CHAPTER 05

MULTIDRUG RESISTANCE IN CLOSTRIDIA

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INTRODUCTION

Anaerobic bacteria including Genus Clostridium have been showing an increasing trend in resistance patterns in recent years against routinely used antimicrobial agents. There are many resistance mechanisms, which have been evolved either intrinsically or acquired from the external environment by bacteria to overcome these agents (Munita and Arias 2016). The most notable being the production of the enzyme β-lactamase (Kuriyama et al. 2000). Other types of resistances include decreased drug accumulation (either through decreased membrane permeability or more active efflux of antibiotic), drug inactivation or modification (enzyme production), alteration of drug binding and/or target sites (alteration of binding proteins), and alteration in metabolic pathways (Li et al. 2016; Munita and Arias 2016; Brook 2017). Many drugs have become ineffective, especially against infections caused by *C. difficile*, *C. perfringens,* and some other species. Meanwhile, there are still some drugs having 80-100% sensitivity against most clostridia which include chloramphenicol, metronidazole, or even ceftriaxone and penicillin in combination with other drugs (Brook 2017). This chapter discusses mechanisms of resistance development including resistance rates reported by various studies against important clostridial species including C. *difficile*, *C. perfringens*, *C. tetani*, *C. sordellii,* and *C. chauvoei*.

Resistance against β-lactams

Beta-lactams are among the most recommended antibiotics which are routinely used. They inhibit bacterial cell wall synthesis and possess four-membered core-lactam ring. Based on adjoining structures, beta-lactams have four groups i.e., penicillin group, cephalosporin group, monobactams, and carbapenems (Petri 2011).
Resistance against beta-lactams most commonly occurred via the enzyme, beta-lactamase. There are four classes of these enzymes namely A, B, C, and D. A, C and D are serine hydrolases while B class is metallohydrolase (Majiduddin et al. 2002). Class D lactamases have been found in gram positive bacteria which also include the one conserved in *C. difficile* species (Toth et al. 2016).

Clostridioides (Clostridium) difficile

The genetic basis of resistance in *C. difficile* included the production of inducible lactamase (Toth et al. 2016). BlaCDD is the gene responsible to produce this enzyme, CD0457 encoding putative membrane-protein *bla X* is usually co-transcribed with *bla CDD*. *bla X* and *bla CDD* confer resistance against ampicillin and have a signal sequence associated with the cell membrane (Zhang and Shen 2017; Armenteros et al. 2019). Bla operon regulated by BlaR1 exhibits dose mediated expression in lactams. However, the resistance may vary depending upon the geographical situation and is thought to be directly related to the clinical use of cefoxitin. Beta-lactams are relatively ineffective against many clostridial species.
A study reported from Texas in swine production groups has shown all 131 isolates resistant to cefoxitin, imipenem, and ciprofloxacin whereas susceptibility was noticed for amoxicillin/clavulanic acid, piperacillin/tazobactam and vancomycin. Isolates were having intermediate resistance to ampicillin (Norman et al. 2009). In another study, an antimicrobial resistance pattern was studied using 523 *C. difficile* isolates, in an integrated population comprising humans and swine. Swine isolates were those collected from farrows, nurseries, breeding-place, and other production places, while isolates of human origin were obtained from workers at swine raising places and non-workers also. The majority of the strains were resistant to ciprofloxacin and cefoxitin while all of them were susceptible to amoxicillin/clavulanic acid, and vancomycin. The non-significant association was found among these studied groups indicating that transmission is unlikely to occur in an integrated population as proven by the results (Norman et al. 2014). Different studies reported susceptibility to co-amoxicillin using ≥16/8mg/L. Out of 2803 isolates, only 4 were

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found resistant; WPR was 0% having low heterogeneity. However, subgroup analysis was not carried out. The piperacillin/tazobactam susceptibility was studied applying breakpoint (≥128/4mg/L). Out of 3041 isolates, 8 were found resistant; the WPR was 0 % and further subgroups were not compared. The meropenem susceptibility was investigated in 17 studies at breakpoint (≥16mg/L). From total 275 *C. difficile* isolates, 20 were found resistant. No significant difference was found in data from 1992-2014 and the data from 2015-2019 (Sholeh et al. 2020a).

Clostridium perfringens

The most commonly used antibiotics in humans and animals belong to a β-lactam class of antibiotics (Price et al. 2019). Due to this prolonged usage, resistance against β-lactams has been found most frequently in bacteria, which has posed a difficulty in treating and overcoming these bacterial infections. Most of these bacteria produce beta-lactamase enzyme, which hydrolyzes the beta-lactam ring present in these compounds, thereby making them ineffective (Bush and Bradford 2016). The situation is worrisome not only for aerobes but also in case of anaerobes like *C. perfringens*, due to the existence of *bla 2* gene which has been reported in some of the studies from clinical settings (Mishra et al. 2016). This *bla2* gene was initially reported in Firmicutes, and bacteria like *C. perfringens* have acquired it from the external environment and incorporated it into their genomes.

