Microbiology Spectrum
American Society for Microbiology Press

Antimicrobial Resistance in *Clostridium* and *Brachyspira* spp. and Other Anaerobes

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ABSTRACT This article describes the antimicrobial resistance to date of the most frequently encountered anaerobic bacterial pathogens of animals. The different sections show that antimicrobial resistance can vary depending on the antimicrobial, the anaerobe, and the resistance mechanism. The variability in antimicrobial resistance patterns is also associated with other factors such as geographic region and local antimicrobial usage. On occasion, the same resistance gene was observed in many anaerobes, whereas some were limited to certain anaerobes. This article focuses on antimicrobial resistance data of veterinary origin.

INTRODUCTION

Anaerobic bacteria are unable to grow in the presence of oxygen. However, most clinical isolates grow very well under anaerobic conditions. Anaerobes can be divided into two groups: strict anaerobes, which are killed by exposure to oxygen, and aerotolerant anaerobes, which can tolerate some exposure to oxygen. They colonize many anatomical sites of animals, most notably the oral cavity, the rumen, and the lower intestinal tract, where they are part of the microbiota. They have also been associated with bacteria-rich mucosal surfaces of the respiratory tract, urinary and genital tracts, and even the skin (1). Only a small proportion of anaerobes can cause primary diseases, examples of which include *Clostridium* spp. such as *Clostridium perfringens* and *Clostridium difficile*, enterotoxigenic *Bacteroides fragilis*, *Dichelobacter nodosus*, and some *Brachyspira* spp. Other anaerobes, such as *Actinobaculum suis*, *Prevotella* spp., *Porphyromonas* spp., *Fusobacterium* spp., some *Clostridium* spp., *Peptococcus* spp., and *Streptopeptococcus* spp., are considered mostly opportunistic pathogens. Their disease onsets usually require predisposing factors such as inoculation into a normally sterile site through local trauma or any other conditions that permit bacterial entry and colonization. Anaerobes are often associated with clinical conditions involving necrotic and suppurative lesions such as abscesses and cellulitis. These opportunist infections are frequently multiple and commonly involve mixtures of aerobic and anaerobic bacteria, the former reducing the environment to allow the anaerobes to flourish. They are also considered a potential reservoir of antimicrobial resistance genes for other bacterial species (2).

The Clinical and Laboratory Standards Institute (CLSI) has established standardized methodologies for antimicrobial susceptibility testing for most anaerobes, which can be found in document M11 (3). This standard

Received: 24 February 2017, **Accepted:** 19 December 2019, **Published:** 23 January 2020

Editors: Frank Møller Aarestrup, Technical University of Denmark, Lyngby, Denmark; Stefan Schwarz, Freie Universität Berlin, Berlin, Germany; Jianzhong Shen, China Agricultural University, Beijing, China, and Lina Cavaco, Statens Serum Institute, Copenhagen, Denmark

Citation: Archambault M, Rubin JE. 2020. Antimicrobial resistance in *Clostridium* and *Brachyspira* spp. and other anaerobes. *Microbiol Spectrum* 8(1):ARBA-0020-2017. doi:10.1128/microbiolspec.ARBA-0020-2017.

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provides reference methods for the determination of MICs of anaerobic bacteria by agar dilution and broth microdilution. However, the document M11 standard was developed with human pathogens using human-specific methods and interpretative criteria. The documents VET01, 01S, and 02 provide the currently recommended techniques for antimicrobial agent disk and dilution susceptibility testing, criteria for quality control testing, and interpretive criteria for veterinary use, but refer to CLSI document M11 for guidance concerning anaerobes (4–6). As of 30 January 2017, document VET06 provides guidance for antimicrobial agent disk and dilution susceptibility testing, criteria for quality control testing, and breakpoints for fastidious and infrequently tested bacteria for veterinary use (7). Document VET06 includes a table on anaerobic bacteria and breakpoints for agar dilution and broth microdilution susceptibility testing. It includes data on *Brachyspira hyodysenteriae* which provides information and breakpoints for agar dilution and broth microdilution susceptibility testing. In most cases in which veterinary-specific breakpoints have not yet been established, human breakpoints have been used when appropriate (see CLSI document M11). The veterinary-specific breakpoints have been established following CLSI document VET02, with particular attention given to product label indications and directions as approved by regulatory authorities. Acceptable quality control ranges of MICs for anaerobic reference strains using agar dilution and broth microdilution are also provided in VET06. The methods described in this document are generic reference procedures that can be used routinely for susceptibility testing by clinical laboratories. They can also be used to evaluate commercial devices for possible routine use. Nevertheless, the data presented in this article have been mainly collected under the umbrella of human-specific methods and interpretative criteria of document M11 or other previously published breakpoints. Other organizations from different countries have been involved with antimicrobial susceptibility testing standardization for anaerobes, such as EUCAST (www.eucast.org) and the World Organization for Animal Health (www.oie.int).

Agar dilution is usually the reference method for most anaerobic bacteria, but other techniques may be used as long as equivalence to the reference methods is established (3). The disadvantages of the agar dilution approach are the laborious, time-consuming steps required to produce testing plates, mainly when the number of antimicrobials to be tested is high or when only a limited number of bacteria are to be tested. The Epsilometer test (Etest) is a rapid commercially available gradient diffusion system for quantitative antimicrobial susceptibility testing routinely used by laboratories for anaerobes such as *C. difficile* (8, 9). The use of disk diffusion tests is not recommended for anaerobic microorganisms because results are not reproducible, presumably due to the varied or insufficient growth rates of anaerobes. In addition, they do not correlate with those of the reference agar dilution method.

Anaerobic bacteria are usually naturally susceptible to most classes of antimicrobial agents, with the exception of aminoglycosides. The natural resistance to aminoglycosides can be explained by their requirement in oxygen for their transport into the bacterial cytoplasm (10). Anaerobes are also naturally resistant to polymyxins and the older fluoroquinolones. Trimethoprim-sulfonamides may not be effective *in vivo* against anaerobes due to the presence of thymidine in necrotic tissue (11). Penicillin, metronidazole, or clindamycin can be used for usual anaerobic coverage. Nitroimidazole antimicrobials such as metronidazole are usually effective because their intracellular reduction to active antimicrobial metabolites occurs under anaerobic conditions. However, due to their genotoxicities, they are not allowed for use in food-producing animals in many countries such as Canada, the United States, and the European Union. The use of chloramphenicol, an active antimicrobial against anaerobes, in treating food-producing animals is also prohibited. Among antimicrobial resistance particularities, the *B. fragilis* group of bacteria of animal origin are frequently resistant to penicillins and some cephalosporins because they produce beta-lactamases (12, 13), although the use of clavulanic acid in combination with beta-lactam antimicrobials may restore susceptibility (14). *Clostridium* spp. are considered naturally resistant to trimethoprim because they have trimethoprim-insensitive dihydrofolate reductases and also have a permeability barrier to trimethoprim (15, 16).

In general, bacterial antimicrobial resistance is acquired on mobile genetic elements (MGEs) such as plasmids, transposons, and/or conjugative transposons. However, for fluoroquinolones and rifampin, resistance-mediating mutations are the main and most efficient resistance mechanisms. These mutations are also acquired, but not located, on MGEs. Although under-investigated, this is also true for many antimicrobial-resistant anaerobic species described in the literature. A mini-review that summarizes what is known about tetracycline and macrolide-lincosamide-streptogramin B (MLS_B) resistance in genera with anaerobic species has

been published (17). It discusses the MGEs associated with acquired tetracycline and/or MLS_B resistance genes. Briefly, various tetracycline resistance efflux genes such as *tet*(B), *tet*(K), *tet*(L), and *tetA*(P) have been found in anaerobic species as well as tetracycline resistance genes coding for ribosomal protection proteins such as *tet*(M), *tet*(O), *tetB*(P), *tet*(Q), *tet*(W), and *tet*(32). Enzymes which inactivate tetracycline have been described, of which *tet*(X) has been identified in *Bacteroides*, though it is not functional under anaerobic growth conditions. This was also observed with the genes conferring MLS_B resistance. The rRNA methylase MLS_B resistance genes *erm*(B), *erm*(C), *erm*(F), *erm*(G), and *erm*(Q) have been identified in anaerobes. Since then, many more resistance genes and mechanisms have been unraveled.

This article describes the antimicrobial resistance known to date of the most frequently encountered anaerobic bacterial pathogens of animals. The following sections show that antimicrobial resistance can vary depending on the antimicrobial, the anaerobe, and the resistance mechanism. The variability in antimicrobial resistance patterns is also associated with factors such as geographic region and local antimicrobial usage. On occasion, the same resistance gene was observed in many anaerobes, whereas some were limited to certain anaerobes. This article focuses on antimicrobial resistance data of veterinary origin.

