PHENOTYPIC AND GENOTYPIC CHARACTERIZATION OF HIGH-LEVEL MACROLIDE AND LINCOSAMIDE RESISTANCE IN *CORYNEBACTERIUM* SPECIES IN CANADA AND THE DISTRIBUTION OF THE *ermX* RESISTANCE DETERMINANT AMONG *CORYNEBACTERIUM* SPECIES

BY

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Finally, for my audience of One… may this be for Your glory alone.

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

LIST OF ABBREVIATIONS

ATP: adenosine triphosphate

BLAST: Basic Local Alignment Search Tool

bp: base pair

caMHB: cation-adjusted Mueller-Hinton broth

CBAB: Columbia Blood Agar Base

cfu: colony forming units

DNA: deoxyribonucleic acid

dNTPs: deoxynucleotide triphosphates

EDTA: ethylenediaminetetraacetic acid

Erm: erythromycin ribosome methylase

HSC: Health Sciences Centre, Winnipeg, MB

ICAAC: Interscience Conference on Antimicrobial Agents and Chemotherapy

LCDC: Laboratory Centre for Disease Control

MBC: minimum bactericidal concentration

MIC: minimum inhibitory concentration

MHBwLHB: cation-adjusted Mueller-Hinton broth with lysed horse blood

ml: millilitre

MLS_B: macrolide-lincosamide-streptogramin B group

mM: millimolar

mRNA: messenger RNA

NML: National Microbiology Laboratory

PCR: polymerase chain reaction

rDNA: ribosomal DNA sequence

RNA: ribonucleic acid

rpoB: DNA dependent RNA polymerase, beta-subunit

TAE: tris-acetate-EDTA

TBSA: tuberculostearic acid

µl: microlitre

µm: micrometre

µM: micromolar

ABSTRACT

Specific bacterial commensals demonstrating multidrug resistance (MDR) are opportunistic pathogens for immunocompromised patients, including *Corynebacterium* species (spp.). Severe infections due to MDR corynebacteria are being increasingly reported where several MDR phenotypes have been described. One such phenotype, the macrolide-lincosamide-streptogramin B phenotype (MLS_B), is characterized by high-level resistance to macrolides, lincosamides, and streptogramin B. Resistance is thought to be attributable to acquisition of the *ermX* gene, a methyltransferase that alters the ribosomal macrolide binding site. Until recently, *ermX* had been reported in only six *Corynebacterium* spp. We have observed other corynebacteria can also display high-level resistance to MLS_B antimicrobials and are *ermX* positive. Hypotheses being tested include: 1) high-level macrolide and lincosamide resistance in *Corynebacterium* spp. is caused by acquiring *ermX*; 2) distribution of *ermX* is more widespread than previously published; 3) *ermX* is associated with transposon *Tn*5432; 4) multidrug resistance has spread to Canadian *C. afermentans* and *C. aurimucosum* strains.

1.0 INTRODUCTION

1.1 Description of *Corynebacterium*

1.1.1 Microbiology

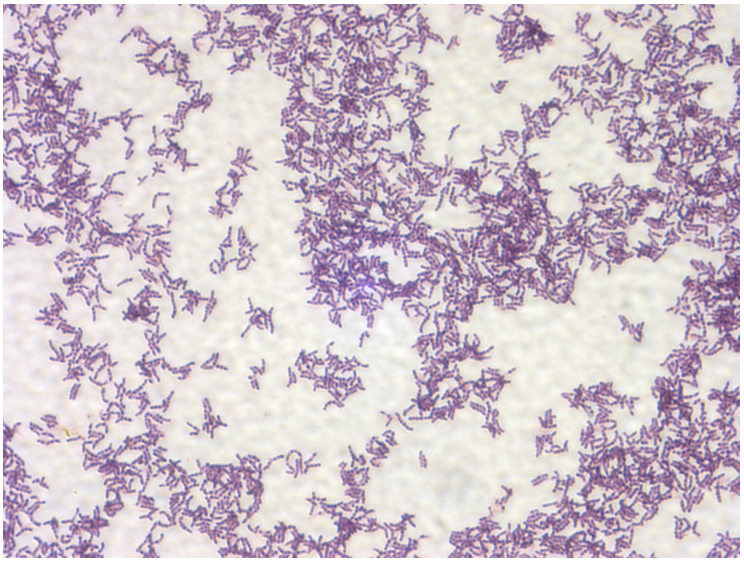
The genus *Corynebacterium* was first proposed in 1896 by Lehmann and Neumann following the characterization of its most recognized species, *Corynebacterium diphtheriae*, now known as the etiological agent for diphtheria (33). Aside from *C. diphtheriae*, little attention has been given to other species in the genus with respect to basic systematics, or to their role as etiologic agent in disease for many years. Consequently, study among antimicrobial susceptibility among these agents has been minimal. In the mid 1980s, interest in *Corynebacterium* species was renewed as several species began to be associated with severe infections in immunocompromised patients (42, 53, 62, 92, 100). Over the next twenty years, the number of species in the genus *Corynebacterium* would more than double (33). The genus is presently made up of 78 valid species, with at least 6 more currently in press.

The genus *Corynebacterium* is assigned to the family *Corynebacteriaceae* in the suborder *Corynebacterineae*, characterized by a genome with high G+C (guanine and cytosine, respectively) content (33, 36, 108). They are small to medium sized Gram-positive, asporogenous pleomorphic rods that divide by binary fission and are arranged in a dispersed angular manner. The genus is represented by species that are fermentative, oxidative, or neither, and by some displaying a lipophilic phenotype, meaning their growth is enhanced in the presence of fatty acids such as Tween 80,

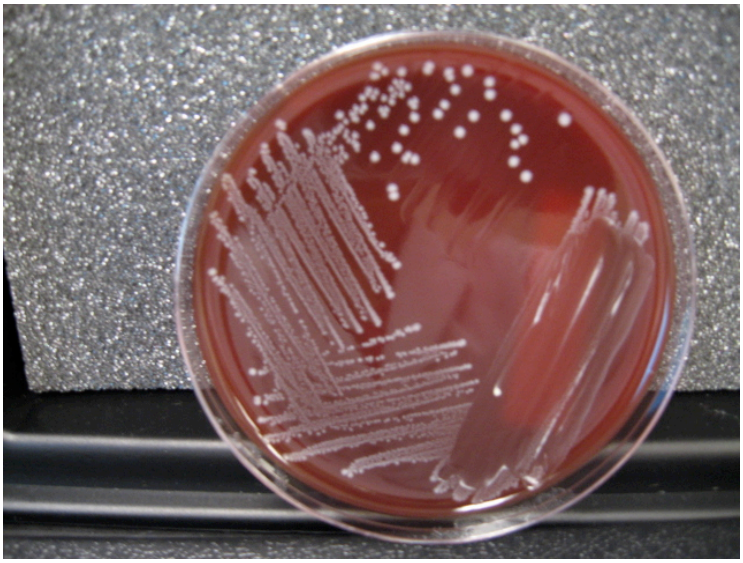
which is an oleic acid derivative. The absence of a fatty acid synthase within the bacterial genome has been attributed as one possible cause for the lipophilic phenotype (87). Chemotaxonomically, *Corynebacterium* species have a meso type IV diaminopimelic acid with arabinose and galactose sugars in the cell wall, as well as short-chained mycolic acids ('mycolates') of 22-36 carbons in length (36). *C. amycolatum, C. atypicum, C. caspium, C. ciconiae* and *C. kroppenstedtii* lack mycolates entirely in their cell wall. Long chained cellular fatty acids are of the straight-chained saturated and monounsaturated types, with significant amounts of palmitic (C16:0) and oleic (C18:1ω9c) acids. Small amounts of tuberculostearic acid (C10me18:0) may be present in some species (2). Products of fermentation may include small volumes of acetic, succinic and lactic acid; however production of propionic acid is species specific (3). Dihydrogenated menaquinones with either eight and/or nine isoprene units are present. Membrane phospholipids include simple, phosphatidylinositol, phosphatidylinositol dimannoside(s), trehalose dimycolates, and other glycolipids (18).

