isolates to imipenem (267). However, a Thailand study reported 24.9% resistance of *C. perfringens* isolates against imipenem, while a study done in Iran reported even higher (37.97%) resistance to imipenem (177,185).

Penicillin G is used for both human (especially soft-tissue infections) and animal infections, as well as prophylaxis (268). In this study 89% of isolates tested were susceptible, as resistance was only observed in 2/19 isolates, which is similar to rates reported elsewhere (177,179,267). This represents a decrease from 100% to 89% susceptibility to penicillin when compared to the

last survey done in Cape Town, RSA, although the last published study was done more than 20 years ago (237).

Multiple isolates in this study exhibited phenotypic drug resistance against clindamycin and/or metronidazole. While not used as widely compared to previous decades, clindamycin is used in patients with a contra-indication for other preferred antibiotics (e.g., for patients with beta lactam allergies or drug therapy failure), and in cases where better antibiotic infiltration (into tissues) is needed to improve outcomes (269,270). Clindamycin resistant isolates in this study were from the brain, blood, and liver. This is concerning since infections of the brain require drugs that effectively can cross the blood-brain barrier (e.g., clindamycin). The resistance rate to clindamycin amongst strains isolated in this study was 5-fold higher than previously reported for isolates from the same region (21% vs 4%) (237). Historically, frequent use and improper antimicrobial stewardship (in healthcare and agricultural sectors) along with the propensity of the underlying resistance mechanisms to be encoded on mobile elements present on the chromosome or on plasmids have promoted the spread of clindamycin resistance. For example, over a two period (2007–2009 to 2010– 2012), increased antibiotic resistance rates among *C. perfringens* isolates were noted (271).

Worldwide, resistance to metronidazole has been observed (177–179). In Iran, 11.39% of *C. perfringens* strains resistant to metronidazole have been reported from human stool samples (177). Whereas in Thailand, Tansuphasiri *et al*. (2005) reported 13.5% of metronidazole resistant isolates from human faeces (185). Meanwhile in RSA, 4% metronidazole resistance has been reported in a clinical setting in Cape Town (237). This shows an increase in resistance of *C. perfringens* isolates to metronidazole from 4% to 21%.

One challenge when comparing local resistance rates is that data are sometimes aggregated (e.g., anaerobes, or *Clostridium* spp. treated as a group). Additionally, not all the results of surveillance end up being published. For example, the data from the last survey of anaerobe microbes done for the same clinical setting prior to 2011 has not been published, neither are there specific data on antibiotic resistance for *C. perfringens* mentioned. Better comparisons could be made by looking at more recently generated data from local diagnostic laboratories. Future studies should look at more current drug susceptibility profiles reported by local diagnostic laboratories, using data where drug resistance tests were requested for anaerobe bacteria, as well as data from unpublished surveys that might have been done. Moreover, a

central database is needed to upload recent data for drug susceptibility profiles. Importantly, standardised methods using the same media and methods using the same media and methods would be advantageous in allowing interstudy comparisons.

In general, increased resistance of *C. perfringens* strains to antibiotics (especially in animals, food, and environment) has been reported in the literature (160,177,259). Identifying the origins of drug resistant isolates requires studies that include patient metadata, as well as patient household addresses and occupations and agricultural practices in the same urban/suburban settings. For example, the use of antibiotics such as penicillin in the local meat and poultry industry assists with the fight against infections in stocks, and act as growth promoters, thereby assisting sustainability (272,273) (274). However, use of these antibiotics can promote the spread of resistance. Improved education of farmers and a change towards improved antimicrobial stewardship is needed in the meat and poultry industry, but a negative impact on cost due to increased infections and death of poultry and animal stocks if antibiotics are removed might influence practices. The meat, poultry and milk industries together make up a billion-dollar industry in RSA (275). Interestingly, a recent study found that methicillin resistant *Staphylococcus aureus* isolates obtained from a survey of municipal abattoirs and meat products did not originate from contamination within facilities, suggesting drug resistance generated elsewhere, rather from agricultural practices (276).