CLOSTRIDIUM

Clostridia are anaerobic Gram-positive rods with a low G + C content that form heat-resistant endospores. They are prokaryotic bacteria of the phylum *Firmicutes*. The genus *Clostridium* belongs to the *Clostridiaceae* family in the order *Clostridiales*. Most *Clostridium* spp. are intestinal commensals or inhabitants of soil or both. Only a few are pathogenic microorganisms (1). Clostridial diseases can be divided into three groups according to the types of infection they cause in animals: (i) enteric diseases are associated with enterotoxin-producing clostridia such as *C. perfringens*, *C. difficile*, and *C. spiroforme*. These species produce toxins that may act locally and/or systematically in the intestinal tract. They have also been involved with antibiotic-associated diarrhea. (ii) neurotoxic diseases are associated with species that produce potent neurotoxins, such as *C. botulinum* and *C. tetani*. These are rarely treated with antimicrobials. Thus, data on their antimicrobial susceptibility are not reviewed in this article. (iii) Histotoxic diseases involve species that produce histotoxins, such as *C. chauvoei*, *C. novyi*, *C. septicum*, *C. sordellii*, *C. haemolyticum*, *C. perfringens*, and *C. colinum* (1).

Enterotoxin-Producing Clostridia: *C. perfringens*

C. perfringens has been recently divided into seven types, A to G, based on the toxins they produce (18). Type A can cause gas gangrene (malignant edema) in several animal species and yellow lamb disease. Type B is responsible for lamb dysentery, while type C is associated with hemorrhagic and necrotizing enteritis mainly in neonatal animals. Type D is the agent of enterotoxemia mainly in small ruminants, and type E is responsible for bovine hemorrhagic gastroenteritis and enterotoxemia in rabbits. *C. perfringens* type F consists of strains responsible for *C. perfringens*-mediated human food poisoning and antibiotic associated diarrhea. *C. perfringens* type G comprises isolates that produce NetB toxin and thereby cause necrotic enteritis in chickens (18).

Susceptibility data in the literature mainly concern *C. perfringens* isolates from broilers because necrotic enteritis is a common and economically significant poultry disease that can be controlled by antimicrobials worldwide. Various antimicrobials such as bacitracin, avilamycin, virginiamycin, and lincomycin are currently used as in-feed medication for prophylactic or treatment purposes against necrotic enteritis in broilers, whereas diseases associated with *C. perfringens* in other animal species are only rarely treated with antimicrobials, with the exception of canine enteritis.

Occurrence of antimicrobial resistance in *C. perfringens*

The occurrence of tetracycline, bacitracin, and virginiamycin resistances has been described worldwide in *C. perfringens* from chicken broilers (19–23). Earlier studies of *C. perfringens* poultry isolates reported resistance to oxytetracycline (MIC, >1 mg/liter) as one of the most frequent resistances in samples from Sweden (76%), Denmark (10%), Norway (29%) (19), and Belgium (66%) (20). Resistance to bacitracin has also been reported in *C. perfringens* poultry isolates from the United States (88%) (21) and Denmark (18%) (19). Virginiamycin resistance has also been described in broiler isolates from Norway (18%) (19) and from the United States (31%) (21). It is believed that the use of these antimicrobials in broilers in many countries reflects the pattern of antimicrobial resistance observed. Earlier studies of poultry and pigs reported on susceptibilities to ampicillin, amoxicillin, penicillin, avilamycin, vancomycin, avoparcin, and ionophores such as

narasin, salinomycin, lasalocid, and monensin (19–21, 24–26).

More recent studies of *C. perfringens* poultry isolates reported resistance to bacitracin and tetracycline as frequently encountered resistances in samples from Canada (22, 27) and Korea (28). Sulfonamides, macrolides, and lincosamide resistances were recently described in a few reports (28–31). In Belgium, resistances to tetracycline (66%) and lincomycin (61%) were the most frequent resistances observed, while bacitracin resistance was not noted (29). This was also reported in a Taiwanese study (31). Elevated MIC_{50} for virginiamycin has been described but rarely documented in chicken isolates (22). High levels of resistances to many antimicrobials have been observed in only one Egyptian report (30), where beta-lactam resistance was found to be a rare event. Globally, recent studies of poultry are still reporting on susceptibilities to beta-lactams, fluoroquinolones, and phenicols (28, 29).

There are only a few reports on antimicrobial susceptibility of *C. perfringens* from other species (25, 32–39). Earlier studies of *C. perfringens* of porcine origin reported resistance to tetracycline, erythromycin, clindamycin, and lincomycin, indicating multidrug resistance, whereas isolates were generally quite susceptible to penicillin and chloramphenicol (25, 33). More recently, reduced susceptibility to clindamycin (28%), erythromycin (31%), and tetracycline was observed in *C. perfringens* isolates of swine origin from Canada (22). In a German study, resistance to linezolid, with simultaneous resistance to florfenicol and erythromycin was reported (34). A Brazilian study of *C. perfringens* isolated from piglets (35) reported susceptibility to amoxicillin and ceftiofur, whereas resistance to tetracycline and lincomycin was quite common. A study in Thailand reported that most of the *C. perfringens* isolates from piglets were susceptible to ampicillin, bacitracin, chlortetracycline, doxycycline, and oxytetracycline, with MIC_{50} values ranging from 0.32 to 8 mg/liter (32). However, high resistance rates were observed for ceftiofur, enrofloxacin, erythromycin, lincomycin, and tylosin, and among resistant isolates, 82% were resistant to more than one type of antimicrobial. *C. perfringens* of bovine origin with reduced susceptibility to clindamycin, florfenicol, and tetracycline was reported in a Canadian study (22). *C. perfringens* isolates from cooked beef sold in the streets of Cote d'Ivoire, Africa, were shown to exhibit resistance rates to tetracycline, doxycycline, chloramphenicol, and erythromycin ranging from 20 to 50% (36). A survey of 50 Swedish *C. perfringens* isolates from individual dogs with acute diarrhea (37) reported that 18% of the isolates showed resistance to tetracycline and 54% showed decreased susceptibility to metronidazole, with an MIC of 4 mg/liter. In this study, all isolates were shown to be susceptible to all beta-lactams tested as well as to chloramphenicol and clindamycin (37). In a study in Costa Rica, multiresistance to clindamycin, chloramphenicol, penicillin, and metronidazole was observed in 5% of *C. perfringens* strains of animal origin (38).

The presence of *C. perfringens* in water is generally regarded as an indicator of fecal contamination, and exposure to waterborne spores is considered a possible source of infection for animals. In a Spanish study, the antimicrobial susceptibility of *C. perfringens* in water sources in a zoological park located in Madrid was investigated (40). Most isolates displayed intermediate susceptibility (57%; MIC, 16 mg/liter) or resistance (5.7%; MIC, ≥32 mg/liter) to metronidazole. In this study, no resistance to other antimicrobials was detected, although some isolates showed elevated MICs to erythromycin and/or linezolid.

Recently, antimicrobial tolerance was shown to be mediated by biofilms in *C. perfringens* (41–44). Biofilms are structured communities of bacterial cells enclosed in a self-produced extracellular polysaccharide matrix which provides increased resistance to environmental stresses (45). Studies have demonstrated that the biofilm formed by *C. perfringens* could protect the cells from an exposure to atmospheric oxygen and to high concentrations of penicillin (41, 42). More recently, antimicrobial tolerance mediated by biofilms in *C. perfringens* was observed for bacitracin, penicillin, lincomycin, virginiamycin, tylosin, and the anticoccidial agents salinomycin, narasin, and monensin (44).

Another interesting area of research concerning *C. perfringens* is the impact of antimicrobial resistance on fitness and virulence (46–49). Recently, using comparative transcriptomic analysis, a study demonstrated that *C. perfringens* exposure to fluoroquinolones affected virulence (toxin production) in addition to drug resistance (46). In another study, it was observed that both the genetic background of the strain and the fluoroquinolone which induced resistance affected the fitness of *C. perfringens*-resistant mutants (47). Also, a ciprofloxacin-resistant mutant of *C. perfringens* with stable mutations in the topoisomerase genes was shown to accumulate less norfloxacin and ethidium bromide than the wild type via an ABC transporter protein (NP_562422) which was also associated with reduced susceptibility to norfloxacin and ciprofloxacin (49). It will be interesting to follow these new areas of research in regard to antimicrobial resistance.

Genetic basis of antimicrobial resistance in *C. perfringens*

The most common genetic antimicrobial resistance determinants described to date are associated with bacitracin, tetracyclines, MLS, and chloramphenicol antimicrobials (Table 1). Bacitracin resistance has been associated with genes encoding for an ABC transporter and an overproduced undecaprenol kinase in *C. perfringens* of poultry origin (23). These two mechanisms were both shown to be encoded by a *bcr*ABD operon under the control of a regulatory gene, *bcr*R (23). These genes were shown to be located on the chromosome and expressed under bacitracin stress (23). More recently, it was demonstrated that the *bcr*RABD locus was also localized to an 89.7-kb plasmid, pJIR4150, on a novel genetic element, ICECp1, which is related to the Tn*916* family of integrative conjugative elements (50). It was shown to be conjugative and associated with the pCW3 family of conjugative antimicrobial resistance and toxin plasmids from *C. perfringens* (50).