Usually, penicillin G has been used as a drug of choice and most Clostridium strains have also been found susceptible to penicillins until recently. The exceptions include *C. ramosum*, *C. innocuum,* and *C. clostridioforme*. Clinically management of infections caused by anaerobes had been good with penicillin G. Semisynthetic penicillins are less effective as compared to penicillin, ampicillin, and amoxicillin. However, due to the production of BLs by anaerobes, these drugs have limited use. The first generation cephalosporins have activity similar to penicillin G, however, they have been found effective against *C. perfringens*. Additionally, the biofilm formation by *C. perfringens* might protect the cells from atmospheric oxygen and higher concentrations of the antibiotic penicillin. The resistance mediated by biofilm formation in *C. perfringens* has been found against penicillins, virginiamycin, lincomycin, tylosin, and even salinomycin, monensin and narasin (Charlebois et al. 2017). In a study carried out to find the antimicrobial susceptibility of *C. perfringens* against different antibiotics, the resistance rate was 26% against ampicillin, 3.7% (chloramphenicol), 15.2% (Ciprofloxacin), 6% (clindamycin), 32.9% (erythromycin), 45.4% (gentamicin), 0% (metronidazole), 52.5% (nalidixic acid), 10% (vancomycin), 21.8% (penicillin), 32.1% (trimethoprim-sulfamethoxazole), 19.3% (amoxicillin), 38% (imipenem) and 2.5% against ceftriaxone respectively. The resistance rate to cloxacillin was 100%, to cephalexin was 0%, to oxacillin 45.6%, to cephalothin 8.6%, bacitracin 89.1%, and colistin 40% respectively. Overall high dosage of penicillin is an effective therapy against soft tissue infections caused by *C. perfringens* in humans. Penicillin resistant *C. perfringens* strains are rare, as no strain was found resistant to it, in studies carried out in Brazil (Silva et al. 2009), Canada (Leal et al. 2008), and New Zealand (Roberts et al. 2006). In Iran, however, the resistance rate reported was higher, i.e., 21.8%. This difference might be due to the difference in the type of samples used. It has been known that *C. perfringens* is a normal flora of the GIT of animals and humans and may lead to foodborne diseases. These microorganisms also can transfer resistance genes via mobile elements to other gut microbiota (Hosseinzadeh et al. 2018).

Resistance against Cephalosporins

Anaerobes have evolved three major mechanisms conferring resistance to them against beta-lactam antibiotics. Firstly, inactivating enzymes i.e., β-lactamases including penicillinases and cephalosporinases (Kuriyama et al. 2000); secondly, low-affinity penicillin binding proteins (PBPs); thirdly, decreased permeability via alterations in porin channels. Cephalosporinases are quite often belonging to subgroup 2e and may inhibit BL inhibitors like clavulanic acid, tazobactam, sulbactam). Cephalosporins have either a class or specific beta-lactamase enzymes, which can inactivate them (Bui and Preuss 2021). The aerobic anaerobic i.e., polymicrobial infections usually require metronidazole in addition to beta-lactam, cephalosporin, or fluoroquinolones for treating anaerobes as BLs are becoming ineffective against anaerobes. There has been an emerging trend in the resistance of anaerobes against penicillins, cephalosporins, clindamycin, and fluoroquinolones.

Anaerobic microorganisms can be tested for enzyme beta-lactamase by using chromogenic cephalosporin test like nitrocefin disks (Papanicolas et al. 2014). Many anaerobes possess cephalosporinase enzyme, which is the reason for limited efficacy of cephalosporins against anaerobes. First generation cephalosporins have activity much like penicillin G against anaerobes. The second-generation drugs like cefoxitin have efficacy against anaerobes however this efficacy varies geographically and also these drugs a relatively less effective against Clostridia with the exception of *C. perfringens*. Third generation drugs have also raised concerns regarding antimicrobial resistance and are of limited use against clostridia.

Clostridioides (Clostridium) difficile

C. difficile is usually evaluated routinely for antibiotics, having an association with CDI. Cephalosporins and clindamycin are considered high risk agents for CDIs. Cephalosporin has been known to be resistant to *C. difficile*; even studies have reported its overgrowth following CFs therapy. The mechanism of resistance to these drugs is not known in-depth and they are termed as constitutively resistant to cephalosporin (Spigaglia 2016). The resistance may be strain dependent also and it is known that antibiotic degrading enzymes and modification of the target site are mainly involved in making these drugs ineffective.

C. difficile resistance pattern against β-lactam antibiotics has been found variable in different studies. Against cephalosporins resistance rates are 14.3%(ceftriaxone), 3.5% (cefoperazone), 10.5% (Cefepime) which are low as compared to 76% against ceftazidime and 95% for cefotaxime. Recent studies have shown that lactamase enzyme in *C. difficile* imparts resistance to penicillins, cephalosporins and monobactam class of lactams (Banawas 2018). Ceftriaxone susceptibility was also reported in various studies. From 3476 isolates used in various studies, 1289 were having resistance. The percentage was 37.1 at breakpoint ≥64mg/L. WPR for this drug was 47% with substantial heterogeneity.

Resistance against Chloramphenicol

Anaerobes were not found to have resistance against CH, although few clinical studies have reported treatment failure.

Lack of resistance might be due to its infrequent use clinically. In the USA, this drug has been used rarely. Resistance against chloramphenicol is rare however some strains have MICs clustered nearby breakpoints. This resistance is because of the drug inactivation due to nitroreduction and/or acetyltransferase. It is a bacteriostatic agent having good susceptibility against anaerobes, MICs of this drug is usually clustered near the susceptibility range. Some reports do show treatment failure using chloramphenicol but on the other hand, this drug has been in use for the last 65 years against anaerobic infections.