In the past, reference to members of the genus *Corynebacterium* have been described as "diphtheroids" or "coryneforms," however these terms are quite vague and encompass the majority of genera in the suborder *Corynebacterineae*, or genera found in other families of Gram-positive rods, such as the *Microbacteriaceae* (*Microbacterium* spp.) or the *Cellulomonadaceae* (*Cellulomonas* spp.) rather than just *Corynebacterium* species. A more accurate representation of the genus *Corynebacterium* is the term "corynebacteria," which will be used synonymously with "members of the genus *Corynebacterium*" throughout.

Figure 1: Gram stain of *Corynebacterium resistens* CCUG 50093^{T}, 1000x magnification, 24h growth (A); *Corynebacterium resistens* CCUG 50093^{T}, 48h growth on CBAB (B). Images taken by Special Bacteriology staff at the National Microbiology Laboratory (NML).



A.



B.

1.1.2 Historical Significance

Historically speaking, the most relevant *Corynebacterium* species is *C. diphtheriae*, since it is the etiological agent of diphtheria. *C. pseudotuberculosis* and *C. ulcerans* are also capable of causing diphtheria-like illness in humans, and can also possess and express the *tox* gene (40). These two species are primarily animal commensals, but are capable of causing disease in humans. Diphtherial disease has likely been present since ancient times; however it was not formally classified as an infectious disease until the 1820s as being distinguishable from other causes of a sore throat. Known as "the strangling angel of children," large outbreaks of diphtheria took place throughout the 18^{th} and 19^{th} centuries, ravaging populations and having the greatest effect on children, primarily those less than 10 years of age. During the 1880s, *C. diphtheriae* was visualized for the first time in a specimen taken from a pseudomembrane of a diseased individual and later was proven to be the cause of diphtheria (109). Two scientists in 1888 were able to show that *C. diphtheriae* was capable of producing a potent toxin that was able to cause disease in animals when distributed in very small doses. Reprieve from the disease began in the 1890s, when the discovery was made that the serum of animals previously inoculated with diphtheria toxin was able to confer immunity to the toxigenic traits of disease in susceptible animals (40). This treatment was implemented in Canada as early as 1914; however, incidence of diphtherial disease remained high. The discovery of diphtheria toxoid, or inactivated toxin by Gaston Ramon in France in 1924 was the key to eliminating the disease (70). Canadian field trials held in the late 1920s and early 1930s were the first of

their kind and led the way to virtually eliminating diphtherial disease among Canadians during this time. Other populations such as the British and the Americans were not as quick to realize the effectiveness of treatment with the toxoid, and subsequently diphtheria rates within these countries remained high until the same strategy was adopted in the mid to late 1930s. Antimicrobials used to combat diphtheria took longer to develop, and were not readily available until the production of sulfonamide antimicrobials for clinical use in the 1940s. In the present day, case reports of diphtheria in developed nations are low. In spite of the vaccine being in circulation for more than 80 years, however, outbreaks of diphtheria are still seen in different countries, the most recent one occurring in countries newly independent from the Soviet Union (21, 45, 88).

Other *Corynebacterium* species have also been described since the early 20th century, including *C. xerosis* and *C. striatum* (33). These species were first described close to the same time that *C. diphtheriae* was first characterized, and had to be differentiated from *C. diphtheriae* when diagnosing a true case of diphtheria. For years these species were misidentified as each other; however phenotypic and molecular studies conducted in the 1990s showed that some strains, including reference strains classified as *C. xerosis* were actually misidentified *C. striatum* and *C. amycolatum* strains (34). Aside from these two strains and a small number of other species, interest in further characterization of *Corynebacterium* species was low for the greater part of the 20th century. Beginning in the mid to late 1980s, interest in the corynebacteria slowly began to increase due to the emergence of severe infections in immunocompromised patients, coinciding with the advent of advances in more precise genetically-based

characterization methods. With the introduction of antimicrobials into clinical practice halfway through the 20th century, the problem of antimicrobial resistance also began to be associated with *Corynebacterium* species. In the span of approximately 20 years, over 50 new *Corynebacterium* species have been described in the literature. Not all recently described *Corynebacterium* species have been clinically relevant, yet the vast majority of those isolated were from active human infections.

1.1.3 Medically-Relevant vs. Non-Medically Relevant Corynebacteria

The members of the genus *Corynebacterium* can be loosely divided into two groups: medically relevant and non-medically relevant species. The non-medically relevant species include animal commensals, industrially relevant and environmental isolates. The medically relevant species include those that have been isolated from active human infections, as well as four species considered to be zoonotic agents – bacterial species that are usually benign, but can still cause disease in the animal host, and are capable of causing disease in humans. These four include *C. bovis, C. mastitidis, C. pseudotuberculosis,* and *C. ulcerans. C. pseudotuberculosis* and *C. ulcerans* are of particular importance due to the fact that they are capable of possessing and expressing the gene found in toxin positive *C. diphtheriae* isolates (33). *C. mastitidis, C. renale, and C. ulcerans* are three species capable of causing disease in animals. The medically relevant *Corynebacterium* species comprise approximately 55-60% of all *Corynebacterium* species. *Corynebacterium* isolates in hospitals and laboratories have largely been regarded as specimen contaminants because of the preconceived notion

that they are benign commensals (33, 36). However, when a positive culture is isolated from normally sterile body sites multiple times, such as blood, or if it is the predominant organism isolated from a nonsterile site, further characterization is warranted. Clinical significance is also increased if multiple specimens are positive for the same *Corynebacterium* species and coryneform bacteria are seen in the direct Gram stain. Aside from *C. diphtheriae*, a number of medically relevant species have emerged in recent years, isolated from various areas of the human body (35, 59, 105-107). Several *Corynebacterium* species occupy a particular niche in the body. For example, *C. auris* occupies the ear, *C. durum* is frequently found as a commensal in the oropharynx region, and *C. macginleyi* is frequently found in ocular samples (25, 36). In addition to these, there are more than 35 additional *Corynebacterium* species that are defined as medically relevant. They have either been isolated from sterile body sites, or as the predominating organism from nonsterile sites. The type of infections caused by these species range from minor wounds to severe cases of endocarditis and osteomyelitis, and sometimes requires large, prolonged doses of antimicrobials to successfully treat the infection.