Previous studies that have reported on reduced susceptibility to tetracycline in poultry *C. perfringens* have identified the *tetA*(P), *tetB*(P), and *tet*(M) genes as the most common genetic determinants (19, 51–53). The *tet*(P) gene was first identified in the conjugative *C. perfringens* R-plasmid pCW3, which demonstrated two functional overlapping tetracycline resistance genes, *tetA*(P) and *tetB*(P) (54). The *tetA*(P) gene encodes for a transmembrane protein which mediates active efflux of tetracycline from the cell, while *tetB*(P) encodes a protein which has significant similarity to Tet M-like tetracycline resistance proteins associated with ribosomal protection (54). While *tetA*(P) seems to be associated with all tetracycline-resistant strains, it was demonstrated that most of the isolates carried a second tetracycline resistance gene, *tetB*(P) or *tet*(M) (53). The *tetB*(P) gene was shown not to disturb the MIC of tetracycline in *C. perfringens* isolates already carrying *tetA*(P) (19) and was only associated with low-level tetracycline resistance (54). Other studies have also reported tetracycline resistance genes such as *tet*(Q), *tet*(K), *tet*(L), *tet*(O), and *tet*(W) (20, 29). The conjugative tetracycline resistance plasmids are relatively common in *C. perfringens* and are closely related to the originally isolated pCW3 (55–57). More recently, a conjugative 49-kb tetracycline resistance plasmid which is very similar to pCW3 was recently described in a *netB*-positive necrotic enteritis-derived *C. perfringens* strain (58). In a study from the United-States, susceptibility to tetracycline and minocycline in *C. perfringens* was most common in strains isolated from chickens, followed by those from soils, clinical samples, and foods (59). The most common resistance genes in this study were *tetA*(P) and *tetB*(P), with only one tetracycline-resistant food isolate with an intact *tet*(M) gene (59). Fragments with high degrees of identity to parts of the *tet*(M) sequences were also found in other strains, mainly of clinical origin, and often in isolates with *tetB*(P) (59). Interestingly, in this study, no correlation was observed between the level of susceptibility to tetracycline or minocycline and the presence of *tetA*(P), *tetB*(P), or part of *tet*(M) (59). More recently, all bacitracin-resistant *C. perfringens* poultry isolates in a Canadian study were found to carry both *tetA*(P) and *tetB*(P) (23). Plasmid curing experiments revealed the loss of the *tet* genes, indicating, as expected, plasmid localization of these genes (23).

Macrolide resistance is usually mediated by *erm*, erythromycin resistance methylase, genes (17). The proteins encoded by the *erm* genes confer N^6 dimethylation of a specific adenine residue (A2058) of the 23S rRNA molecule (17). This alteration of the macrolide target site is catalyzed by an rRNA methyltransferase. This resistance mechanism confers cross-resistance to macrolides, lincosamides, and streptogramin B (MLS_B phenotype) (17). The *erm*(B), *erm*(F), and *erm*(Q) genes have been described in *C. perfringens* (17). This resistance has been shown to be plasmid-mediated (17, 60). In a recent Canadian study, only one bacitracin- and tetracycline-resistant *C. perfringens* poultry isolate was shown to harbor an MLS_B resistance gene, *erm*(B) (23). A mutational analysis of the Erm(B) protein from *C. perfringens* indicated that nine mutants with single point mutations in the *erm*(B) gene produced stable but nonfunctional Erm(B) proteins (61). All of the mutants had amino acid changes within conserved methyltransferase motifs that were important for either substrate binding or catalysis, indicating that the point mutations all involved residues important for the structure and/or function of this rRNA methyltransferase (61). Lincomycin resistance in *C. perfringens* is also common and is usually encoded by *erm* genes that confer MLS_B resistance (62). In a 2006 study of *C. perfringens* in dogs, a relatively high prevalence of tetracycline resistance was reported, where 96% (119/124) of the isolates were positive for the *tetA*(P) gene, and 41% (51/124) were positive for both the *tetA*(P) and *tetB*(P) genes (63). In this study, only one isolate was positive for the *erm*(B) gene, and another was positive for the *erm*(Q) gene (63). Of the 15 tested isolates, 2 (13%) demonstrated transfer of tetracycline resistance via bacterial conjugation (63).

In contrast to the MLS_B phenotype, specific resistance to lincosamides is due to enzymatic inactivation

TABLE 1 Overview of the genes or the mutations in genes associated with acquired antimicrobial resistance so far identified in the different anaerobes of animal origin

Resistance mechanism	Resistance gene(s)	Gene product(s)	Resistance phenotype	Anaerobes involved	References
Chemical modification	*Cat*(P,Q)	Acetyltransferases	Chloramphenicol	*C. perfringens*	16, 67
	lnu(A, B, P)	Nucleotidyl transferases	Lincosamides	*C. perfringens*	28, 62
	tet(X)	Oxidoreductase	Tetracyclines	*Bacteroides*	17
Efflux: decreased intracellular drug accumulation	*tet*(B), *tet*(K), *tet*(L), or *tetA*(P)	Efflux system of the major facilitator superfamily	Tetracyclines	*C. perfringens*, *C. difficile*, *C. septicum*, *C. sordellii*	20, 29, 52, 54, 108
	*bcr*RABD	ABC transporter and an overproduced undecaprenol kinase	Bacitracin	*C. perfringens*	23, 50
	mef(A)	Efflux system of the major facilitator family	14-,15-Membered macrolides	*C. perfringens*	72
Hydrolytic degradation	bla_{OXA-63} group genes	Beta-lactamases	Beta-lactam antibiotics	*B. pilosicoli*	163, 164
	cep(A)	Beta-lactamases	Beta-lactam antibiotics	*Bacteroides*	12
Methylation of the target site	*erm*(B, C, F, G, or Q)	rRNA methylase	MLS_B	*Bacteroides*, *C. perfringens*, *C. difficile*	17, 97
Mutational modification		Mutation in the gene *rplD*, encoding protein L4 of the 50S ribosomal subunit	Linezolid, florfenicol and erythromycin	*C. perfringens*	34
		Mutation in the 23S rRNA gene	One or more of these drugs: macrolides, lincosamides, streptogramins, pleuromutilins, tetracyclines	*Brachyspira*	158–161
		Mutation in the genes coding for ribosomal proteins, L2, L3, L4, and L22	Tiamulin	*Brachyspira*	159, 161
		Mutation in the 16S rRNA gene	Doxycycline	*Brachyspira*	162
Protection of the target site	*tet*(M, O, Q, W, 32, or *B*(P))	Ribosomal protection proteins	Tetracyclines	*Bacteroides, C. perfringens*, *C. difficile*, *C. septicum*, *C. sordellii*	12, 20, 29, 52, 54, 97, 106

of those antibiotics, usually via phosphorylation and nucleotidylation of the hydroxyl group at position 3 of lincosamides (64). Lincosamide nucleotidyltransferases encoded by the *lnu*(A) and *lnu*(B) genes (formerly *lin*) have been observed in *C. perfringens* from Belgium broilers (29). The *O*-nucleotidyltransferases encoded by these genes inactivate lincosamides by adenylation (65, 66). An Australian study, using a *C. perfringens* lincomycin-resistant but erythromycin-susceptible strain, demonstrated that the lincomycin resistance *lnu*(P) gene was plasmid borne (plasmid pJIR2774) and could be transferred to other *C. perfringens* isolates by conjugation (62). This plasmid did not harbor tetracycline resistance. The *lnu*(P) gene was shown to encode for a putative lincosamide nucleotidyltransferase and was located on tISCpe8, a functional transposable genetic element and member of the IS*1595* family of transposon-like insertion sequences (62). This element was reported to have significant similarities to the mobilizable lincomycin resistance element tISSag10 from *Streptococcus agalactiae* (62). Like tISSag10, tISCpe8 carries a functional origin of transfer-like region within the resistance gene, allowing the element to be mobilized by the conjugative transposon Tn*916* (62). Recently, a new mutation was detected in a *C. perfringens* strain isolated from pig manure, which was shown to be resistant to linezolid, florfenicol, and erythromycin (34). This mutation was described in a highly conserved region of *rplD*, encoding protein L4 of the 50S ribosomal subunit (34).