Arising of MDR resistant pathogens are posing a serious risk to both human and animal health (277,278). MDR in *C. perfringens* strains is a serious concern as it encodes virulence factors such as toxins, that are associated with severe infections (1,67). This study revealed 21% (4/19) of isolates (GSH6, GSH24, GSH40 and GSH48) to be MDR resistant. They were resistant to either metronidazole, clindamycin, and / or beta lactam, which are the commonly used drugs in clinical settings. MDR resistant isolates in human have been previous reported (185,279,280). One study reported MDR resistant strain to a wide range of antibiotics, approximately 40 antibiotics (279). Tansuphasiri *et al*. (2005) reported 63.8 % of MDR *C. perfringens* strains from faecal samples of patients admitted to Bangkok hospitals (185). While Tayez *et al.* (2020) reported varying degrees of MDR from samples isolated from dromedary camels, pastures, and herders (281).

Intermediate resistance was also observed in other isolates. Clinical metadata was not available for the samples studied; thus, no conclusions can be made regarding how resistance could have

developed: was the patient infected with a drug resistant isolate, or did it develop due to prolonged hospitalisation, what was the comorbidities associated with patients whose samples were analysed, *etc*. Nevertheless, the resistant isolates were from infected deep tissues (brain abscess, fluid/aspirate (liver), as well as from blood culture. *C. perfringens* from blood cultures usually originates from an infected focus somewhere in the human body, thus we can assume the patient also had an infected organ potentially. Nevertheless, it is reasonable to conclude that the patients either were infected with drug resistance isolates from the environment (soil, water, infected red and white meat, vegetables). Alternatively, it could be the consequence of repeated or prolonged exposure to antibiotics, or infection with drug resistant strains emerging due to inappropriate use of antimicrobials in humans and animals resulting in the emergence of MDR (MDR resistant) strains. One needs to do further studies to discern drug resistance amongst isolates from these food sources.

To date, there have only been a limited number of whole genome sequence analyses for strains of South African origin. Whole genome sequence analysis of five isolates in this study revealed genome sizes ranging from 3.28-3.46 million base pairs which is comparable to publicly published *C. perfringens* genomes, ranging from 2.9-4.1 million base pairs (47). The *in-silico* analyses also confirmed the PCR-based toxin, such as alpha toxin (*plc*), and revealed the presence of the *cpb2* gene in three strains (GSH 21, GSH 46 and GSH 48). Other widely known elements such as alpha-clostripain *(cloSI*), the sialidases encoded by *nanJ* and *nanH*, collagenase (*colA*), hyaluronidases encoded by *nagJ, nagL* and *nagH* were identified in all five strains. Perfringolysin O (*pfoA*), sialidase (*nanI*), hyaluronidases *nagI* and *nagK* were also common and identified in all but one of the isolates. All these toxins and enzymes are known for contributing to the pathogenicity and colonisation of *C. perfringens* (282).

The sialidase (neuraminidase) enzymes play a significant role in the pathogenesis of *C. perfringens*-mediated tissue infection. The hydrolyse host mucosal sialic acids and play a role in colonisation during intestinal infections. Together they promote the activity of other *C. perfringens* toxins, assisting with destruction and invasion (51,247,283). The *C. perfringens* collagenase (*colA*) aids with attachment, and degradation of collagens, thereby also contributing to disease progression. Perfringolysin O, encoded by the *pfoA* gene, plays a notable role in the pathogenesis of gas gangrene, acting synergistically with the alpha toxin. Hyaluronidase, described in this study, also has been reported previously in *C. perfringens* isolates from soil and is known to assist *C. perfringens* spread into deeper tissues (48,284).

Whole genome analysis indicated that some of the sequenced isolates (GSH 21, GSH 46 and GSH 48) from unidentified origin, brain, and unidentified origin, respectively were positive for the gene encoding the beta2 toxin. The beta2 toxin is encoded by the *cpb*2 gene and has been found located on a variety of plasmids of different sizes (89,285). This toxin was first identified from a piglet that had necrotic enteritis in 1997 (89), but its role is unclear as it has since been isolated from both diseased and healthy gastrointestinal and non- gastrointestinal human body sites (286,287). Strains harbouring the beta2 toxin have been found in the faecal matter of autistic children (288,289), although the relevance of this association remains unknown. It is considered as an accessory toxin and as a potential contributor to *C. perfringens* related enteric diseases and may act synergistically with other major toxins (89,290). The translated beta2 toxin is smaller than the beta toxin (27.6 kDa and 34.8 kDa respectively) and shares only 15% similarity with the beta toxin at the amino acid level (89). However, it exhibits structural similarity to typical pore-forming toxins (89) and has recently been demonstrated to highly form cation-selective channels in lipid bilayers *in vitro* (291). This toxin has been implicated in the pathogenesis of enteritis in different livestock (89). Thus far two types of beta2 toxins have been described, namely a consensus and atypical form of this toxin (232). Each of the beta2 toxin positive isolates included in the WGS harboured the atypical form of the toxin. Interestingly, even though these genes encode full length toxins that when recombinantly expressed show activity in tissue culture, they appear not to be expressed *in vitro* raising questions regarding their physiological role *in vivo* (232,292). In animals studied the consensus *cpb2* genes are mostly observed in *C. perfringens* isolated from pigs, while the atypical form appears to predominate in other animals (232).