1.1.4 Multidrug Resistance: An Overview

The introduction of antimicrobials into routine clinical care was initially seen as the final solution to the problem of bacterial infections. However, no sooner did antimicrobials like penicillin and erythromycin become part of clinical care than resistance to these drugs was reported. This led to the discovery and development of several antimicrobial classes, with some being more clinically successful than others. Resistance to nearly all antimicrobial classes is now reported in cases worldwide, with several strains being classified as "multidrug resistant." However, the classification of a strain as being multidrug resistant is not a universal statement. For example, strains of *Mycobacterium tuberculosis* classified as multidrug resistant (MDR-TB) must fulfill a set of established criteria, which includes resistance to at least two first-line antimicrobials used in the treatment of active tuberculosis, such as isoniazid and rifampin (12). The term multidrug resistant has also been applied to other bacterial species, including *Pseudomonas aeruginosa* and others. These reports all indicate that the strains in question are resistant to several antimicrobials, yet the antimicrobials they are resistant to vary, as well as the number. Reports of multidrug resistant *Corynebacterium* species also exist, however multidrug resistance in these reports is also not defined specifically (10, 23, 87). In this study of *Corynebacterium* species, multidrug resistance will be defined as resistance to at least one antimicrobial within three different antimicrobial classes. Today, bacterial strains known as "superbugs" exist, displaying susceptibility to only a few of the most costly and toxic medications. The acquisition of resistance genes over the course of time enabled bacteria to survive, as there are resistance genes for

nearly every antimicrobial class developed for clinical use. These genes were first acquired through plasmid uptake, yet now they are located within bacterial chromosomes due to the acquisition of transposable or mobile genetic elements (54). Antimicrobials such as erythromycin and tetracycline currently have over 40 different resistance genes characterized in a widespread number of aerobic and anaerobic bacterial genera, both Gram-positive and Gram-negative (48, 65, 67). In addition to the acquisition of resistance mechanisms, the bacterial host can also modify specific antimicrobial targets within its structure to disrupt activity, such as introducing mutations within the *gyrA* and *parC* genes, conferring fluoroquinolone resistance (77). These types of mutations are known as intrinsic mutations. Another form of resistance is seen with enzymes such as phosphorylases, acetyltransferases, and glycosylases actively targeting and modifying the antimicrobial, eliminating its effectiveness due to the addition of an extra acetyl group or phosphate molecule. Also, certain antimicrobials are ineffective against certain bacterial pathogens due to their structure or chemical composition. For example, the antimicrobial vancomycin is a large molecule, and because of its size it is unable to penetrate the outer membrane of a Gram-negative organism, making it suitable only for use against Gram-positive pathogens (104).

1.1.5 Frequently Isolated Multidrug Resistant *Corynebacterium* species

1.1.5.1 *Corynebacterium jeikeium*

In recent years, the association between the genus *Corynebacterium* and a multidrug resistant phenotype has increased in significance. While many *Corynebacterium* species are capable of causing infection in humans, there are a few species that are frequently isolated clinically. One clinically significant species, if not the most clinically significant is *Corynebacterium jeikeium*, a lipophilic species that is part of the normal skin microflora (33, 36). Previously known as Coryneform CDC Group JK according to a naming method developed in the 1980s for provisionally naming *Corynebacterium* isolates, *C. jeikeium* has been linked to many different diseases, including various skin and soft tissue infections, septicaemia, osteomyelitis, meningitis, and endocarditis (39, 42, 55, 56, 58, 90, 100). Isolates of *C. jeikeium* often display reduced susceptibility to at least three or more antimicrobial classes, such as the beta-lactams, aminoglycosides, cephalosporins, tetracycline, rifampin, sulfonamides, and the MLS_B group (macrolide-lincosamide-streptogramin B). Treatment of *C. jeikeium* infections usually involves combination therapy, or utilizing large doses of glycopeptide or lipopeptide antimicrobials such as vancomycin or daptomycin for extended periods of time (55, 90).

1.1.5.2 *Corynebacterium amycolatum*

Another species frequently isolated within the clinical setting is *Corynebacterium amycolatum*, also considered as part of the skin microflora (33). First described in 1988, *C. amycolatum* has also been linked to cases of both moderate and severe types of infections, including skin infections, peritonitis, endocarditis and others (13, 19, 53). *C. amycolatum* isolates were misidentified for a number of years as belonging to one of three species, *C. minutissimum, C. striatum,* or *C. xerosis* (34, 110). Certain *C. amycolatum* isolates were also misidentified as either Coryneform CDC Group F-2 or I-2, according to the older naming method devised in the 1980s (39). These particular groups were classified as such because they lacked mycolates within their cellular membrane, yet differed in one or two biochemical tests from what was characterized to be a 'true' *C. amycolatum*, such as the nitrate reduction test. The majority of strains previously classified as Group F-2 or I-2, however ended up being renamed as *C. amycolatum* isolates. *C. amycolatum* can be differentiated from *C. minutissimum, C. striatum,* and *C. xerosis* based on colony colour, morphology, and through several other phenotypic and molecular tests. *C. amycolatum* colonies are white and "sugary" in appearance, while *C. xerosis* colonies are yellowish, dry and wrinkled, more so than *C. amycolatum* colonies (95). Also, *C. xerosis* isolates very rarely display reduced susceptibilities to antimicrobials, while *C. amycolatum* isolates frequently display resistance to several antimicrobial classes (33, 36, 77). Treatment of *C. amycolatum* infections is similar to treatment of *C. jeikeium* infections, particularly if the strain is multidrug resistant.

1.1.5.3 *Corynebacterium striatum*

Corynebacterium striatum is a skin commensal that has been identified for decades, and has been defined as a pathogen of clinical significance since the early 1990s (66). Like *C. jeikeium* and *C. amycolatum*, *C. striatum* has been linked to cases of osteomyelitis and endocarditis, as well as meningitis (26, 75, 99). Infections such as pneumonia and septicemia have also been attributed to this organism (51, 81). *C. striatum* isolates have also been misidentified in the past as either *C. minutissimum* or *C. xerosis*, based on phenotypic testing alone rather than incorporating molecular tests into the identification process. Isolates are also frequently multidrug resistant. *C. striatum* has also established itself as a nosocomial pathogen. There was a recent case in which a multidrug resistant *C. striatum* clone was responsible for a nosocomial outbreak in Japan, and another single multidrug resistant *C. striatum* clone was responsible for an extensive nosocomial outbreak in Italy (10, 60). Other outbreaks due to *C. striatum* within the hospital setting have occurred in the past as well (7).