Clostridioides (Clostridium) difficile

Multidrug resistance has been observed in *C. difficile* isolates obtained from animal sources including chloramphenicol. In a study on swine population, the strains were found resistant to clindamycin, intermediate susceptible to ampicillin, and susceptible to chloramphenicol and tetracycline (Norman et al. 2009). In another study carried out on an integrated population of humans and swine, the human isolates were obtained from wastewater collected from workers and non-workers. All the strains of *C. difficile* isolated from human subjects were susceptible to chloramphenicol (Norman et al. 2014). In *C. difficile* resistance against chloramphenicol has been mediated by catD gene encoding CAT enzyme , present on mobilizable transposans Tn4453a and Tn4453b, having structural and functional relatability with *C. perfringens* transposan Tn4451 (Lyras et al. 1998).

Chloramphenicol antibiotics are still recommended against *C. difficile* infections. In Iran, a total of ten studies have reported resistance patterns of *C. difficile* against various antibiotics. The fixed effect model was used for studying some antibiotics including Chloramphenicol. A resistance pattern against this drug was observed in 6.2% isolates. In Europe resistance reported was 3.7%. Data have indicated that Chloramphenicol can still be recommended for CDI.

Clostridium perfringens

Previously isolates of *C. perfringens* from swine have shown multidrug resistance against clindamycin, erythromycin, and tetracycline, but these isolates were susceptible to chloramphenicol. On the other hand, isolates identified from African (Cote d'Ivoire) cooked beef have shown resistance to chloramphenicol including some other drugs (Kouassi et al. 2014). In another study multidrug resistance to chloramphenicol along with some other drugs was found in 5% isolates of *C. perfringens* from animal origin (Mau-Inchaustegui and Rodriguez-Cavallini 2011). It is notable that that commonly reported resistance determinants to date have been found associated with bacitracins, MLS, tetracyclines, and chloramphenicol drugs.

Resistance against CH has been mediated via acetyltransferase enzymes encoding cat(P) and cat(Q) genes. Cat(Q) gene has been shown to have variation from *C. perfringens* cat(P) gene. Moreover, cat(Q) monomer has 53% sequence conservation with cat(P) and 39-53% with other cat proteins at amino acid level. Phylogenetic analysis has shown that cat(Q) is closer to Cat proteins from *S. aureus* and *C. coli* like cat monomers from Clostridial species. In the case of *C. perfringens*, mobilizable transposans including Tn4451 and Tn4452 have been identified (Adams et al. 2002) conferring resistance against chloramphenicol antibiotic, but these genetic elements are not conjugative, which is taken care by co-resident elements thereby facilitating transfer to the cells. Tn4451 mobilizable transposan has elicited transposition dependent upon unusual resolvase enzyme. Conventionally transposition has been dependent upon transposase or integrase enzymes (Adams et al. 2002). PIP401,53kb plasmid has been the first conjugative plasmid identified from *C. perfringens* which imparts resistance against chloramphenicol as well as tetracycline. It was obtained from human isolate of *C. perfringens* type A strain (CP590).

Resistance against Macrolide–Lincosamide–Streptogramin (MLS_B)

Clostridioides (Clostridium) difficile

C. difficile has acquired resistance against clindamycin, erythromycin. *C. difficile* isolates became resistant to MLS_B family i.e., Macrolide-lincosamide-streptogramin B, through ribosomal methylation process. Erythromycin ribosomal methylase B (ermB) has been the most widespread resistance gene detected in *C. difficile* isolates (Schmidt et al. 2007; Spigaglia et al. 2005). The erm class B is usually present on mobile genetic elements, best known of them is Tn 5398, a 9.6 kb mobilizable transposan sequence (Farrow et al. 2001). This element has two copies of ermB and is known to be transferable *in vitro* from *C. difficile* to *S. aureus* and/or *B. subtilis*. Transfer of Tn5398 from donor to recipient is carried out by other conjugative transposans responsible for Integration and/or excision in the donor genome as Tn5398 does not encode gene for producing recombinase enzyme (Mullany et al. 2015). Integration process into recipient may occur through homologous recombination or site-specific recombinase enzyme of the recipient cell. It is also recently known that a portion of genome having Tn5398 integrate in to the recipient cell through homologous recombination (Wasels et al. 2015b). Resistance against erythromycin, clindamycin or erythromycin alone has also been reported in erm negative *C. difficile* strains. Some of these strains have alterations in 23RrDNA/ribosomal proteins (L4 or L22). However, these alterations also exist in the susceptible isolates which exclude their role in imparting resistance (Spigaglia et al. 2011). It is further noticed that resistant erm-negative strains when treated using 2 pump inhibitors (reserpine and carbonyl cyanide m-chlorophenyl hydrazone–CCCP), didn't reduce MICs, thereby indicating non-involvement of efflux mechanisms in mediating resistance (Spigaglia et al. 2011). In this perspective, in the absence of *erm* genes, other determinants might have role in *C. difficile* resistance to MLS_B. The other determinants including cfrB and/or cfrC, encoding 23SrRNA methyltransferase impart resistance against phenicols, lincosamides, oxazolidinones, pleuromutilins and streptogramin A (PhLOPSA) have also been detected in several *C. difficile* strains (Candela et al. 2017). The cfr gene denominated as Tn6218 is a non-conjugative element, which is also associated to Tn916. A study from Japan reported more than 96% resistance in *C. difficile* isolate against lincosamide and macrolides (Senoh et al. 2015). Prevalence data from Iran shows 61.5% of isolates are resistant to erythromycin (Khademi and Sahebkar 2019).

Clostridium perfringens

Cross resistance has been observed in macrolide, lincosamide, and streptogramin B groups. MLS_B antibiotics inhibit protein

synthesis in gram positive as well as gram negative bacteria and resistance against these antibiotics is because of methylation of 23SrRNA encoding *erm* genes, thus preventing MLS_B antibiotics binding with ribosomes. The resistance mechanism in anaerobes against MLS_B antibiotics has been conferred by five genes including *erm*(Q), *erm*(B), *erm*(C), *erm*(F) and *erm*(G). However, not even a single gene has been identified in these bacteria that encode MLS resistant efflux proteins and/or inactivating enzymes (Roberts 2003).