Tetracycline-resistance has been the most common antibiotic resistance trait reported in *C. perfringens* genomes (195). The genes involved in tetracycline resistance are both chromosomally encoded and present on plasmids. The two functional genes [*tetA*(P) and *tetB*(P)] have been reported to overlap by 17 bp (193). The data presented in this study show that all the isolates harboured at least one gene with the potential to confer resistance to tetracycline. Two isolates (GSH 21, GSH 60) encoded both *tetA*(P) and *tetB*(P), while GSH 40, GSH 46 and GSH 48 appeared to only harbour the *tetB*(P) gene. While some *tetB*(P)-positive, *tetA*(P)-negative strains have been reported (23,46,293), further experiments will be necessary to confirm the genotype of the isolates from this study. Importantly, for the GSH 40, GSH 46 and GSH 48 assemblies, the *tetB*(P) genes were located at the extreme end of contigs. Further investigation revealed the presence of truncated *tetA*(P) gene open reading frames (ORFs) at

the extreme beginning of other contigs in the assembly suggesting the possibility that the *tetA*(P) gene was present in these isolates, but poor assembly in the region may have masked the presence of full-length genes. (7). In this case, targeted Sanger sequencing of the relevant loci will be used to confirm the lack of a *tetA*(P) gene for these isolates.

Alternative tetracycline resistance determinants not identified in this study include the *tet*(M) resistance gene, which encodes the TetM protein. This is a cytoplasmic protein that protects the ribosome from the action of tetracyclines (294). Interestingly, the *tet*P(B) gene encodes a protein that has significant similarity to TetM-like tetracycline resistance proteins and is responsible for a low-level resistance to tetracycline (295).

Genome analysis further revealed the presence of a potential clindamycin resistance gene in a clindamycin resistant strain, GSH 40. This drug belongs to the family of the macrolide-lincosamide and streptogramin (MLS) antibiotics. In recent studies it has been shown there is an increase of drug resistance (296). Clindamycin resistance determinants in anaerobes are encoded by erythromycin resistance methylase (*erm* (A), *erm* (B), *erm* (F), *erm* (C), and *erm* (Q)) (189,297). Two of MLS_B genes, *erm*(B) and *erm* (Q), have been reported to cause clindamycin resistance in human and animal *C. perfringens* (188,190). Some studies reported that few *C. perfringens* isolates harbored the *erm* (Q) gene (23,194). In contrast, in Berryman *et al*. (1994), found 79% of *C. perfringens* carried the *erm* (Q) gene, suggesting that it may be widespread in resistant isolates (188). An additional two strains (GSH 40 and GSH 48) showed full phenotypic resistance to clindamycin while only GSH 40 encoded a clindamycin genotypic resistance determinant. Therefore, the resistance in GSH 48 may be due to overexpression of efflux pumps or novel resistance mechanisms. A role for active efflux pumps can be tested in future studies by determining clindamycin MICs in the presence and absence of efflux pump inhibitors (e.g., carbonyl cyanide *m*-chlorophenylhydrazone [CCCP] or reserpine).

The presence of antimicrobial resistance genes is relevant as most antimicrobial agents are extensively used in both human and animal infections as prophylaxis and therapy potentially selecting for drug resistance. Not only are these antimicrobial agents used as therapy but also used in animal feed as growth promoters (6). The continuous misuse of these antibiotics has facilitated the emergence of microbial resistance which has limited the antimicrobial effectiveness. Resistance can occur due to mutations of the targeted site or can be acquired through horizontal gene transfer of extrachromosomal elements such as insertion sequences, phages, and plasmids amongst *C. perfringens* isolates in the environment (46,47,263,294) (294). Some pathogens acquire new genes that inhibit the action of these drugs (such as those

encoding energy-dependent efflux of tetracycline or for a protein that protects bacterial ribosomes from the action of tetracycline); usually these are on plasmids or other mobile elements (294). While isolated from human patients, the phylogenetic analysis of the drug resistant isolates indicated that some of the isolates clustered with foodborne pathogens, as well as potential non-human origins. This warrants further studies to discern environmental sources of human infection and studies of antibiotic use in those environments (for example agriculture and meat producers).