Chloramphenicol resistance has been shown to be mediated by chloramphenicol acetyltransferase (CAT) enzymes encoded by *cat*(P) and *cat*(Q) (67). The *cat*(Q) gene was shown to be distinct from the *C. perfringens* *cat*(P) gene. The deduced CATQ monomer had considerable amino acid sequence conservation compared with CATP (53% similarity) and other known CAT proteins (39 to 53%). The amino acid sequence of CATP was significantly similar to CAT monomers from *Vibrio anguillarum* and *Campylobacter coli*, whereas phylogenetic analysis revealed that the CATQ monomer was as closely related to CAT proteins from *Staphylococcus aureus* and *C. coli* as it was to CAT monomers from the clostridia (67). Chloramphenicol resistance has been located on mobilizable transposons in *C. perfringens* (68). Mobilizable transposons are transposable genetic elements that also encode mobilization functions but are not in themselves conjugative (69). To conjugate, they rely on coresident conjugative elements to facilitate their transfer to recipient cells. *C. perfringens* mobilizable transposons include Tn*4451* and Tn*4452*, which are closely related (68, 69). The Tn*4451* group of elements encodes resistance to chloramphenicol with an unusual transposition which is dependent upon a large resolvase protein rather than a more conventional transposase or integrase (69). This group also encodes the mobilization protein TnpZ, which acts at the RS(A), an upstream palindromic sequence, or origin of transfer site located on the transposon (69). In the presence of a coresident conjugative element, this promotes the movement of the nonreplicating circular intermediate and of plasmids on which the transposon is located (69).

The *mef*(A) gene encodes an efflux pump associated with resistance to macrolides in the absence of resistance to lincosamides and streptogramin B and was first described in *Streptococcus pneumoniae* (70, 71). This gene has been observed in *C. perfringens* recovered from water, soil, and sewage from 14 U.S. states (72). In this study, the antimicrobial resistance genes *tet*A(P), *tet*B(P), *tet*(M), *erm*(B), and *erm*(Q) were also observed, indicating that environmental *C. perfringens* organisms are capable of acting as reservoirs for these antimicrobial resistance genes.

Enterotoxin-Producing Clostridia: *C. difficile*

C. difficile is the agent of necrotizing enterocolitis, often antibiotic-associated, in several mammalian species (9, 73–77). The disease is especially observed in animals with large or expanded bowels such as horses, swine, rabbits, and guinea-pigs (1). It has also been described in calves, foals, piglets, and dogs (1). In human medicine, *C. difficile* is a major nosocomial pathogen that also causes antibiotic-associated diarrhea, often referred to as a pseudomembranous colitis (9, 73, 77). Healthy carriers have been described in humans and animals (78). Oral treatment with broad-spectrum antibiotics is reported as a risk factor for the disease to occur (9, 73). Erythromycin has been reported as an antimicrobial associated with *C. difficile* colitis in horses (79, 80). Other antimicrobials often associated with this disease in horses are trimethoprim-sulfonamides, beta-lactams, clindamycin, rifampicin, and gentamicin (74, 76). Other factors such as hospitalization and changes in diet may also contribute to the development of *C. difficile* infection (81).

The virulence of *C. difficile* is essentially mediated by two toxins of the large clostridial cytotoxin family named toxin A (TcdA), an enterotoxin, and toxin B (TcdB), a cytotoxin (1). The genes *tcd*A and *tcd*B are located on a large pathogenicity locus in the chromosome (1). An important increase in incidence of human *C. difficile* infection has been observed across the United States, Canada, and Europe over the past decade due to the emergence of highly virulent (or hypervirulent)

strains of *C. difficile* (9). The most prominent hypervirulent type is categorized as PCR ribotype 027 (RT027), North American pulsed field gel electrophoresis type I, and restriction endonuclease analysis group B1 (9). Strain RT027 is characterized by severe infection, a high rate of recurrence, mortality, and resistance to traditional therapy (9). In addition to RT027, a number of emergent highly virulent ribotypes, correlated with RT027 or not, have recently been identified (82). The hypervirulent RT078 has been recognized as a cause of infections in humans in hospitals (83) and in the community (84) and in animals (85–89). The epidemic of RT027 infections marked an antimicrobial resistance turning point for *C. difficile* with the arrival of fluoroquinolone resistance, most likely due to overuse of this antimicrobial in human medicine (9). Since then, many reports have been published on the antimicrobial susceptibilities and resistance genes of *C. difficile* of human origin. Far less documentation is readily available in veterinary medicine. A recent review on the phenotypic and genotypic traits of antimicrobial resistance in *C. difficile* taking into consideration the most recent data has been published (9).

Occurrence of antimicrobial resistance in *C. difficile*

Acquired resistance in *C. difficile* isolates of animal origin to a diverse range of antimicrobials including chloramphenicol (80), rifampin (90, 91), metronidazole (91), tetracyclines (75, 80, 92), erythromycin (75, 80, 92), and vancomycin have been described (91). Earlier studies of *C. difficile* isolates reported on susceptibility to penicillin (80, 90, 92), but acquired resistance was described to be between 10 and 25% in a prospective study on equine diarrhea (92). In a Western Australian investigation over a 24-month period (2007 to 2009), *C. difficile* was isolated from 14 (23%) of 62 diarrheal horses (including 10 foals), and all isolates were reported as susceptible to metronidazole and vancomycin (93). A survey of 777 horses of different breeds, age, and sex and their environment revealed that all 52 strains of *C. difficile* recovered were susceptible to metronidazole (MIC, ≤4 mg/liter) and vancomycin (MIC, ≤2 mg/liter) (90). A cross-sectional observational study of *C. difficile* recovered from diarrheic and nondiarrheic foals (n = 153) resulted in 7 (4.6%) positive samples for *C. difficile* A/B toxin, all from diarrheic foals. All of the *C. difficile* isolates were susceptible to metronidazole and vancomycin (94). Resistance to metronidazole was reported in some strains of *C. difficile* from a prospective study of horses admitted to an intensive care unit for acute gastrointestinal tract disease with loose feces (n = 130) (95). Horses infected with these strains were 10 times more likely to have been treated with metronidazole prior to the onset of diarrhea than horses infected with other strains. The duration from onset of diarrhea to discharge was longer, systemic inflammatory response syndromes were more pronounced, and the mortality rate was higher in horses infected with these strains, indicating that metronidazole-resistant strains may be associated with severe disease (95). Also, in a retrospective study of 28 foals with *C. difficile*-associated diarrhea, 10 of 23 (43%) *C. difficile* isolates were resistant to metronidazole (96). In this study, molecular fingerprinting revealed marked heterogeneity among isolates, except for the metronidazole-resistant isolates (96). A Canadian study of *C. difficile* from horses admitted to a veterinary teaching hospital over a 7-month period recovered 10 isolates with high-level resistance to clindamycin and ceftiofur (81). Among these isolates, seven PCR ribotypes were identified, including RT014 (81). In a *C. difficile* study of horses with colitis in Sweden, all 36 clinical and 14 environmental isolates were shown to be susceptible to vancomycin and avilamycin, but 50% had bimodal MIC distributions of erythromycin, virginiamycin, spiramycin, and oxytetracycline (80). All isolates in this study were resistant to rifampin (80).

An earlier study of *C. difficile* isolated from diarrheic neonatal piglets from the United States reported a high occurrence of resistance to bacitracin and ceftiofur ($MICs_{90}$, ≥256 mg/liter) (75). In this study, the MIC_{90} (64 or ≥256 mg/liter) for erythromycin, tilmicosin, and tylosin suggested resistance of a proportion of *C. difficile* isolates, while susceptibility to tetracycline varied widely among isolates with MIC_{50} and MIC_{90} of 8 and 32 mg/liter, respectively. In this study, The $MICs_{90}$ for tiamulin (8 mg/liter) and virginiamycin (16 mg/liter) suggested moderate susceptibility in those isolates. More recently, an investigation of antimicrobial resistance determinants of *C. difficile* isolated from swine raised in Ohio and North Carolina revealed that 19% (119/609) of *C. difficile* isolates were resistant to tetracycline, and 7% (44/609) were resistant to both erythromycin and tetracycline (97). In this study, the majority of *C. difficile* isolates (80.5%) were shown to have a MIC of >32 mg/liter for ciprofloxacin (98). An investigation of *C. difficile* among different age and production groups of swine in a vertically integrated swine operation in Texas revealed that all isolates (n = 131) were resistant to cefoxitin, ciprofloxacin, and imipenem, whereas all were susceptible to metronidazole, piperacillin/tazobactam, amoxicillin/clavulanic acid, and vancomycin (99). The majority of

isolates were resistant to clindamycin, resistant or intermediate to ampicillin, and susceptible to tetracycline and chloramphenicol (99). In a study comparing the antimicrobial resistance patterns of *C. difficile* isolated from a closed, integrated population of humans and swine, antimicrobial susceptibility testing was performed on 523 *C. difficile* strains (100). Swine isolates originated from a vertically flowing swine population consisting of farrowing, nursery, breeding, and grower/finisher production groups, while human wastewater isolates were collected from swine worker and nonworker occupational group cohorts. All of the swine and human strains were susceptible to amoxicillin/clavulanic acid, piperacillin/tazobactam, and vancomycin (100). In addition, all of the human strains were susceptible to chloramphenicol (100). The majority of the human and swine strains were resistant to cefoxitin and ciprofloxacin (100). Statistically significant differences in antimicrobial susceptibility were found among the swine production groups for ciprofloxacin, tetracycline, amoxicillin/clavulanic acid, and clindamycin (100). No significant differences in antimicrobial susceptibility were found across the human occupational group cohorts. This study reported metronidazole resistance in 8.3% of the swine strains and in 13.3% of the human strains (100). The authors concluded that the finding of differences in susceptibility patterns between human and swine strains of *C. difficile* provides evidence that transmission between host species in this integrated population is unlikely (100).