Mef(A) gene encoding efflux pump associated resistance to macrolides alone (while lincosamides and streptogramin B were still effective) was first identified in *S. pneumoniae*. This gene was later on identified in *C. perfringens*. The sample source was soil, sewage and water across 14 US states (Soge et al. 2009). Besides other antibiotic resistance genes, the study also reported *erm*(Q) and *erm*(B) highlighting that environmental *C. perfringens* isolates might act as reservoir for these resistance genes.

Macrolides have medium to good in vitro activity against most anaerobes, but they cause little toxicity also. The most effective macrolide against gram positive oral anaerobic microflora is Clarithromycin. Similarly, erythromycin is effective to some extent against severe soft tissue infections caused by anaerobes, providing good adequate cleaning and drainage of the infected tissue. Erythromycin resistance has been observed in 32.9% of samples in a study from Iran (Khademi and Sahebkar 2019). A study on three *C. perfringens* genomes revealed *erm*(Q) gene that encodes resistance to MLS_B. The *erm* gene i.e., *erm*(Q) has been first detected in *C. perfringens*.

Resistance against Clindamycin

Clindamycin, a broad-spectrum antibiotic has proven clinical effectivity against anaerobes as shown in the previous research/clinical trials. It has been used against dental infections in people allergic to penicillin as well as against aspiration pneumonia. Clindamycin hydrochloride has rapid complete absorption from GIT and also reaches all body tissues including sputum, saliva, soft tissues, respiratory tissue, prostate, semen as well as bones and joints**.** Resistance is conferred by macrolide-lincosamide-streptogarmin (MLS) type 23 S methylase, usually encoded by one of the *erm* gene expressed in higher amounts. This antibiotic is not recommended nowadays against intraabdominal infections. Several species of clostridia have become resistant to this drug. 20% of *C. ramosum* isolates have been found resistant to clindamycin (Goldstein and Citron 2011).

Clostridioides (Clostridium) difficile

C. difficile has shown variable susceptibility against clindamycin. Resistance has been on the increasing side in treating skin and soft tissue infections. *C. difficile* isolates from equine origin identified over a period of seven months have shown high levels of resistance to clindamycin. A Spanish study reporting 144 isolates of porcine origin reported a higher incidence of RT078 (94.4%) having multidrug resistance in 49.3% of isolates tested (Peláez et al. 2013). This study reported more than 27.8% resistance to clindamycin. Similarly, Italian isolates of *C. difficile* sampled from swine and dogs have shown 10 and 6 ribotypes from both species respectively (Spigaglia et al. 2015). The major strain found was RT078 (50% in swine isolates), whereas non-toxigenic canine strain RT010 was found in 64% isolates. 15% resistance was reported against clindamycin in pigs and 51% was found for isolates of canine origin (Spigaglia et al. 2015). The isolates belonging to ribotype 027FQR had 75 -100% resistance against clindamycin. A detailed study from 15 regions of Italy has shown MDR in more than 59% of isolates and 100% resistance in PCR ribotypes 365/607 & 018. MDR pattern in this species is often linked to resistance against clindamycin (Spigaglia 2016).

A meta-analysis of the data included 64 studies reporting around 20,000 *C. difficile* isolates; 6685 (34%) were found resistant to clindamycin. The WPR to clindamycin was 59% having no significant difference on the basis of time of study when it was conducted (Sholeh et al. 2020a). Significant differences were found when continent wise data was compared. The highest resistance was found in isolates from Asia followed by South America. When compared on the basis of the quality of studies set using different parameters, the high, moderate, and low quality data reported 63%, 57%, and 17% respectively (Sholeh et al. 2020a). The method used for antimicrobial susceptibility testing was also having significant differences among them.

Clostridium perfringens

Studies carried out on samples of porcine origin of *C. perfringens* have reported resistance against erythromycin, tetracycline as well as clindamycin. Some recent studies also reported 28% resistance against *C. perfringens* isolates of porcine origin reported from Canada. The *C. perfringens* isolates from bovine origin have also reported reduced susceptibility to clindamycin. 5% of *C. perfringens* isolates of animal origin have shown resistance clindamycin in other studies (Mau-Inchaustegui and Rodriguez-Cavallini 2011).

Resistance against Metronidazole

Metronidazole usually gives well anaerobic coverage. Nitroimidazoles are effective against anaerobes as intracellular reduction of the drugs into active antibacterial metabolites takes place anaerobically. However, these are genotoxic and therefore not used in food animals in various countries.

Resistance against metronidazole is attributable to nitroimidazole reductase (nim) enzyme, which converts 4-,5-nitroimidazole into 4-,5-aminoimidazole and avoid toxic radical formation required for antimicrobial effect of drug. Nim has been found in aerobic as well as anaerobic bacteria. Nim genes are usually located on mobilizable plasmids and can lead to 5-Ni drugs ineffectiveness. However, resistance sometimes exists even in nim negative strains due to the sub-MIC concentrations of metronidazole.