Three isolates (GSH 21, GSH 46 and GSH 48) showed phenotypic evidence of metronidazole resistance, but this could not be associated with specific antimicrobial resistance determinants. Resistance may have been due to a decrease in drug permeability, decrease in drug activation and an upregulation of enzymes involved in DNA repair, all of which are methods that have been identified for other bacteria (298,299). However, metronidazole resistance in *C. perfringens* is not clearly understood (300). Resistance in other anaerobe bacteria such as *Bacteroidetes fragilis* and *Helicobacter pylori*, has been reported to be conferred by *nim* genes, alteration of the genes encoding nitroreductase (*rdxA* and *frxA*) and overexpression of DNA repair genes (301–303). The antimicrobial activity of metronidazole depends on the reduction of the prodrug either by gaining an electron from ferredoxin or from reduced flavodoxin by pyruvate-oxidoreductase (POR) (304,305). Therefore, *H. pylori* studies showed that any alteration in the genes encoding nitroreductase may lead to failure in activating metronidazole in the cell thereby causing metronidazole resistance (301,304,306). Additionally, *recA* genes play a crucial part in repairing the damage in the DNA caused by metronidazole and in SOS responses in both *H. pylori* and *B. fragilis* (307,308). Mutation and overexpression of RecA proteins in *H. pylori* and *B. fragilis* may cause resistances to metronidazole. In other *Clostridium* spp. it has been reported that a multifactorial process which involves alteration in different metabolic pathways such as nitroreductases, iron uptake and DNA repair play a part in metronidazole resistance (309–312). Similar to *B. fragilis recA* genes play a crucial part in repairing the damage in the DNA and in the SOS responses of *Clostridium* spp.

Multi-peptide resistance factor (*mprF*) was detected in all the selected genome isolates. This gene is usually conserved in some Gram-positive and Gram-negative bacteria (46,313). It confers resistance through modifying surface molecules of antimicrobial peptides (AMPs) and thereby evades the host defenses in the gut (46,313,314). The *mprF* gene was first described in *S. aureus* mutants, with modified lipid constituents in the membrane, which resulted in

increased susceptibility to AMPs (314). Subsequently, the two *mprF* genes (*cmprF1*, *cmprF2*) from *C. perfringens* were expressed in *S. aureus* and appeared to also play a role in AMP resistance. Therefore, the presence of *mprF* in *C. perfringens* could suggest that it plays a role in evasion of the host defense mechanisms in the gut.

Putative MDR efflux pumps were also found in all the genomes of this study. ABC transporters were previously described in the *C. perfringens*, where they play a major role in causing intrinsic resistance to broad range antibiotics (23,46). Previous studies reported that the genes that are usually found to cause the MDR in *C. perfringens* include *mep*A and *vga*B, which encode a MDR and toxic compound extrusion (MATE) family efflux pump and an ABC transporter respectively (23,46). However, the putative ABC transporters identified in this study did not show significant homology to either of these proteins. Therefore, further characterization is necessary to propose a role for these proteins in phenotypic resistance.

An investigation based on MLST developed by Deguchi *et al.* (2009) (65) was performed to determine the genetic relatedness of *C. perfringens* in human isolates. Two isolates (GSH 46 and GSH 48) from the brain and an unidentified site belonged to a single MLST, ST274 (315). This could potentially indicate that the two isolates from this study were from a single source, and this was supported by further core genome SNP analyses discussed later. Three isolates (GSH 21, GSH 40 and GSH 60) were assigned to new STs that have not previously been described. MLST revealed that isolate GSH 21 was closely related to two isolates (with ST143 and ST144) that were isolated from a riverbed in South Africa, one isolate from Asia (Japan) and two clinical stool isolates from China (23,316,317). GSH 40 was closely related to two STs (ST339 and ST401), from clinical diarrheal patients in China (317). Previous study indicates that ST401, ST339 and ST274 were associated with human stool and blood samples (315,317). Interestingly, GSH 60 appeared to be genetically divergent, harbouring three novel alleles and differing from the closest match in the PubMLST database (Strain 13, isolated from soil) by a total of six alleles (316,318).