In a survey of antimicrobial susceptibility of 144 Spanish *C. difficile* swine isolates, a high prevalence of the toxigenic RT078 (94.4%) was observed along with multidrug resistance (49.3%) among isolates tested (101). In this study, resistance to clindamycin, ertapenem, erythromycin, and moxifloxacin was common (≥27.8% in all cases). Also, all isolates were resistant to ciprofloxacin but susceptible to daptomycin, linezolid, meropenem, rifampicin, teicoplanin, tigecycline, metronidazole, and vancomycin. It was found that erythromycin and moxifloxacin resistance was associated with the geographic origin of the isolates and that metronidazole heteroresistance was observed. A study of commercial pigs at the preharvest food-safety level (68 sows and 251 young pigs from 5 farms) in North Carolina and Ohio (3 farms) revealed ciprofloxacin resistance as predominant in young pigs (91.3% of isolates) and sows (94%) (102). In this study, the ciprofloxacin-erythromycin-tetracycline resistance profile was detected in 21.4% and 11.7% of isolates from young pigs and sows, respectively. Also, erythromycin and tetracycline resistances were both significantly associated with toxin gene profiles (102). In a Japanese study, *C. difficile* from neonatal piglets less than 20 days of age recovered during June to August 2012 were shown to be susceptible to vancomycin and metronidazole (103). In this study, resistance against clindamycin, ceftriaxone, erythromycin, and ciprofloxacin were found in 59, 6, 46, and 75% of the isolates, respectively. Also, of the 61 toxigenic *C. difficile* isolates (toxin A^+B^+), the incidence of resistance to clindamycin, ceftriaxone, erythromycin, and ciprofloxacin was 71%, 10%, 43%, and 74%, respectively. It was also observed that the percentage of resistant isolates derived from piglets against all antimicrobials, particularly ceftriaxone, was lower than that clinically isolated from humans (8, 103). In a North Carolina study of free-ranging feral swine in areas with extensive commercial swine production, antimicrobial resistance was detected in *C. difficile* isolates for six of the eight antimicrobials tested (104). Briefly, isolates exhibited a high frequency of resistance to tetracycline (57%) and levofloxacin (30%), whereas none of the isolates exhibited resistance to metronidazole and vancomycin.

Characterization of *C. difficile* isolates from Italian swine and dogs revealed 10 PCR ribotypes in porcine strains and 6 in canine strains (105). The predominant type found in porcine strains was RT078 (50%), whereas canine strains carried the nontoxinogenic RT010 (64%). Among swine, resistance to erythromycine (60%), moxifloxacin (35%), clindamycin (15%), and rifampin (5%) was observed, whereas all isolates were susceptible to metronidazole or vancomycin. Among dogs, 51% of strains were resistant to clindamycin, 46% to erythromycin, 21% to metronidazole, and 5% to moxifloxacin or rifampin, but all isolates were susceptible to vancomycin. In this study, five porcine strains (10%) and nine canine isolates (41%) were multidrug resistant, and some multidrug-resistant canine strains (n = 8) were highly resistant to metronidazole, with MICs of ≥32 mg/liter. Also in this study, using the EUCAST cutoff for metronidazole (MIC, >2 mg/liter), 13 canine isolates and 1 porcine strain were found to have reduced susceptibility to metronidazole (MICs ranging from 3 to ≥256 mg/liter). Those strains belonged to RT010 and RT078, which have also been associated with reduced susceptibility to metronidazole in humans (105). A Cote d'Ivoire study in Abidjan looked at the antimicrobial susceptibilities of *C. difficile* in cooked beef sold in the streets, with a total of 395 kidney and meat samples from vendors (36). A prevalence of 12.4% for *C. difficile* (11.04% in kidney and 13.45% in meat) was determined, with resistance rates to tetracycline, doxycycline, chloramphenicol, and erythromycin against *C. difficile* isolates ranging from

2.05% to 8.16% (36). In this study, metronidazole and vancomycin were the most potent antimicrobial agents against *C. difficile*.

Genetic basis of antimicrobial resistance in *C. difficile*

Studies looking into the genetic basis of *C. difficile* antimicrobial resistance are available in the human medicine literature, but there is a paucity of comparable data from the veterinary discipline (Table 1). Multiple mechanisms for the acquisition of antimicrobial resistance have been described in *C. difficile*, mostly of human origin, such as mobilizable and conjugative transposons, other MGEs, and various mutations (9). To our knowledge, plasmids encoding antimicrobial resistance seem to not have been described in *C. difficile*.

Tetracycline resistance is commonly due to protection of the ribosomes, and the most widespread *tet* class in *C. difficile* is *tet*(M), usually found on conjugative Tn*916*-like elements such as Tn*5397* (9, 106, 107). Other *tet* genes have been identified such as *tet*(P), *tet*(K), and *tet*(L) (108). The presence of both *tet*(M) and *tet*(W) has been described in *C. difficile* isolates from humans and animals (97, 109). Indeed, a study of *C. difficile* isolated from swine raised in Ohio and North Carolina revealed that tetracycline resistance was mainly associated with *tet*(M) (97%), followed by *tet*(W) (32%) genes, with a subset of 31% (37/119) of these isolates carrying both *tet*(M) and *tet*(W) genes (97). In this study, isolates that carried both genes had a wide range of MICs within the category "resistance," indicating no benefit in carrying both genes. Also in this study, the majority (97%; 66/68) of isolates with an erythromycin MIC of >256 mg/liter were found to carry the *erm*(B) gene (91%, 68/75), and the majority of isolates that were resistant to both erythromycin and tetracycline tested positive for both the *erm*(B) and *tet*(M) genes. Both genes have been previously associated with a Tn*916*-like element in *C. difficile* of human origin (107), and this could also be the case in animal isolates. Ribosomal methylation is reported as the most widespread mechanism of resistance of the MLS_B family in *C. difficile* and is mediated by the *erm*(B) gene (109). Characteristics of *C. difficile* strains with reduced susceptibility to metronidazole from 16 studies published between 2012 and 2015 have recently been reviewed (9). Generally, the percentage of *C. difficile* strains resistant to metronidazole is low. However, a number of *C. difficile* strains with MICs of >2 mg/liter, the EUCAST epidemiological cut-off, have been reported in both humans and animals (9). Genes conferring resistance to metronidazole have not yet been described in *C. difficile* (9).

The following reports on antimicrobial resistance genes are of human origin, and these genes have not yet been identified, to our knowledge, from animal sources (110–126). Briefly, the Tn*6164* containing the *tet*(44) and *ant*(6)-Ib genes, predicted to confer resistance to tetracycline and streptomycin, respectively, was described in a *C. difficile* human isolate (110). Resistance to fluoroquinolones in *C. difficile* has been reported to be due to alterations in the quinolone-resistance-determining region of either GyrA or GyrB; the DNA gyrase subunits and several amino acid substitutions have been identified in both GyrA and/or GyrB (9). The majority of *C. difficile* fluoroquinolone-resistant strains have shown the substitution Thr82Ile in GyrA (115–117). For rifampin and rifaximin resistance, mutations in the beta-subunit of the RNA polymerase, *rpoB*, have been described (9). Among the amino acid substitutions identified, Arg505Lys is the most common, particularly in *C. difficile* strain RT027 (118). Fusidic acid resistance has been associated with mutations in the *fusA* gene, encoding for a protein elongation factor (119). Chloramphenicol resistance in *C. difficile* has been identified as mediated by the *cat*D gene, which encodes a CAT enzyme (120). This gene was found on mobilizable transposons Tn*4453a* and Tn*4453b* on the chromosome, and these are structurally and functionally related to the *C. perfringens* mobilizable element Tn*4451* (121). In humans, resistance to linezolid in *C. difficile* has been associated with a *cfr*-like gene, *cfr*(B), that encodes a Cfr RNA methyltransferase causing multiple resistances to peptidyl transferase inhibitors by methylation of A2503 in the 23S rRNA (122, 123). In addition to phenicol and linezolid, the *cfr*(B) gene has been shown to encode resistance to lincosamides, pleuromutilins, and streptogramin A (122). The vancomycin, metronidazole, and cephalosporins resistance mechanisms in *C. difficile* are still unclear (9, 124–126).

Histotoxic Clostridia: *C. chauvoei*, *C. novyi*, *C. septicum*, and *C. sordellii*

C. chauvoei is the causative agent of blackleg, a highly fatal disease characterized by a myonecrosis in cattle and more rarely in sheep. The infection has also been described in other animal species (1). It was suggested that *C. chauvoei* susceptibilities to antimicrobials reflects the fact that blackleg is not treated with antimicrobials due to its virulent appearance leading to rapid death of the animal (127). The MICs for *C. chauvoei* strain JF4335 using the microdilution method according to the CLSI

guidelines (3) have been described as low, suggesting susceptibility to these antimicrobials.