Clostridioides (Clostridium) difficile

C. difficile infections have been treated commonly using oral metronidazole, fidaxomicin etc. (Spigaglia 2016). However, this microorganism possesses many resistant mechanisms, like, metabolic pathway changes, biofilm formation, *ermB* gene (Resistance against MLS_B). *C. difficile* ribotype 027 has been shown to have unusually high resistance against metronidazole drugs (Peng et al. 2017). Although this type of resistance has been quite uncommon. Recent studies on ribotypes 027 and 010 have shown the resistance to metronidazole a complex process. Alterations in metabolic pathways involve

nitroreductases activity, iron uptake, DNA repair as well as biofilm formation are thought to play a vital role (Chong et al. 2014). It is predicted that biofilm matrix alters the physiological state of bacteria thereby acting as a protective barrier as well as imparting more resistance against antibiotics (Vuotto et al. 2016).

A study carried out in Western Australia from 2007 to 2009 found 23% of diarrheal horse isolates susceptible to metronidazole (Thean et al. 2011). A cross sectional study including diarrheal and non-diarrheal foals reported 7 samples positive for *C. difficile* A/B toxin (from diarrheal foals), having susceptibility to metronidazole (Silva et al. 2013). Resistance against metronidazole was however observed in horses having an acute gastrointestinal disease (Magdesian et al. 2006). Studies involving human patients have been reporting high resistance against metronidazole. There has also been an increase in geometric mean of MICs i.e., for RT027 (1.1–1.42mg/L), RT001/072, RT106 and RT356 (0.6 mg/L), RT010 (1.5mg/L) and other RTs (0.13–0.4mg/L) (Freeman et al. 2015). Metronidazole susceptibility to 19645 isolates of *C. difficile* was investigated in more than 100 studies. EUCAST breakpoint 2mg/L was taken as standard in 32 studies reporting 5900 isolates. About 190 were reported to be resistant; WPR to metronidazole was 1%. When the data were compared based on the difference in time range; there was an increase in resistance during time period 2015-2019 as compared to 1992 to 2014. Highest resistance was seen in isolates of Asian origin. The CLSI breakpoint (32mg/L) when applied, 129 out of 13207 isolates were found resistant. There was non-significant association when different parameters were compared like time period and geographical location (Sholeh et al. 2020a).

Clostridium perfringens

Resistance against metronidazole has rarely been reported in *C. perfringens* isolates obtained from humans and/or animals. A study from Sweden included 50 *C. perfringens* isolates obtained from acute diarrheal dogs reported 54% of isolates had decreased susceptibility to metronidazole, having MIC 4mg/L (Gobeli et al. 2012). Another study from Costa Rica reported multiple resistance to several drugs including metronidazole in 5% of *C. perfringens* isolates obtained from animal sources (Mau-Inchaustegui and Rodriguez-Cavallini 2011). Most of these isolates possessed intermediate susceptibility to the drug, metronidazole (57% susceptible; MIC:16mg/L). Similarly, an isolate obtained from a dog has reported a metronidazole resistant strain of *C. perfringens* (Marks and Kather 2003).

Resistance against Tetracyclines

The second most commonly used broad spectrum antibiotic after β-lactams is tetracycline, which nowadays has limited use against anaerobic infections because of the rapid development of resistance against it. Tetracycline analogs including minocycline and doxycycline also have limited use owing to the significant resistance and therefore require susceptibility testing before use to confirm effectiveness. Tigecycline has been found active against anaerobic gram positive bacteria (Frampton and Curran 2005).

Clostridioides (Clostridium) difficile

In *C. difficile*, resistance to tetracycline is mediated by tet genes including *tet(M)*, *tet(P)*, *tet(K)*, *tet(L)*, *tet(W)* and *tet(X)*. The most commonly widespread is tet(M), which is usually carried by conjugative Tn916 like elements (Spigaglia 2016). Both tet(M) and tet(W) have been identified in human and animal isolates of *C. difficile* (Fry et al. 2012). The latest detected tet(X) genes or mutations in the existing *tetM* and *tetW* classes might increase the resistance to tigecycline (He et al. 2019).

This transposan family is responsible for conferring antibiotic resistance to pathogens not only against tetracycline but also other classes of antibiotics. The commonly well-known element in this family is a 21kb Tn5397 which has *in vitro* capability of transfer between *C. difficile* and *B.subtilis*/*E.faecali* (Jasni et al. 2010). Tn5397 element having *tndX* genes encodes for serine recombinase enzyme inserts DNA predicted filamentation processes induced by cAMP (Fic) domain (Wang et al. 2006). Group II intron and a variable excision/insertion module distinguish Tn5397 from Tn916. Tn916 containing xisTn and intTn, encodes excisionase and tyrosine integrase enzyme inserts at multiple regions into the genome of *C. difficile* and carries tetM alleles (Mullany et al. 2012). Around 31 studies indicating the susceptibility of 4861 *C. difficile* isolates to tetracycline have reported 886 isolates under the resistant category (breakpoint 16mg/L). The weighted pooled resistance was 20% having substantial heterogeneity. The continental categorization was having significant differences while there was non-significant difference in two different points of time in the same region. The resistance patterns against tetracycline were 34%, 26% and 16% in Oceania, Asia, and Europe respectively. Categorization on the basis of quality of articles gave resistance rates as 22%, 16% and 40% for high, moderate and low quality data (Sholeh et al. 2020b).

C. difficile isolates from swine kept in the US have shown resistance against tetracycline indicating the presence of tet(M) gene in 97% of isolates, tet(W) in 32%, and a subset of 31%, having both of these genes. However, these 31% isolates showed different MIC values within the "resistance" category (Fry et al. 2012). Besides tetM, various tet genes also existed in *C. difficile*. The presence of both *tetM* and *tetW* has been found in human as well as animal origin isolates (Fry et al. 2012). Tn6164, a 106kb element was reported in M120 *C. difficile* strain. This element contains parts from different bacteria like *S. pneumoniae* etc also predicted to confer resistance to tetracycline etc. Since this M120 strain is susceptible to tetracycline class, Tn6164 is not seemed to be involved in resistance. However, it has been found to enhance virulence of this strain, which results in mortality in more patients as compared to the people infected with strains not having this element.