1.1.5.4 *Corynebacterium urealyticum*

Corynebacterium urealyticum, formerly known as Coryneform CDC Group D-2 is a pathogen of the urinary tract (62). *C. urealyticum* is lipophilic and asaccharolytic, and is the etiological agent of alkaline encrusted cystitis and other urinary tract infections in patients. It possesses potent urease activity, which may contribute to its pathogenicity in the urinary tract by raising the pH, facilitating entry into the organ system (87). It is thought that the increase in pH due to the hydrolysis of urea contributes to the

formation of struvite stones (ammonium magnesium phosphate). In addition to its affinity for the urinary tract, there is one report that *C. urealyticum* is capable of causing infections such as pericarditis (57). It was recently determined that *C. urealyticum* has the ability to form biofilms, and as a result, display significantly reduced susceptibilities to several commonly used antimicrobials, such as ciprofloxacin, erythromycin and vancomycin (78). This is a growing concern, as most *C. urealyticum* isolates are multidrug resistant.

1.1.5.5 Other Emerging Clinical Species

In addition to these four species, there are others that are emerging as significant pathogens within the immunocompromised, including the lipophilic species *C. tuberculostearicum*, a pathogen first described in 1984, but not formally recognized until 2004 (8, 30). Most strains of *C. tuberculostearicum* can now be identified to the species level, but were previously classified as Coryneform CDC Group G or G-2 prior to formal nomenclature being introduced (39). Other species include *C. macginleyi*, a lipophilic species that has an affinity for ocular regions, *C. coyleae*, a species recently described for causing several infections, and *C. ureicelerivorans*, another lipophilic *Corynebacterium* species possessing potent urease activity, making it another likely pathogen targeting the urogenital tract (25, 27, 28, 30, 94). *C. resistens*, a lipophilic species first described in 2005, was so named due to its multidrug resistance phenotype. The type strain of this organism, CCUG 50093^{T}, displays resistance to the beta-lactams,

cephalosporins, imipenem, amikacin, the macrolides, lincosamides, fluoroquinolones and tetracycline (59).

1.1.6 Treatment of infections caused by *Corynebacterium*

Antimicrobials designed to target Gram-positive pathogens are effective in treating infections caused by *Corynebacterium* species. However, when these species are suspected or confirmed to be multidrug resistant, therapy becomes complicated. Combination therapy is useful in certain instances, such as the pairing of a glycopeptide such as vancomycin with gentamicin, an aminoglycoside, as this combination is synergistic in effect (55). Vancomycin used on its own is also effective in treating *Corynebacterium* infections. Studies performed with newer antimicrobials such as tigecycline, telithromycin and daptomycin show that these are also effective choices for treatment (29, 37, 71). Adverse effects with these antimicrobials are minimal for the most part, with the exception of the risk of nephrotoxicity when using combination therapy with vancomycin and an aminoglycoside (104). The risk of hearing loss due to ototoxicity is also seen with glycopeptide usage.

1.2 Antimicrobial Development

1.2.1 Macrolides

As pathogen-disease relationships were established, compounds designed to assist the immune system in fighting these infections began to emerge. The discovery of what would become penicillin in 1928 by Alexander Fleming heralded the beginning of

the antibiotic era (67). During the next 25 to 30 years, several different antibiotics emerged that were based on pre-existing compounds naturally made by fungi and other microbes. One of several antimicrobial classes discovered during this time were the macrolides. The first macrolide, erythromycin, was discovered in 1952, and became available for clinical use the following year (24, 50, 67). The compound is produced naturally by the soil microbe *Saccharopolyspora erythraea*. Their activity stems from a large, macrocyclic lactone ring, the characteristic structure for a macrolide. Macrolides can be 14, 15, or 16-membered rings that are highly substituted, and are attached to two sugar molecules, desosamine and cladinose (104). The target site of the macrolide antibiotics is the 23S rRNA molecule within the 50S ribosomal subunit. The proposed mechanism involves the disruption of the formation and subsequent release of the nascent peptide from the P site of the ribosome. Erythromycin is the only macrolide antibiotic produced naturally; the other members are semi-synthetic derivatives that usually have replaced side chains attached to the lactone ring, such as azithromycin, clarithromycin, and roxithromycin (65, 67). Erythromycin is, in most cases, bacteriostatic, but can be bactericidal when high concentrations of drug are reached *in vivo*, or if there is a low density of bacterial organism present during infection (24, 67). Macrolides are effective against a wide range of bacterial pathogens, both Gram-positive and Gram-negative, and anaerobic pathogens, making them one of the most important antimicrobial classes globally for treatment of a variety of diseases, including skin and soft tissue infections and respiratory infections (65).

1.2.2 Lincosamides

The lincosamide group of antibiotics was discovered not long after the macrolides, in the early 1960s. The first lincosamide, lincomycin, was isolated from the fermentation products of *Streptomyces lincolnensis* var. *lincolnensis*, a soil dwelling microbe (9). The semi-synthetic derivative clindamycin followed soon after, and is presently a more popular choice in the treatment of various bacteria-caused illnesses, including those caused by anaerobic pathogens (67). The common structure of the lincosamides is an amino acid linked to an amino sugar (104). In spite of being structurally unrelated to the macrolides, the mechanism of action of the lincosamides is similar to the action of macrolides in that they also bind to the 23S rRNA molecule in the 50S subunit of the ribosome and prevent elongation of the amino acid chain. Lincomycin is the only naturally produced lincosamide; all other lincosamides are synthetically produced. However, none equate to clindamycin in the areas of potency and low toxicity (50). Like the macrolides, lincomycin and clindamycin are bacteriostatic, but can be bactericidal if used in high enough concentrations or against a low inoculum of bacteria. The spectrum of activity for the lincosamides is broad, including Gram-positive and Gram-negative pathogens, both aerobic and anaerobic. In combination with primaquine, clindamycin is also effective against the fungus *Pneumocystis jirovecii*, a common cause for pneumonia in severely immunocompromised patients (104).

1.2.3 Streptogramins

The streptogramins are cyclic peptides that are naturally made by several *Streptomyces* spp., including *S. pristinaespiralis*, *S. virginae*, and *S. graminofaciens* (11). There are two forms of streptogramins: group A and group B, formerly known as virginiamycin or pristinamycin. Group A streptogramins are polyunsaturated macrolactones made up of lactam and lactone linkages with an oxazole ring, while group B streptogramins includes the compound quinupristin, which has a cyclic hexadepsipeptide structure (104). Quinupristin also binds to a similar region of the 50S subunit as the macrolides and lincosamides, although it is structurally unrelated to both the macrolides and clindamycin (67). The method of action for quinupristin is prevention of protein elongation and causing the early release of incomplete peptide chains. Clinically, quinupristin is seen paired in combination with dalfopristin, a member of the group A streptogramins. While each agent is bacteriostatic on its own, the combination of the two in a 30% to 70% ratio (quinupristin to dalfopristin) results in synergy, making the combination bactericidal (104). It was reported that the addition of dalfopristin to quinupristin increases the binding affinity of the drug to the ribosome 40 fold. The primary target of the streptogramins is Gram-positive bacteria, but is also effective against certain Gram-negative and anaerobic pathogens.