Gas gangrene, otherwise known as malignant edema, is a necrotizing soft tissue infection. It is usually an acute, often fatal, toxemia affecting all species and ages of animals, with a higher incidence in ruminants and horses (1). This disease can be caused by *C. septicum*, *C. chauvoei*, *C. novyi* type A, *C. perfringens* type A, and *C. sordellii* (1). A study in 2001 reported low penicillin and ampicillin MICs in Japanese isolates of *C. septicum* and *C. sordellii* from cattle with gas gangrene (52). In this study, all *C. sordellii* isolates were reported as resistant to oxytetracycline, whereas 22% of *C. septicum* isolates were identified as resistant to this antimicrobial. Also, low MICs were reported for enrofloxacin, erythromycin, vancomycin, and chloramphenicol in these Japanese cattle isolates. In humans, resistance to sulfamethoxazole and trimethoprim but susceptibility to imipenem and cefoxitin have been reported in *C. sordellii* (128–130).

In sheep and cattle, *C. septicum* also causes a disease known as braxy, a necrotizing abomasitis, where affected animals are generally found dead (1). This highly fatal infection is characterized by toxemia and inflammation of the abomasal wall. A study in the 1980s in California that looked at *C. septicum* isolates from humans and animals demonstrated relatively good susceptibilities to many antimicrobials (131, 132), such as beta-lactams, macrolides, lincosamides, phenicols, nitroimidazoles, rifamycins, bacitracin, and tetracyclines.

C. novyi type B causes acute hepatic necrosis, commonly known as black disease, in animals grazing in fluke-infested pastures (133). No information is available on antimicrobial resistance in this bacterium from animals, but data is available from humans (134).

Genetic basis of antimicrobial resistance in *C. chauvoei*, *C. septicum*, and *C. sordellii*

An earlier study on the antimicrobial resistance determinants of *C. septicum* and *C. sordellii* from cattle affected with gas gangrene in Japan identified the *tetA*(P), *tetB*(P), and *tet*(M) genes in tetracycline-resistant isolates (52) (Table 1). This study also reported on the sequences of the tetracycline resistance genes of some *C. septicum* strains which were completely or almost completely identical to those of strains belonging to other clostridial species. More recently, the draft genome of *C. chauvoei* JF4335 was revealed to contain 2,630 predicted open reading frames, of which 1,935 protein sequences could be assigned, along with 632 open reading frames representing hypothetical proteins that could not be assigned (127). Of the assigned proteins, 44 genes were shown to be involved in antibiotic and metal resistance. It was shown that *C. chauvoei* strain JF4335 harbors a genetic potential for penicillin resistance, a beta-lactamase (EC 3.5.2.6), as well as an elongator factor EF G type tetracycline resistance gene potentially involved in protection of ribosomes from tetracycline and catalysis and release of tetracycline [*tet*(M) and *tet*(O) analogue] and a vancomycin B-type resistance protein gene, *van*(W), with no further genes potentially involved in vancomycin resistance.

BRACHYSPIRA

Brachyspira are Gram-negative, fastidious, microaerophilic anaerobic spirochetes which inhabit the colon and cecum (135). There are currently seven species of *Brachyspira* with standing in nomenclature: *B. aalborgi*, *B. alvinipulli*, *B. hyodysenteriae*, *B. innocens*, *B. intermedia*, *B. murdochii*, and *B. pilosicoli* (136). A number of other taxa without standing in the nomenclature, including *B. hampsonii*, *B. canis*, *B. suanatina*, and *B. pulli*, have been described in the literature (137, 138).

Brachyspira-Associated Disease in Pigs

Swine dysentery, characterized by muco-hemorrhagic diarrhea, is the most economically significant disease caused by *Brachyspira* in domestic animals. Swine dysentery was first described in 1921 and has historically been caused by *B. hyodysenteriae*; since the late 2000s a clinically indistinguishable syndrome associated with a novel taxon proposed to be called "*B. hampsonii*" has been described in North America and Europe (139, 140). Spirochetal colitis is a milder syndrome caused by *B. pilosicoli* and is associated with nonhemorrhagic, loose stools and production losses (141, 142).

Brachyspira-Associated Disease in Avian Species

Avian intestinal spirochetosis in domestic poultry species, characterized by diarrhea and mucosal thickening, is most often associated with *B. pilosicoli*, although *B. intermedia* and *B. alvinipulli* have also been implicated (143). Necrotizing typhlitis in juvenile rheas has been reported in association with *B. hyodysenteriae*. Colonization of wild birds with *Brachyspira* spp. is well recognized; phylogenetically diverse microorganisms including recognized pathogens and species of unknown clinical significance have been described in wild birds, including ducks and geese (138, 144).

Brachyspira-Associated Disease in Other Species

Brachyspira spp. have been reported from other domestic animal species, although their significance in disease has not been clearly defined. In people, intestinal spirochetosis associated with *B. aalborgi* and *B. pilosicoli* is an infrequently encountered chronic syndrome most often associated with immunosuppression (135, 145).

Antimicrobial Susceptibility Testing Methods and Challenges

Brachyspira-associated diseases in animals are primarily treated with the pleuromutilins, macrolides/lincosamides, and in the United States, carbadox (146). Treatment is generally empiric, since antimicrobial susceptibility testing of *Brachyspira* is not routinely conducted by most diagnostic laboratories. Although descriptions of the susceptibility of these organisms have been reported in the literature, the lack of standardized testing methods is a critical limitation to the interpretation of these studies. The methods used for antimicrobial susceptibility of other bacteria are described in exquisite detail in highly prescriptive standards published by the CLSI and EUCAST (4, 147). These standards dictate test factors including incubation temperature and time, bacterial concentration, atmosphere, and test media composition. The application of these methods to *Brachyspira* is hindered by the "nonstandard" growth conditions required by *Brachyspira* spp. Furthermore, because *Brachyspira* spp. do not reliably produce surface growth (colonies) on solid media and grow unreliably in liquid media, defining reproducible test endpoints to allow MICs to be determined is challenging. Laboratories performing these assays therefore rely heavily on in-house-developed methods, which while consistent within the facility, may differ substantially between laboratories. Studies reporting the use of agar dilution-based testing describe methods utilizing highly variable inoculum sizes (10^4 to 5×10^5 CFU/spot), incubation temperatures (37 to 42°C), and incubation time (48 to 120 hours) and utilize 5% blood-containing media with variable bases (trypticase soy agar, Wilkins-Charlgren, or Mueller-Hinton) (148–151). The effects of the variability of test methods on diagnostic outcomes was highlighted by a 2005 multicenter ring trial which described inconsistent results between participating laboratories (152). The introduction of the commercially prepared VetMIC *Brachyspira* microdilution panel in the early 2000s was an important step toward standardization of test methods. This panel has helped to improve the uniformity of the test media used by laboratories (153). The VetMIC panel includes tiamulin, valnemulin, doxycycline, tylvalosin, lincomycin, and tylosin.

The paucity of validated interpretive criteria for categorizing *Brachyspira* susceptibility test results is another critical limitation to the application of laboratory data to clinical practice. While breakpoints based on the pharmacokinetic properties of various drugs have been proposed by researchers (Table 2), the CLSI has just started to publish some interpretive criteria for classifying isolates as susceptible or resistant (7) (Table 2). CLSI document VET06 provides guidance for antimicrobial agent disk and dilution susceptibility testing, criteria for quality control testing, and breakpoints for fastidious and infrequently tested bacteria for veterinary use (7). Document VET06 includes a table on anaerobic bacteria and breakpoints for agar dilution and broth microdilution susceptibility testing. It includes a table on *B. hyodysenteriae* which provides information and breakpoints for agar dilution and broth microdilution susceptibility testing. Acceptable quality control ranges of MICs for anaerobic reference strains using agar dilution and broth microdilution are also provided in VET06. The methods described in this document are generic reference procedures that can be used routinely for susceptibility testing by clinical laboratories (7).

Emergence of Antimicrobial Resistance

The treatment of *Brachyspira*-associated diseases relies heavily on mechanistically similar drugs (primarily lincosamide- and pleuromutilin-type compounds) which inhibit protein synthesis by binding to the 50S ribosomal subunit. It therefore stands to reason that there is a high selection pressure for the development of resistance to

TABLE 2 Proposed breakpoints for the interpretation of *Brachyspira* MICs

	Proposed resistance breakpoint[a] or wildtype cutoff[a] (mg/liter)								
Author	Valnemulin	Tiamulin	Lincomycin	Tylosin	Tylvalosin	Doxycycline	Genatmicin	Carbadox	Reference
Burch	>0.125–0.25	>0.5–1	>50–100	>1632	>16–32				219
Pringle	>0.12	>0.25	>1	>16	>1	>0.5			156, 220
Duhamel		≥2	≥75				≥10	≥1	221

[a]Resistance breakpoint, Burch and Duhamel; wildtype cutoff, Pringle.

these drugs. A number of research groups have reported longitudinal studies of diagnostic isolates from their regions, and it is clear that resistance to drugs critical for treating *Brachyspira*-associated disease is emerging. Decreases in the susceptibility (increasing MICs) of *B. hyodysenteriae* to tiamulin and valnemulin have been reported when isolates from as early as the late 1980s are compared to isolates from the mid- to late-2000s. These trends have been described in multiple European countries (Italy, Germany, the Czech Republic, and Sweden) and in Japan (149, 154–157). Fewer longitudinal studies including *B. pilosicoli* have been published, although a Swedish investigation found that in contrast to decreasing pleuromutilin susceptibility among *B. hyodysenteriae*, the susceptibility of *B. pilosicoli* appeared to be stable (156).