The phylogenetic tree from this study aligned the *C. perfringens* strains into the expected five phylogenetic clades (I-V), which is in agreement with previous studies that reported the phylogenetic relationship of *C. perfringens* indicated that *C. perfringens* strains are divided into five phylogenetic groups (clades), which are mostly host-and/or disease-specific

(49,53,69). For example, The JFP *netF*-positive isolates were found to cluster closely in a core genome MLST (cgMLST)-based phylogenetic analysis (319). Another study indicated that isolates from clade I were primarily associated with foodborne disease and darmbrand in humans suggesting that isolates contained within this cluster are host- or disease-specific. (47,61). However, in this study most clades consisted of isolates from diverse backgrounds, hosts, and diseases. For example, Clade I which consists of isolates from this study GSH 21, GSH 46 and GSH 48, was the largest clade, encompassed *C. perfringens* strains isolated from different hosts: avian (the largest group), equine, human, food, environment, ruminant, canine as well as some strains of unknown origin. The variation in isolation source amongst strains in this clade suggests potential spread and transmission between hosts. Phylogroup II-V also contained isolates from different hosts such as animals, humans, and environment, suggesting a potential spread and transmission between hosts (47). Noteworthy, GSH 21, GSH 46 and GSH 48 contained beta2 toxins and were also the only isolates that exhibited phenotypic resistant to metronidazole. Bendary *et al*. (2022) reported a correlation between continuous use of antibiotic, and a selective expression of virulence gene such as *cpb2* gene. Which might be the case in our study, where metronidazole was frequently used, causing a selection of high expression of *cpb2* gene. Therefore, the frequent use of metronidazole led to development of phenotypic resistance noted in these isolates.

Clade V GSH 60 from this study was closely related to isolates of ruminant origin, which could indicate a spread through the food chain, either directly (come in contact with an animal or excretion) or indirectly (consumption of infected food products such as meat, and dairy products). Meanwhile clade II (GSH 40) is primarily associated with food isolates.

Clade I GSH 21 (from this study) was closely related to an environmental *C. perfringens* strain: SC- C24, which was isolated from a river system in the northern region of RSA. The sampling location was downstream from a wastewater treatment plant and most likely was contaminated by faecal matter. While the area is geographically distinct from Cape Town, it is noteworthy that these strains appeared to be genetically related. Two strains, GSH 46 and GSH 48 (ST274) were closely related to a published human strain, type F, which causes sporadic diarrhoea. In addition, these isolates were identical at the core genome level, suggesting that if not clonal, they were very closely related. Although personally identifiable information was not collected as part of the current study, both isolates were collected during a similar period and may have

been isolated from the same patient (personal communication). GSH 40 clustered with isolates in clade II along with human and food isolates. Finally, GSH 60 (clade V) was related to type D (sheep) isolates, although on a fairly deep branch suggesting that it may have diverged quite significantly from these isolates.

11. Conclusion

The presence of drug resistance, notably clindamycin and metronidazole reduced susceptibility and/or outright resistance, in human clinical isolates of *C. perfringens* is worrisome. Also of concern is the potential origin of these drug resistant isolates which in some cases clustered with isolates of non-human origin. This indicates that these patients potentially at some point were exposed to either infected food products or faecal contamination of non-human origin. Due to the speed with which *C. perfringens* infections progress, it is likely that isolates developed antimicrobial resistance prior to rather than during infection. However, we did not collect clinical metadata from patients and thus do not know the length of hospital stay, previous antibiotic exposure or antibiotics used to treat these specific infections (although the standard recommended regime would be either clindamycin or penicillin). The data presented here suggest we need to be concerned about clindamycin and metronidazole resistance in *C. perfringens* as well as its potential origins. Furthermore, the data suggest studies are necessary to discern antibiotic use and drug resistance in the local poultry and other meat-related industries.

12. Future perspectives

The whole genome sequencing of five clinical isolates in this study suggested origins from diverse environments, both human and environment. This indicates that we need to expand our knowledge about the local reservoirs and antibiotic use practices, as drug resistance develops due to drug pressure. More comparative molecular studies should be done on isolates from clinical samples, animals, food, and water in RSA, enabling early interventions where needed, including education regarding inappropriate antibiotic use, hygiene practices notably where informal farming and slaughtering practices occur, as well as the need to cook food properly.