Figure 2: Cartoon schematic of a 70S bacterial ribosome, showing the 30S and 50S subunits. The A and P sites containing tRNA molecules are depicted in blue, with the A site on the left and the P site on the right. The mRNA being translated is in purple beneath the tRNA molecules. The macrocyclic lactone ring of erythromycin is shown in red. A nascent peptide chain is shown in pink. The presence of the macrolide causes the premature release of the nascent peptide.

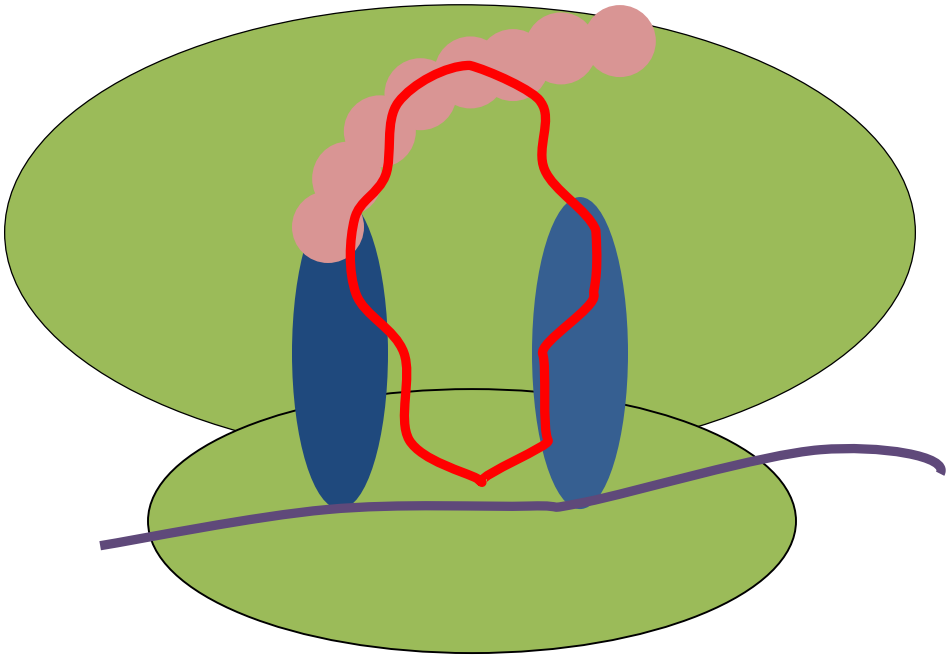


Figure 3: Chemical structures of erythromycin (A), clindamycin (B), quinupristin (pristinamycin I^A) (C), and dalfopristin (pristinamycin II^A) (D).

Images adapted from:

http://img.freebase.com/api/trans/image_thumb/wikipedia/images/commons_id/938104?maxheight=510&mode=fit&maxwidth=510 (A);

http://upload.wikimedia.org/wikipedia/commons/4/48/Clindamycin.png (B);

http://www.pnas.org/content/100/suppl.2/14555.full.pdf+html (C) (11);

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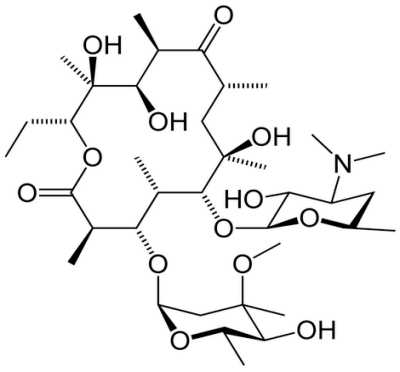
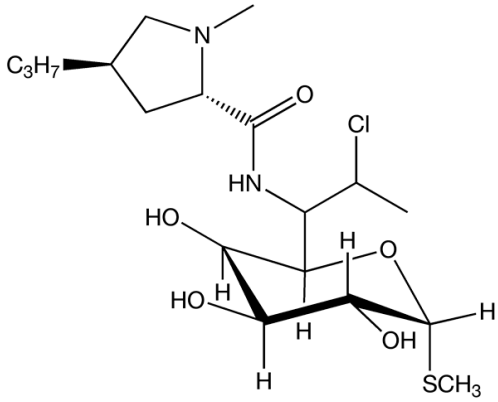
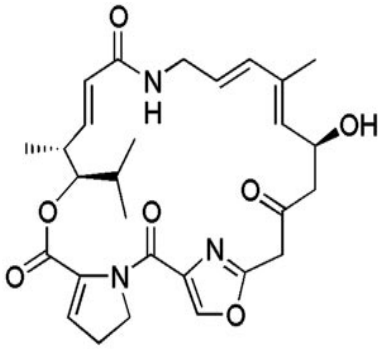


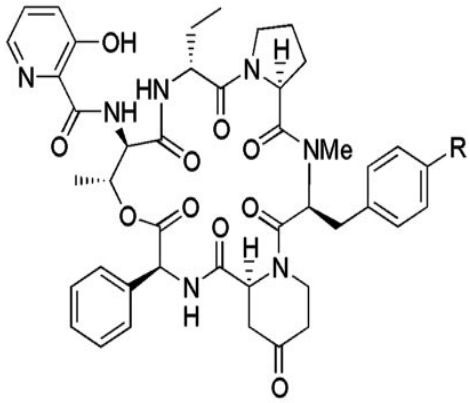
A.



B.



C.



D.

1.3 MLS_B Resistance

1.3.1 Inducible vs. Constitutive Resistance

MLS_B resistance is characterized as resistance to the macrolides, lincosamides, and streptogramin B antimicrobials. Two forms of MLS_B resistance are thought to exist: inducible (MLSi) and constitutive (MLSc) (24, 68). One reason proposed to explain this phenomenon is a mechanism known as translational attenuation of an mRNA leader sequence (67). This attenuation is dependent on the presence or absence of erythromycin. In the absence of erythromycin, the mRNA of the *erm* (erythromycin ribosomal methylase) gene is in an inactive conformation because of a sequestered Shine-Dalgarno sequence. This prevents efficient translation of the *erm* transcripts. Another possible explanation for inducible resistance is also due to the absence of erythromycin. *Bacillus* species possessing *ermK,* another class of *erm* genes which can prevent the complete mRNA sequence from being translated due to rho factor-independent termination (14). Not all strains belonging to the same species of bacteria are capable of being induced; for example, some strains of *Staphylococcus aureus* are capable of possessing inducible resistance to clindamycin, but not all of them, meaning that it is a phenomenon seen on a strain by strain basis (22). Inducible MLS resistance can be observed through the double disk diffusion test, where the presence of an erythromycin antibiotic disk placed in close proximity to a clindamycin antibiotic disk on a suitable agar plate swabbed with a lawn of bacteria will confer resistance to clindamycin. This is characterized by the classic “D-zone” that forms between the two

antibiotic disks (61). Constitutive MLS resistance confers high-level resistance to all macrolides, lincosamides, and streptogramin B. The D-zone test would result in no blunting of zones of inhibition. Inducible resistance is a phenomenon that is seen in several bacterial species and genera, yet is not exclusive to any one genus or species.