Mechanisms of Resistance

Genetic associations with elevated antimicrobial MICs have been identified for a number of species-drug combinations (Table 1). Most of the published studies have focused on either *B. hyodysenteriae* or *B. pilosicoli*. Single nucleotide polymorphisms (SNPs) in rRNA gene sequences have been found to be associated with elevated macrolide, lincosamide, streptogramin, pleuromutilin, and tetracycline MICs. The first SNP found to be associated with decreased drug susceptibility was an A → G substitution at position 2058 of the 23S rRNA gene (158). Subsequently, additional SNPs, including at positions 2032, 2055, 2057, 2447, 2504, 2535, 2572, and 2611, have also been related to decreased susceptibility to one or more of these drugs (159–160). Decreased susceptibility to tiamulin has also been associated with mutations in ribosomal proteins L2, L3, L4, and L22 (159, 161). Ribosomal RNA SNPs have also been associated with decreasing susceptibility to doxycycline in *B. hyodysenteriae* and *B. intermedia*. Specifically, a polymorphism at location 1058 of the 16S rRNA gene has been identified in *B. hyodysenteriae* with decreased susceptibility to doxycycline (162).

A number of narrow-spectrum oxacillinases (Ambler class D beta-lactamases which hydrolyze penicillin, ampicillin, and oxacillin) have been identified in *B. pilosicoli* (163). To date, 14 closely related enzymes (OXA-63, 136, 137, 192, and 470 to 479) have been identified (164). The impact of these beta-lactamases on the treatment of *Brachyspira*-associated disease is questionable, although a deeper understanding of their epidemiology may be helpful for developing a more holistic model of resistance in animal pathogens. To date, there are no publications describing the presence of these enzymes in species other than *B. pilosicoli*, although whether this is reflective of the relative propensity of this species to carry these genes or of researcher/publication bias is unknown.

There is a great paucity of data describing the role of horizontal gene transmission in the epidemiology of resistance among *Brachyspira*. Phage-mediated transfer of resistance to chloramphenicol and tylosin between *B. hyodysenteriae* has been reported following exposure to metronidazole and carbadox (165). Plasmids have not been demonstrated to facilitate the dissemination of resistance, and one of the few studies addressing conjugative elements did not identify an association between the phenotype and the presence or absence of plasmids (166). Further work on understanding the extent and basis of resistance is needed in view of the extensive use of antibiotics to control brachyspiral infection in swine and poultry.

BACTEROIDES

Bacteroides are anaerobic Gram-negative rods and are one of the major groups colonizing the large intestines of animals and humans (167, 168). The *B. fragilis* group species are commonly associated with intra-abdominal abscesses, bacteremia, and soft tissue infections in both animals and humans. Some strains of *B. fragilis* can produce enterotoxins and are involved in diarrhea in lambs, calves, piglets, foals, young rabbits, and children. Antimicrobial resistance data on *Bacteroides* of animal origin is limited, whereas information is far more documented for human strains.

Earlier studies of clinical *Bacteroides* isolates from various animal species have indicated that penicillin, ampicillin, and cephalothin resistance varied between 18 and 24%, whereas tetracycline resistance was found to be between 9 and 20%, depending on the study (169, 170). In these studies, *Bacteroides* strains were usually susceptible to clindamycin and metronidazole. In a study of abscesses in pigs, *Bacteroides* isolates were susceptible to clindamycin, penicillin, ampicillin, minocycline, chloramphenicol, and cefoxitin, with clindamycin being the most active antimicrobial tested, with a median MIC of 0.8 mg/liter (171). Among *Bacteroides* isolates recovered from the uteri of dairy cows with retained fetal membranes and postparturient endometritis, all isolates were found to be susceptible to clindamycin (MIC_{90} of 0.064 mg/liter), and all but two were susceptible to metronidazole (172). In this study, the MIC_{90} of tetracycline was >256 mg/liter, and susceptibility to ciprofloxacin was variable, with a bimodal distribution of MIC values. Earlier studies also reported on *Bacteroides* clini-

cal isolates from dogs and cats, with 29% of isolates being resistant to ampicillin and 16% to clindamycin (173). In this study, all isolates were susceptible to amoxicillin-clavulanic acid and chloramphenicol, while most were susceptible to metronidazole. Beta-lactamase activity was also observed in all ampicillin-resistant isolates.

More recently, in a study of *Bacteroides* isolates (n = 10) recovered from clinical cases of caprine and ovine foot rot, a necrotic pododermatitis, strains were found to be generally resistant to penicillins, first-generation cephalosporins, tetracycline, and erythromycin and expressed a low level of beta-lactamase activity (12). The genes *cep*(A) and *tet*(Q) were the dominant resistance determinants conferring resistance to beta-lactams and tetracycline, respectively (12) (Table 1). In a study that analyzed 161 *B. fragilis* group bacteria isolated from calves with and without diarrhea, MIC values for cefoxitin ranged from 32 to >512 mg/liter, with 47 (29.2%) of them being resistant to cefoxitin using the breakpoint of 16 mg/liter (13). In this study, seven isolates were found to harbor plasmids varying from 6.0 to 5.0 kb, with a 5.5-kb plasmid *B. fragilis* Bc5j which might be associated with cefoxitin resistance. Also, using the nitrocefin method, beta-lactamase was detected in 33 (70.2%) isolates. The *cep*(A) gene was observed in total DNA as well as in the 5.5-kb plasmid (13). In a study of enterotoxigenic and nonenterotoxigenic *B. fragilis* in fecal samples from calves with or without acute diarrhea, 124 and 92 members of the *B. fragilis* group were recovered from 54 diarrheal and 54 nondiarrheal samples, respectively (174). In this study, two enterotoxigenic strains were isolated from two diarrhea samples. All strains were found to be susceptible to chloramphenicol, imipenem, moxifloxacin, piperacillin/tazobactam, metronidazole, and tigecycline.

Bacteroides spp. recovered from human clinical specimens frequently exhibit resistance to several antimicrobials, particularly to beta-lactams, tetracycline, ciprofloxacin, and clindamycin (175–178). It has been reported that resistance to multiple antimicrobials has been increasing in *Bacteroides* spp. for decades in human medicine, primarily due to horizontal gene transfer of a plethora of mobile elements (179). A review that summarizes the mechanisms of action and resistance to antimicrobials used to treat *Bacteroides* spp. infections in humans and that highlights current information on conjugation-based DNA exchange has been published (179). Briefly, in humans, resistance to tetracycline has been associated with the *tet*(Q) gene, which encodes for ribosomal protection (175, 181). Resistance to beta-lactams has been associated with the production of beta-lactamases encoded by the *cep*(A) (175, 180, 181) and *cfi*A (182) genes. Clindamycin resistance has been linked with ribosomal modification (181) via the *erm*(B), *erm*(F), and *erm*(G) (183, 184) genes. The *lin*(A) gene has been associated with MLS_B resistance (184). The *nim* genes of *Bacteroides* have been associated with metronidazole resistance via reduced uptake and altered reduction of the nitro group (183, 185, 186). A study demonstrated that constitutive BmeB, a component of the RND (Resistance-Nodulation-Division) family efflux transport systems (BmeABC1-16), expression is prevalent in *B. fragilis* (187), and this involves the transport of antimicrobials such as beta-lactams, fluoroquinolones, and tetracycline (187). Mutations in *gyr*A have been documented in fluoroquinolone resistance (188). *BexA*, which codes for the fluoroquinolone efflux pump, was represented only in a minor proportion of moxifloxacin-resistant strains (184). Combinations of *cfx*A, *cep*A, *cfi*A, *nim*A, *nim*D, *nim*E, *nim*J, *tet*(Q), *erm*(B), *erm*(F), *bex*B, and possibly, *lin* and *mef* genes were recently identified in multidrug-resistant *B. fragilis* using whole-genome shotgun sequencing (183).