Clostridium perfringens

Bacterial resistance to tetracycline came from one or more of the 36 tet genes which follow any one of the three resistance mechanisms (Sheykhsaran et al. 2019). *tetA*(P) and *tetB*(P) were the first identified two functional overlapping resistance genes in *C. perfringens* R-plasmid pCW3. The reduced susceptibility has also been reported in poultry to *tet(M)* gene, in addition to tet(P) genes. Other studies also reported tet(Q), tet(K), tet(L), tet(O), and tet(W) (Gholamiandehkordi et al. 2009). The tet P determinant commonly associated with conjugative as well as non-conjugative plasmids has been present only in Clostridium spp. and has demonstrated the capability to spread in the whole clostridium genus (Vidor et al. 2019). TetA(P) has been found to be linked to all tetracycline resistant strains. Most isolates

resistant to tetracycline also possess tetB(P) or tet(M) genes. TetB(P) has been shown to be associated with low level resistance and did not disturb MIC of isolates already possessing tetA(P) gene (Johansson et al. 2004).

A study on 124 resistant strains isolated from dogs revealed 96% isolates carrying tetA(P) and 41% isolates having tetA(P) and *tetB*(P) genes. *tet(M)* or alone *tetB*(P) was not observed in these isolates (Kather et al. 2006). Tetracycline resistance commonly observed in *C. perfringens* has been associated with antibiotics used in animal feed. A study conducted on 81 tetracycline resistant strains reported all strains carrying the tetA(P) gene with 43 strains having *tetB*(P) on the tet(P) operon. The other 32 strains were having chromosomally encoded tet(M) gene along with tetA(P) gene. The tetracycline resistance has also been reported worldwide in *C. perfringens* isolates identified from poultry. Previously studies have reported resistance to oxytetracycline (MIC>1mg/L) in samples from countries i-e, Sweden (76%), Denmark (10%), Norway (29%), and Belgium (66%) against *C. perfringens* isolates (Johansson et al. 2004; Martel et al. 2004). The isolates from Canada and Korea have also shown high resistance patterns against tetracycline (Park et al. 2015).

Resistance against Fluoroquinolones

Clostridioides (Clostridium) difficile

There are concerns over the use of fluoroquinolones in treating anaerobic infections because of the rapid increase in merging resistance against bacteroides and anaerobic cocci, as well as the impact of these on increasing infections of *C. difficile*. Anaerobes are having natural resistance against older fluoroquinolones. In *C. difficile* resistance against FQs is mainly because of the alterations in quinolone resistance determining region i-e, QRDR of either GyrA or GyrB. This gyrase subunits i.e., GyrA or GyrB may have several amino acid substitutions. However, the most common substitution in FQs resistant *C. difficile* strains was found to be Thr82Ile in GyrA (Spigaglia et al. 2011; Kuwata et al. 2015). This substitution however did not affect *C. difficile* strains *in vitro*, indicating that it can be sustained even in situations, where antibiotic selective pressure is not present at the population level (Wasels et al. 2015a). Repeated exposure to moxifloxacin and levofloxacin also gave rise to mutant resistant strains of *C. difficile* (Spigaglia et al. 2009). As the drug concentration in the intestine of humans is not inhibitory at earlier treatment stages, therefore there are chances that mutation may be acquired in the bacteria against FQs. Ciprofloxacin, a second generation FQ was observed to have 0-1% susceptibility against *C. difficile* strains (Kuwata et al. 2015). Resistance in fourth generation antibiotics like moxifloxacin and gatifloxacin was observed against 36% and 68% *C. difficile* strains respectively (Freeman et al. 2015; Kullin et al. 2017).

The *C. difficile* hypervirulent 027 PCR ribotype has shown frequent alterations in GyrA and/or GyrB subunits, imparting resistance against fluoroquinolones. In a study conducted in Virginia USA on 3118 isolates, resistance against fluoroquinolone and MDR 027 ribotype was frequently seen and reported in 32% of *C. difficile* isolates (Carman et al. 2018). Another study carried out in Japan has shown susceptibility in *C. difficile* isolates against metronidazole and vancomycin, however more than 96% of ribotypes 018 and 369 were having resistance against fluoroquinolones, lincosamides and macrolides (Senoh et al. 2015). Moxifloxacin is not recommended for CDI treatment however resistance against this drug is an important marker for spread of *C. difficile* in health care setups (Dingle et al. 2017). When ciprofloxacin and moxifloxacin were used as representative fluoroquinolone drugs against *C. difficile* isolates, the former showed highest resistance (95% WPR), and the latter was having 32% (CLSI standard) and 49% (EUCAST standard) resistance (Sholeh et al. 2020a).

The usage of antibiotics including clindamycin and cephalosporins along with fluoroquinolones, ampicillin, and amoxicillin are associated with a high risk of CDI. The resistance rate may vary in different places. In Iran, *C. difficile* were found to have high resistance i.e., 69.5% against ciprofloxacin, 93.4% levofloxacin, 92.9% against nalidixic acid, and 67.9% against moxifloxacin. High resistance against moxifloxacin were also found in *C. difficile* strains from countries including China, Korea, and Germany reporting 61.8%, 62.6%, 68% resistance respectively, and 100% resistance against isolates from the Czech Republic and Poland. Low resistance rates were seen in Brazil (8%), France (8%), Hungary (41.2%), Israel (4.7%), Japan (0%), New Zealand (0%), Sweden (15%), Spain (43%), and United States (36%) (Banawas 2018). in addition over usage of fluoroquinolones has been found associated with hypervirulent 027/BI/NAP1 *C. difficile* strain (Peng et al. 2017).