1.3.2 Other Resistance Phenotypes

In addition to the MLS_B phenotype, other variations of the phenotype exist. Resistance to only the macrolides has been reported in certain bacterial species, including Gram-positive pathogens such as *Streptococcus pyogenes*, and is commonly known as M resistance (15, 49). Resistance to only streptogramin A or streptogramin B has also been reported (50, 67). Resistance to the lincosamides and streptogramin A has also been reported in strains of *Streptococcus agalactiae* in New Zealand (50). It is not known presently whether these phenotypes are able to be induced, like the MLS_B phenotype.

Figure 4. Schematic of proposed mechanism of inducible clindamycin resistance. Panel A shows the secondary structure of the *erm* gene in the absence of macrolides. The stem loop structure of the mRNA prevents ribosome binding, which prevents translation of the methylase. Panel B shows the secondary structure after a macrolide-bound ribosome forms a complex with the short peptide and stalls. Other ribosomes that are not bound by macrolides are able to translate the mRNA and form the methylase, protecting other ribosomes from being bound by macrolide molecules. Panel C highlights an example of a mutation where the short peptide and inverted repeat 2 (IR2) are deleted, leading to constitutive methylase translation. Adapted from Woods (102).

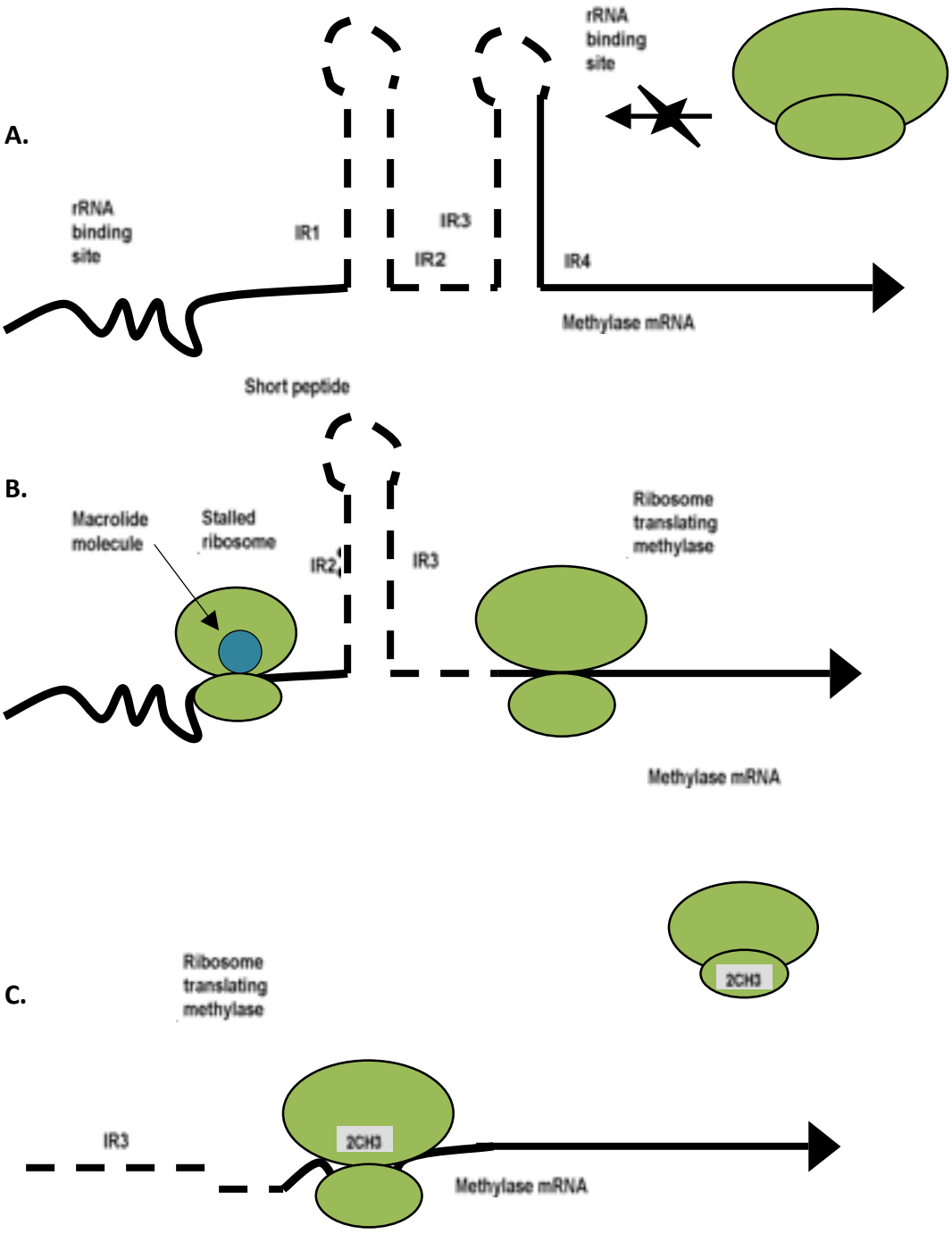
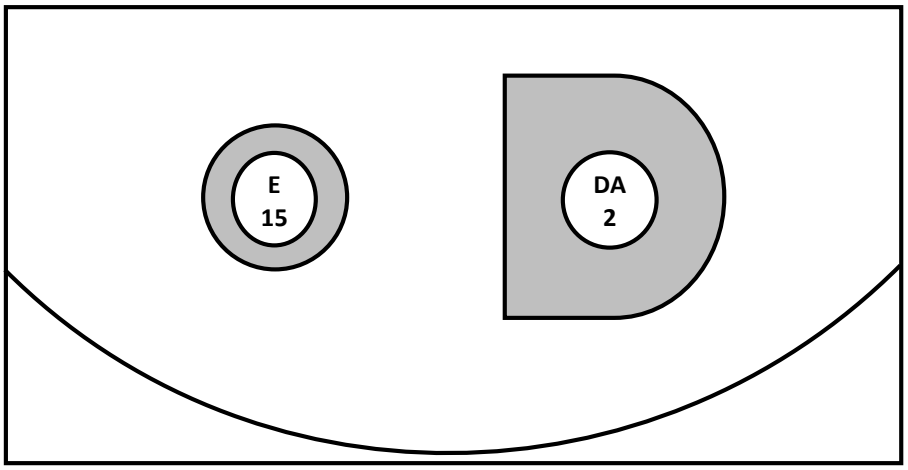

A.
rRNA binding site
IR1
IR3
IR2
IR4
rRNA binding site
Methylase mRNA
Short peptide
B.
Macrolide molecule
Stalled ribosome
IR2
IR3
Ribosome translating methylase
Methylase mRNA
C.
Ribosome translating methylase
2CH3
IR3
2CH3
Methylase mRNA

Figure 5. Cartoon schematic of classic “D-Zone” formation due to inducible clindamycin resistance in the presence of erythromycin. The erythromycin antimicrobial disk is on the left, while the clindamycin disk is on the right. Adapted from: Woods (102).