Furthermore, as plasmids are major drivers for dissemination of some virulence factors and drug resistance genes, studies on plasmids and phages circulating in local strains could be useful. Discernment of plasmids borne in environmental isolates, notably those present in the agricultural food industry is important as the use of antibiotics in food animal and bird rearing potentially could be selective for drug resistance isolates which, in turn, pose a risk via contact of foods (meat and vegetables). Our future studies aim to characterise *C. perfringens* plasmids (if any) in our clinical isolates and determine what genes of relevance (virulence and drug resistance) are carried on these. Also, a weakness of this study was the lack of access to positive controls (those toxin types of animals and bird origin). Future studies should include these. Future studies need to include WGS of all isolates to confirm the absence of these toxins, thus far only five isolates were characterised by analysis of genome data.

13. Appendix

13.1. Preparation of a brain heart infusion (BHI) broth

A total of 18.5 g of brain heart infusion agar was added into 500ml of distilled water. Followed by yeast extract (2.5g), cysteine (0.25), Sodium carbonate (Na_2CO_3, 2g), and 5ml of Hemin-menadione. The media was stirred and heated to dissolve the media. Before the media was poured into sterilised petri dishes, it was autoclaved for 20 min at 121°C.

Table 13.1*:* Media and reagents for preparing a brain heart infusion (BHI) broth.

Media and reagents	
Brain heart-infusion (BHI) broth	18.5 g
Yeast extract	2.5 g
Cysteine	0.25 g
Sodium carbonate (Na_2CO_3)	2g
Distilled water	500 ml
Hemin-menadione	5 ml

13.2. Preparation of 1.5% agarose gel

To prepare the 1.5% agarose gel, 1.5g of agarose powder was added into 100ml of 1X TAE and to dissolve the agarose powder it was melted in a microwave oven. Three microliters (3µl) of ethidium bromide per 100ml of TAE was added prior to solidifying in order to visualise the DNA post-electrophoresis using a ChemiDoc XR imager (Bio-Rad).

13.3. Cultural characterisation

Phenotypic characterisation of *C. perfringens* was recorded based on the growth characteristics of the organism on blood agar: Colony characterisation showed complete hemolysis of the red

blood cells in the blood agar and a sour smell of the culture. Cell morphology characteristics: Gram positive, rod shaped.

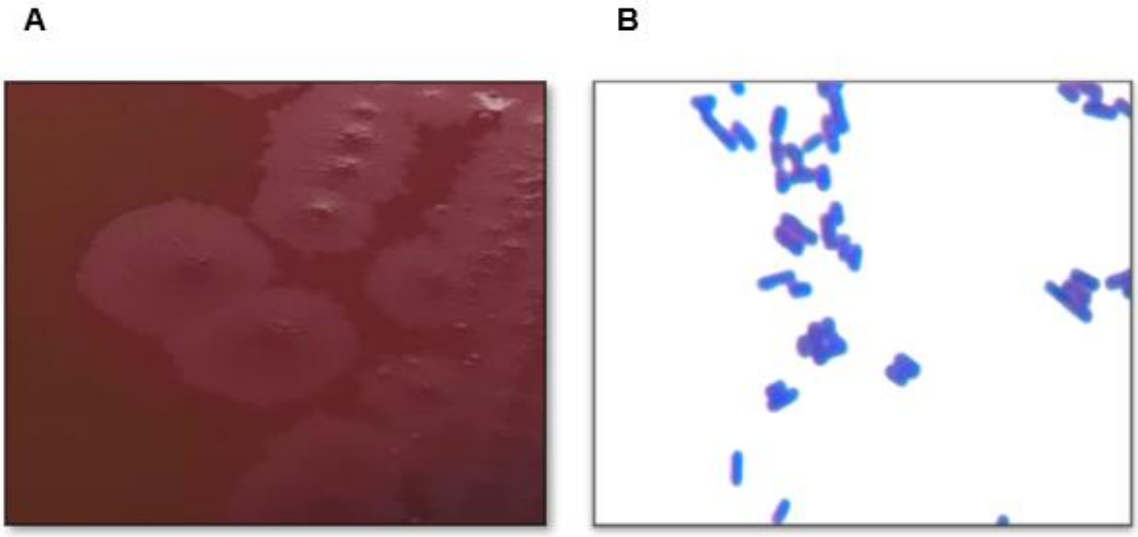

Figure 13.1: *C. perfringens* on blood agar: **A** large, flat colonies with irregular edges (fried egg appearance) and exhibiting a double zone of hemolysis of the red blood cells. **B** Gram stain showing a Gram-positive bacilli *C. perfringens*.

14. References

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