FUSOBACTERIUM

Fusobacterium are non-spore-forming Gram-negative rods that are usually found as normal flora in the mouth, the intestines, and the urogenital tract (189). *F. necrophorum* is considered the most virulent species, followed by other species such as *F. nucleatum*, *F. canifelinum*, and *F. varium* (189–191). *Fusobacterium*can cause a plethora of necrotic infections in animals, such as necrotic stomatitis, foot rot (interdigital necrobacillosis), gangrenous dermatitis, and pulmonary, hepatic, and jaw abscesses (189). These infections are often polymicrobial, and some of them (hepatic necrobacillosis, necrotic laryngitis, and foot rot) have important economic impacts in cattle (189). Antimicrobials can be used to reduce the risk of *Fusobacterium*-associated liver abscesses in cattle (192). The antimicrobials most commonly reported for usage in prophylaxis against this disease are bacitracin, chlortetracycline, oxytetracycline, tylosin, and virginiamycin. In humans, *Fusobacterium* is associated with clinically distinctive, severe septicemic infections variously known as necrobacillosis, postanginal sepsis, or Lemierre's syndrome (193). It may also occur after accidental trauma such as animal bites (194, 195). In earlier studies of *Fusobacterium* isolates from human bite wounds, susceptibility was reported to a range of beta-lactam antimicrobials as well as metronidazole; resistance to penicillin, clindamycin, and ciprofloxacin

was seldom seen (194, 195). In contrast, a study in Taiwan reported a higher level of resistance (196). In humans, resistance to penicillin associated with beta-lactamase production has been described, and there is widespread resistance to erythromycin and other macrolides (193, 197).

Earlier studies of *Fusobacterium* from various animal species revealed susceptibility to penicillin, ampicillin, cephalothin, chloramphenicol, clindamycin, tetracycline, and metronidazole (169, 173). A Spanish study in the 1990s that investigated *F. necrophorum* isolates from hepatic abscesses in cattle and sheep described broad susceptibility to spiramycin, lincomycin, tylosin, oxytetracycline, chlortetracycline, metronidazole, cotrimoxazole, sulfadimethoxine, virginiamycin, and fosfomycin but resistance to clindamycin (42%) (198). In this study, out of the 13 beta-lactam antimicrobials tested, only 1, cefotetan, was not active against all isolates. In a U.S. study of *F. necrophorum* from bovine hepatic abscesses, isolates were generally found to be susceptible to penicillins, tetracyclines (chlortetracycline and oxytetracycline), lincosamides (clindamycin and lincomycin), and macrolides (tylosin and erythromycin) (199). In this study, bacitracin and virginiamycin were also active against *F. necrophorum*. A Spanish and Portuguese study of *Fusobacterium* (n = 108) from 90 cases of foot rot in sheep described that most isolates were susceptible to beta-lactams (benzyl penicillin, ampicillin, cloxacillin, cefadroxil, cefuroxime, and cephalexine), chloramphenicol, clindamycin, and metronidazole, but resistance to erythromycin and spiramycin was observed in 77% and 60% of isolates, respectively (200). In this study, doxycycline was also quite active against *Fusobacterium*.

More recently, a survey of *Fusobacterium* strains isolated from caprine and ovine foot rot revealed that most isolates were generally susceptible to beta-lactams, whereas resistance to tetracycline and/or erythromycin was observed (12). In a study of subgingival plaque from dogs with and without periodontitis, *F. nucleatum* and *F. canifelinum* were susceptible to most of the antimicrobials tested; however, different resistance rates to clarithromycin, erythromycin, and metronidazole were observed (191). Intrinsic resistance (MIC, >4 mg/liter) to levofloxacin and other fluoroquinolones has been observed in *F. canifelinum* originating from cats or dogs (201). In this study, it was found that Ser79 was replaced with leucine, and Gly83 was replaced with arginine when compared to the quinolone resistance-determining region within *gyr*A of susceptible strains of *F. nucleatum*. A survey of clinical isolates of *Fusobacterium* (n = 23) recovered at necropsy over a 2-year period from the respiratory tracts of white-tailed deer indicated that susceptibility to antimicrobials was markedly different for *F. varium* compared to *F. necrophorum* and *F. funduliforme* (202). In this study, all isolates were susceptible to ampicillin, florfenicol, and trimethoprim-sulfamethoxazole, whereas fewer *F. varium* isolates were susceptible to chlortetracycline, clindamycin, oxytetracycline, tiamulin, tilmicosin, tulathromycin, and tylosin compared with *F. necrophorum*. Also, intermediate or variable susceptibilities were observed to ceftiofur, danofloxacin, penicillin, and sulfadimethoxine. Almost all isolates were resistant to enrofloxacin, indicating intrinsic resistance. In summary, despite observed resistance to macrolides and tetracyclines, *Fusobacterium* spp. of animal origin remain quite susceptible to antimicrobials, including those of the beta-lactam class. To date, to our knowledge, there has been no analysis of the genetic basis of acquired antimicrobial resistance.

DICHELOBACTER NODOSUS

D. nodosus, a Gram-negative non-spore-forming bacterium, is the principal etiologic agent of foot rot. It is a transmissible disease that involves the epiderma of the feet and results in lameness. It is an economically important and worldwide endemic disease affecting the sheep industry in terms of production losses and costs for treatment and prevention (203). Other animal species may also develop foot rot but in a milder form. Foot rot is a contagious infection involving the invasion of the epidermal tissues of the interdigital space of the foot and of the soft horn of the hoof (203). Eventually, the hoof and its underlying dermal tissues become separated. This hoof underrunning process can range from mild to severe, but the latter only occurs when virulent strains of *D. nodosus* are involved. Host and environmental factors can influence the disease (203–205). Virulent and benign variants of *D. nodosus* have recently been demonstrated by whole-genome sequencing, and they have been shown to correlate with the presence of *aprV2* and *aprB2*, respectively (206–208). These genes encode for distinct extracellular proteases with only a single amino acid substitution (206). The infection can also be polymicrobial and may involve *F. necrophorum* as well as other anaerobes, but the presence of *D. nodosus* seems to be mandatory for true foot rot to occur. When treatment is attempted, a footbath with bactericidal agents such as copper sulfate or zinc sulfate can be used after removal of necrotic debris and horn (209). Antimicrobials have been successfully used to control foot rot in particular settings, and penicillin-streptomycin (210), eryth-

romycin (211), oxytetracycline (212), and lincomycin-spectinomycin (213) have been shown to be efficient.

A U.S. study performed in the 1980s of the antimicrobial resistance of *D. nodosus* reported penicillin as the most effective antimicrobial along with susceptibility to cefamandole, clindamycin, tetracycline, chloramphenicol, erythromycin, cefoxitin, tylosin, and nitrofurazone (214). Two Spanish studies of goats and sheep in the 1990s revealed more penicillin and tetracycline resistances in *D. nodosus* (215, 216). Their results also indicated that chloramphenicol, metronidazole, and rifampin were effective *in vitro*, but chloramphenicol and metronidazole are both restricted worldwide in food-producing animals. More recently, in a survey in Portugal and Spain of 90 clinical cases of ovine foot rot, 69 strains of *D. nodosus* were recovered, and 90% were susceptible to penicillin, ampicillin, erythromycin, spiramycin, tylosin, chloramphenicol, and enrofloxacin (200). In this study, resistance to metronidazole, oxytetracycline, doxycycline, and trimethoprim was observed in 17.5%, 42%, 14%, and 10% of the strains, respectively (200). In another survey, where *D. nodosus* was isolated from 48% of the sampled animals (n = 25) with foot rot, the susceptibility of 99 isolates to 5 antimicrobials (penicillin G, amoxicillin, spiramycin, erythromycin, and oxytetracycline) indicated that resistance was in all cases higher than 30% (217). In this study, the efficacy of erythromycin and oxytetracycline in the treatment of ovine foot rot using an intramuscular injection at the beginning of the treatment demonstrated that 75% of animals were cured within 15 days with one or the other antimicrobials used (217). More recently, in a Spanish survey of *Dichelobacter* and other anaerobes obtained from clinical cases of foot rot, beta-lactams, tetracyclines, and metronidazole were shown to have the highest *in vitro* efficacy (218). In this study, *D. nodosus* showed no resistance either to penicillins (penicillin G) or to cephalosporins (cefadroxil, cefuroxime, and cephalexin). To date, to our knowledge, there has been no analysis of the genetic basis of acquired antimicrobial resistance.

CONCLUSION

Antimicrobial resistance has been described in the most frequently encountered anaerobic bacterial pathogens of animals. However, because animal data are too limited in certain cases, this article focused not only on antimicrobial resistance data of veterinary origin, but also included antimicrobial resistance identified in anaerobes from humans. Many studies describe point prevalence but give little data about trends in the development of resistance over time. Overall, antimicrobial resistance can vary depending on the antimicrobial, the anaerobe, and the resistance mechanism. On occasion, the same resistance gene was observed in many anaerobes, whereas some genes were limited to certain anaerobes. Surprisingly, in some anaerobes, mechanisms of antimicrobial resistance have not been studied, and further attention should be given to the investigation of molecular mechanisms of resistance. This may be facilitated by the increasing use of routine genome sequencing as well as a shift of CLSI approaches to gene-based analysis of resistance. Additional work is indeed required to increase our knowledge about antimicrobial resistance in anaerobes.

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