1.4 Macrolide-Lincosamide-Streptogramin B Resistance Mechanisms

1.4.1 Methyltransferases

As is the case with each new antimicrobial introduced, resistance development soon occurs after the drug is made available. Several resistance genes have been identified that confer resistance to one or all three of these mechanism-related drug classes. The first mechanism to be identified was due to the post-transcriptional modification of the 23S rRNA caused by an N-6-methyltransferase that transfers one or two methyl groups to a specific adenine residue located within the peptidyltransferase region of the 23S rRNA molecule (96, 98). The function of methylation has classified this group of genes as the *erm,* or erythromycin ribosomal methylase genes. There are presently at least 33 *erm* genes validly published in both Gram-positive and Gram-negative genera (65). The function of this enzyme is well-conserved; the methylation of the adenine residue (corresponding to A2058 of the 23S rRNA sequence in *E. coli*) is sufficient to alter the MLS_B binding site (93). The methylation of this nucleotide renders the macrolide binding site ineffective, and in turn alters the binding sites of the lincosamide and streptogramin B antimicrobials, resulting in cross-resistance. Methylation does not appear to have an effect on the host other than altering the resistance phenotype. This form of resistance is usually associated with a transposon, but can be plasmid-associated as well (82, 87, 96).

1.4.2 Efflux

A second mechanism of resistance to the MLS_B antimicrobials is by efflux pump, where bacteria utilize energy driven pumps to remove the drug from their cytoplasm. These efflux proteins do not alter the drug, but rather attempt to prevent the binding of it to the ribosome (67). The most common efflux system known for macrolide resistance is the *mef* (macrolide efflux) family of transporter proteins, which are homologous to the major facilitator superfamily (MFS) of efflux proteins. Their energy is derived from the hydrolysis of ATP. There are additional efflux proteins that share homology with the ABC transporter superfamily. The most common macrolide efflux system is encoded by the gene *mefA*, which is present in a variety of Gram-positive genera, including *Enterococcus, Micrococcus, Staphylococcus,* and *Corynebacterium* (49). These pumps do not confer cross-resistance to other drugs such as the lincosamides or streptogramin B, but only confer low-level resistance to the macrolide antibiotics. An efflux pump encoded by the *lsaA* and *lsaB* genes facilitates lincosamide efflux, but no other drug class. *Streptomyces lincolnensis*, the producer of lincomycin also possesses a lincosamide efflux pump encoded by the gene *lmrA* (24, 67).

1.4.3 Drug Modification

A third mechanism is drug modification or inactivation. *ereA* and *ereB* genes both encode an esterase, an enzyme capable of hydrolyzing the macrolide lactone ring (67). This esterification of the antimicrobial renders it ineffective. This is a common mechanism utilized among the *Enterobacteriaceae*, including the genera *Enterobacter,*

Escherichia, Klebsiella, and *Proteus*, and is not seen in Gram-positive genera (1, 54). Phosphorylases also targeting the macrolide antimicrobials exist in some Enterobacteriaceae, known as the *mphA* and *mphB* genes (97). Macrolide glycosylation also occurs in *Streptomyces lividans* via the *mgt* gene. These genes are limited to function only on erythromycin, and not the 16-membered macrolide lactone rings. Nucleotidyltransferase or *lnu* genes confer resistance to lincomycin and clindamycin by transferring an adenine residue to the position three hydroxyl group on lincomycin and clindamycin, altering its conformation, making it unable to bind to the appropriate site within the ribosome (24, 67).

1.4.4 Other Resistance Mechanisms

Another form of MLS_B resistance is an intrinsic mutation specific to the bacterial strain. It involves the mutation of the adenine residue within the 23S rRNA that is methylated by the *erm* gene to a different nucleotide. Reports of this type of resistance were first seen in isolates of *Mycobacterium intracellulare* 23S rRNA and was noted by the conversion of the adenine residue to cytosine, guanine or uracil (54). Mutations at the adenine residue preceding A2058 or the guanine residue following A2058 (A2057G; G2059A, respectively) were seen in a group of *Propionibacterium* spp. isolates (69). Another form of macrolide resistance is due to ribosomal mutations within the 50S subunit. This phenomenon has been seen in *Streptococcus pneumoniae* isolates, where the ribosomal proteins L4 and L22 incur a mutation that renders the macrolide binding site ineffective (101). A similar phenomenon was also seen in *Turicella otitidis* isolates

(6). Mutations within bacterial 23S rRNA alleles are also seen with mutations in the L4 and L22 proteins.

There are two gene classes responsible for conferring resistance to the streptogramins; the *vgb* (virginiamycin factor B hydrolase) gene hydrolyzes streptogramin B, rendering the drug ineffective. Genes conferring resistance to streptogramin A are known as *vat*, or virginiamycin factor A acetylation genes, and function by adding an acetyl group to the streptogramin A molecule (67). The *vat* genes are usually plasmid borne.

1.5 MLS_B Resistance and *Corynebacterium*

1.5.1 Discovery of *ermX*

Macrolide and lincosamide resistance in *Corynebacterium* species was first reported in Canada in the early 1970's in a strain of *C. diphtheriae* isolated from Canada's North, yet no insight into the mechanism for this resistance phenotype was provided at that time (41). Several years later it was determined that MLS_B resistance in *C. diphtheriae* was plasmid-mediated, and the plasmid was later designated pNG2 (72, 73). It was noted at that time that resistance to the MLS_B antimicrobials could be spontaneously lost, making the bacterial strain susceptible again. This was attributed to a recombination event within the bacterium resulting in the excision of the resistance gene. The discovery of a proposed mechanism, however, explaining resistance was not determined for more than a decade. The discovery of a methyltransferase within *Corynebacterium* species was first noted in 1990 in a strain of *C. diphtheriae*, and was

termed *ermCd* (66). One particular study took place in the early 1980s, focusing on *Corynebacterium* strains possessing R, or resistance plasmids (46). This one particular strain of what was identified at that time as *C. xerosis* came to be known as M82B, and its resistance plasmid was termed pTP10 (46). At this time further work was not done to genetically determine what genes were responsible for conferring resistance to these strains. In the mid-1990s, this strain was the subject of several publications, and had its resistance plasmid sequenced (84, 85). From that study, it was determined that erythromycin and clindamycin resistance was caused by an erythromycin resistance gene termed as *ermCx*. The gene *ermCx* was part of a larger transposon that came to be known as *Tn*5432 which was flanked by two identical insertion sequences known now as *IS*1249 (84). At this time it was also determined that strain M82B was in fact a strain of *C. striatum*, not *C. xerosis.* This gene was nearly identical to the one discovered in *C. diphtheriae* years before. In 1999, a universal naming scheme was introduced for all MLS_B resistance determinants discovered thus far, and the methyltransferase was subsequently renamed *ermX*, for its initial discovery in what was thought to be a strain of *C. xerosis* (67). Since initially being published in *C. diphtheriae*, *ermX* is also presently published as being detected in *C. coyleae, C. jeikeium, C. amycolatum, C. striatum,* and *C. urealyticum* (27, 60, 68, 87, 103). Researchers have also indicated that a similar gene also designated *ermX* is present in bacterial genera such as *Propionibacterium* and *Bifidobacterium*, yet the majority of species within these genera are anaerobic, and genetically are otherwise distantly related to corynebacteria (69, 91). There is also one report indicating that *ermX* has been found in *Arcanobacterium pyogenes*, a genus

within the family *Actinomycetaceae* which is another family within the order *Actinomycetales*, like the *Corynebacteriaceae* (43).