Clostridium perfringens

Anaerobes are no more susceptible against first generation FQs. The newer class of quinolones, however, has significant activity against anaerobes like *C. perfringens*. Low susceptibility quinolones include levofloxacin, ofloxacin, ciprofloxacin, enoxacin, pefloxacin, fleroxacin, and lomefloxacin. gatifloxacin, grepafloxacin. Moxifloxacin, Sparfloxacin, and Trovafloxacin have intermediate anti-anaerobic activity. Trovafloxacin has restricted use as it is hepatotoxic. Highly susceptible drugs include clinafloxacin and sitafloxacin, as they show the highest *in vitro* activity against anaerobes (Stein and Goldstein 2006). FDA has approved moxifloxacin usage and it has been successfully used against the skin and mixed intraabdominal infections caused by anaerobes including *C. perfringens*.

Resistance against Aminoglycosides

Anaerobic bacteria have a natural resistance to aminoglycosides, owing to their requirement of oxygen for their movement to the cytoplasm of the cell.

Clostridioides (Clostridium) difficile

Bacitracin antibiotic has BcrA,-B, and –C; an ATP-dependent ABC type efflux system responsible for its non-effectiveness against *C. difficile* isolates. Resistance against kasugamycin is mainly because of KsgA gene producing dimethyl transferase enzyme (Duffin and Seifert 2009).

Clostridium perfringens

Aminoglycosides aren't able to reach the target site in the bacterial cell. In a cell free environment streptomycin and gentamicin can bind and stop protein synthesis in *C. perfringens* ribosomes. Uptake of these drugs is either energy dependent

or energy independent. When energy dependent, it will be available from O_2 or N_2 dependent electron transport system. However strict anaerobes lack this system and don't have the capability to import these drugs (Ricci and Piddock 2003). These drugs, therefore, don't accumulate inside *C. perfringens.* Trimethoprim-sulfamethoxazole also is ineffective against anaerobes.

Antibiotic Resistance in other Species of Clostridium

Clostridium tetani

Clinical isolates of *C. tetani* have been found susceptible to penicillin, although most studies have targeted antitoxin and vaccine developments against this bacterium. Penicillin has been considered the standard treatment (Campbell et al. 2009) but the efficiency of penicillin depends on its efficiency to reach the infection site effectively. In most studies all strains were found sensitive to penicillin, highest MIC was found 0.25ug having zone of 29mm using 10ug discs commercially available (Campbell et al. 2009). A study from Pakistan also reported all *C. tetani* isolates were sensitive to penicillin. However, another report from Canada has reported alive *C. tetani* in wounds treated for two weeks using high penicillin doses. Some patients have prolonged recovery time which lasted for 16 days in a study using penicillin as a treatment option.

A study on 45 clinical *C. tetani* isolates reported none of the strains were resistant to penicillin. In this way unlike other clostridial species e.g., *C. tetani* has not been found to acquire resistance against commonly used antimicrobial drugs (Sebaihia et al. 2006). The results, therefore, highlighted the fact that penicillin can still be used for treating tetanus along with additional therapeutic agents. The inefficiency to treat with penicillin is sometimes due to the reason that the appropriate therapeutic dose administered intravenously could not be able to reach the infected tissue in required amount.

Metronidazole has nowadays been considered as the first line of treatment against *C. tetani* infections as an alternative to penicillin. Other drugs effective against tetanus includes cephalosporin, chloramphenicol, clindamycin, macrolides, and tetracycline (Sebaihia et al. 2006). Metronidazole is an alternative drug against CT after penicillin has the highest MIC of 1.0ug having zone of 26mm using 5ug discs. A study on clinical *C. tetani* isolates reported none of the strain resistant to Metronidazole (Campbell et al. 2009). *C. tetani* has been found resistant against erythromycin in studies.

Clostridium chauvoei

The virulent *C. chauvoei* strain gives potential insights into the genome of this microbe and revealed its replication in infected tissues of the host and the role of various virulence genes during that process. The chromosomal region of this microorganism has resistance genes conferring resistance against antibiotics. Nevertheless, *C. chauvoei* has been found sensitive to many antibiotics. The MICs for JF4335 strain using CLSI standard on Mueller Hinton broth were 2ug/ml for Cephalotin, 0.5 ug/ml for Clindamycin, 0.25ug/ml for Enrofloxacin, 0.25ug/ml for Erythromycin, <0.012 ug/ml for Penicillin, 1ug/ml each for Vancomycin and Tetracycline and <4ug/ml for chloramphenicol (Frey and Falquet 2015). These MICs have given clear indication of failure of treatment of blackleg using antibiotics which lead to rapid death of animals. The strain JF4533 has also been found to have a gene for resistance against penicillin and an elongation factor G type gene for tetracycline resistance. The Vancomycin B type (vanW) gene has also been found to be present along with other resistance genes. Besides these, there are multi-antimicrobial extrusion proteins that confer resistance against antibiotics. While all these genes have been present in JF4533, they are either not expressed or are producing non-functional proteins doing functions other than exporting antimicrobial agents (Frey and Falquet 2015).

Table 1: Antibiotic activity against *C. difficile*

Resistant	Intermediate	Susceptible
Beta lactam	Ampicillin	Metronidazole
Tetracycline	Moxifloxacin	Vancomycin
Lincosamide	Rifampicin	Fidaxomicine
Microlides	Gatifloxacin	Chloramphenicol
Fluoroquinolones	Clindamycin	Cefoperazone
Ciprofloxacin		Ceftriaxone
Cephalosporin		Cefepime
Erythromycin		
Ceftazidime		
Ceftaxime		
Aminoglycosides		

Table2: Antibiotic activity against *C. perfringens*

Resistant	Intermediate	Susceptible
Beta lactam	Semi-synthetic Penicillin	Penicillin
Cloxacillin	Cephalosporin	Ampicillin
Oxacillin	Sparfloxacin	Amoxicillin
Cephalothin	Grepafloxacin	Metronidazole
Bacitracin		Trovafloxacin
Colistin		Gatifloxacin
Tetracycline, Doxycycline		Moxifloxacin
Viirginiamycin		Chloramphenicol
Macrolides		Vancomycin
Lincosamide		Ceftriaxone
Ciproflaxacin ,Ofloxacin		
Levofloxacin, Fleroxacin		
Pfloxacin, Enoxacin,		
Lomefloxacin		
Clindamycin		
Erythromycin		
Aminoglycosides		

Table 3: Antibiotic activity against *C. tetani*

Resistant	Intermediate	Susceptible
Co-Trimoxazole	Macrolides	Penicillin
Erythromycin	Clindamycin	Metronidazole
Ofloxacin		Chloramphenicol
		Tetracycline
		Cephalosporin
		Cefaperazone

Table 4: Antibiotic activity against *C. chauvoei.*

Resistant	Intermediate	Susceptible
Lincomycin	Neomycin	Chloramphenicol
Metronidazole	Kanamycin	Tetracycline
Bacitracin	Ampicillin	Baquiloprin/ Sulphadimidine
		Erythromycin
		Gentamicin
		Sulphonamides
		Penicillin

Table 5: Antibiotic activity against *C. sordellii*

Resistant	Intermediate	Susceptible
Aminoglycosides	Clindamycin	Benzyl Penicillin
Streptomycin	Lincomycin	Ampicillin
Kanamycin		Carbenicillin
Neomycin		Co amoxiclav
Tobramycin		Cefoxitin
Gentamicin		Erythromycin
Tetracycline		Metronidazole
Oxytetracycline		

The susceptibility of different drugs to *C. chauvoei* has been 100% against chloramphenicol, 93.7% for baquiloprim/ sulphadimidine and tetracycline, 93.5% for erythromycin, 87.5% for gentamicin, 87.7% for sulphonamides and 75% for penicillin. The less susceptible antibiotics included 55.2% for neomycin, 43.7% for kanamycin, and 62.5% for ampicillin. The species showed resistance against bacitracin, metronidazole, and lincomycin (Rais et al. 2016). Another study reported sensitivity against penicillin, oxytetracycline and chlortetracycline. Moreover, it is important to administer the drug both locally and systemically during the early stages of disease onset.

Clostridium. sordellii

C. sordellii cause severe infections which lead to death in a very short duration. The only way is to have antibiotic therapy at the earliest. A study has shown *C. sordellii* is sensitive to B-lactams including ampicillin, benzyl penicillin, carbenicillin, cefoxatin and coamoxiclav and resistant against cephalothin (Sasaki et al. 2001). Another study using 12 isolates for susceptibility testing has reported all isolates susceptible to Erythromycin and metronidazole except one which has been found resistant. Resistance has also been observed against aminoglycosides i-e, gentamycin, kanamycin, neomycin, streptomycin, and tobramycin (Nakamura et al. 1986). Nakamura and other colleagues in 1986 have shown a *C. sordellii* isolate with high MIC for vancomycin. Clindamycin and lincomycin also behaved differently in different studies. Perhaps one reason might be the difference in the methods used for susceptibility testing. Brazier et al., have used the disc diffusion method for sensitivity testing and found that 50% of the tested isolates were resistant to clindamycin. The other studies carried out on a panel of 12 and 24 isolates have used the agar dilution method and have found no resistance against clindamycin. Similarly, Nakamura has reported complete sensitivity to lincomycin against all 24 isolates of *C. sordellii* (Dornbusch et al. 1975; Nakamura et al. 1986). The other two studies however reported resistant strains. The resistance pattern has also been seen in case of tetracycline antibiotic. Similarly, 100% sensitivity to doxycycline has also been found in a study.

Isolates of *C. sordellii* obtained from malignant edema in cattle were also tested for susceptibility against oxytetracycline. A high resistance pattern was observed in all three confirmed *C. sordellii* isolates. Molecular analysis revealed tetracycline resistance genes namely tetA(P) and *tetB*(P) previously reported only in *C. perfringens* and consist of tetracycline resistance determinant TetP, arranged in a distinctive 17bp pattern and linked transcriptionally. The *tetA*(P) is 46kDa tetracycline efflux protein that causes active efflux of the tetracycline drug from the prokaryotic cell (Bannam and Rood 1999). The *tetB*(P) is 72.5kDa ribosomal protection protein, which dissociates tetracycline from the target and binds to bacterial ribosomes. *tetP* in *C. perfringens* is found on pCW3 (47kb) or other conjugative plasmids (Bannam et al. 2011). There is a lot more to know about the location of tetA(P) and *tetB*(P) genes found in *C. sordellii* isolates.

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