1.5.2 Other Genes

As previously mentioned, the *mefA* gene has been reported in *Corynebacterium* isolates, including *C. jeikeium* and CDC Coryneform Group G-2, now known as *C. tuberculostearicum* (49). There are presently no published reports of *ere*, *lnu*, or other MLS_B resistance genes being found in *Corynebacterium* species. There is also no published evidence presently of streptogramin resistance genes *vat* or *vgb* being present in *Corynebacterium* species as well.

1.6 Purpose

There are four hypotheses being tested in this study: 1) high-level macrolide and lincosamide resistance in *Corynebacterium* spp. in Canada is caused by the acquisition of *ermX*; 2) to confirm that the distribution of the *ermX* resistance determinant among *Corynebacterium* species is more widespread than previously published; 3) determine the genetic location of the *ermX* resistance determinant and possibly establish linkages between other resistance determinants, such as tetracycline resistance genes; and 4) confirm that multidrug resistance has spread to Canadian isolates of *C. afermentans* and *C. aurimucosum*. Successfully sequenced *ermX* gene products were compared to each other to determine if they were genetically identical. Isolates of *C. afermentans* and *C. aurimucosum* were subjected to susceptibility testing to confirm multidrug resistance.

This work was presented in part at the 48th Interscience Conference on Antimicrobial Agents and Chemotherapy (ICAAC) / Infectious Diseases Society of America 46th Annual Meeting, Washington, D.C., October 25-28, 2008.

2.0 MATERIALS AND METHODS

2.1 Bacterial Strains and Culturing Conditions

Clinical strains submitted to the National Microbiology Laboratory (NML), Winnipeg, Manitoba, Canada (formerly known as the Laboratory Centre for Disease Control (LCDC), Ottawa, Ontario, Canada) for species identification between 1973 and 2009 (n=171) were studied. Both LCDC and NML staff had/have interest in characterizing these rare pathogens for several decades, and so the group of isolates studied here probably represents one of the best collections of non-diphtherial *Corynebacterium* strains in the world. Prior to the start of this study, a number of historical *Corynebacterium* strains (from the 1970s to the late 1990s) in the NML collection had biochemical and cellular fatty acid composition (CFA) analyses done. This was because genetic identification of bacteria was not being done routinely in those eras, nor did international guidelines for performing, then interpreting antimicrobial susceptibility testing results exist prior to 2006 (16). Therefore, genetic assays on older strains as described below were first done to definitively identify the isolate to the species level, followed by antimicrobial susceptibility testing. More contemporary isolates would otherwise have had such testing done by NML staff. Seven strains studied were previously received and characterized as part of a collaboration with L. Martinez-Martinez *et al* (NML #'s 93-0679, 93-0681, 93-0686, 93-0691, 93-0696, 93-0708, 93-0717) (52), while another seven strains studied were previously published as part of a collaboration with G. Funke *et al* (NML #'s 95-0266, 95-0267, 95-0269, 95-0270, 95-

0271, 95-0280, 95-0281) (34). Eight strains were received as a gift from Drs. George Zhanel and James Karlowsky from the Health Sciences Centre (HSC), Winnipeg, Manitoba, Canada (NML #'s 090337, 090338, 090339, 090340, 090341, 090342, 090343, 090344), and six strains were received as a gift from Dr. Michelle Alfa at the Clinical Microbiology department at St. Boniface General Hospital, Winnipeg, Manitoba, Canada (NML #'s 090384, 090385, 090386, 090387, 090388, 090389). Twelve strains were previously reported as part of another study highlighting the presence of infections caused by newly described or rare *Corynebacterium* pathogens in Canada in 2002, including NML #'s 89-0572, 89-0826, 91-0032, 91-0077, 92-0042, 92-0043, 92-0360, 97-0160, 98-0116, 99-0145, 00-0064, and 88-0199, which is the type strain of *Corynebacterium afermentans* subsp. *afermentans* (CIP 103499^T) (3, 63). Species represented included *C. accolens* (n=2); *C. afermentans* (n=5); *C. amycolatum* (n=40); *C. appendicis* (n=1); *C. argentoratense* (n=1); *C. aurimucosum* (n=8); *C. confusum* (n=2); *C. coyleae* (n=3); *C. diphtheriae* (n=4); *C. durum* (n=3); *C. freneyi* (n=3); *C. imitans* (n=1); *C. jeikeium* (n=19); *C. macginleyi* (n=1); *C. matruchotii* (n=1); *C. pseudodiphtheriticum* (n=1); *C. propinquum* (n=1); *C. pyruviciproducens* (n=4); *C. resistens* (n=2); *C. simulans* (n=2); *C. stationis* (n=2); *C. striatum* (n=12); *C. tuberculostearicum* (n=10); *C. tuscaniense* (n=2); *C. ulcerans* (n=15); *C. urealyticum* (n=3); *C. ureicelerivorans* (n=2); *C. xerosis* (n=2); and 19 strains unidentifiable to the species level based on 16S rDNA and *rpoB* sequencing (NML #'s 92-0371, 92-0415, 93-0481, 93-0607, 93-0612, 94-0380, 96-0244, 97-0160, 97-0186, 98-0058, 98-0116, 99-0018, 99-0020, 00-0156, 03-0027, 080024, 090322, 090341, ATCC 43833). The NML collection contains many more *C. diphtheriae*

strains than the four shown here (n=~140). These isolates were chosen for this study because they displayed reduced susceptibility to certain antimicrobials. The remaining *C. diphtheriae* isolates either displayed full susceptibility to all antimicrobials tested, or displayed an intermediate MIC to the cephalosporins, a phenomenon that has been described previously (32). Two *Corynebacterium aurimucosum* strains used in this study were previously known as "*Corynebacterium nigricans*," as they uniquely produced a black pigment. These two strains (NML #'s 91-0032 and 92-0360) were the subject of two publications in the early 2000s (3, 76), and were reclassified in 2004 as strains of *Corynebacterium aurimucosum* (20). The four *C. pyruviciproducens* strains (NML #'s 94-0264, 95-0358, 96-0085, 00-0179) and two *C. stationis* strains studied (NML #'s 94-0424 and 03-0173) are currently in press as novel species (5, 89). A complete list of clinical strains is shown in Table 1. Reference strains acquired from recognized international culture collections included *C. afermentans* CIP 103499^T, *C. ammoniagenes* ATCC 6871^T, "*C. ammoniagenes*" ATCC 6872, *C. amycolatum* ATCC 49368^T, *C. appendicis* CCUG 48298^T, *C. confusum* CCUG 38267^T, *C. jeikeium* ATCC 43734^T, *C. jeikeium* ATCC 43217, *C. resistens* CCUG 50093^T, *C. stationis* ATCC 14403^T, *C. tuscaniense* CCUG 51321^T, *C. urealyticum* CCUG 18158^T, *C. urealyticum* ATCC 43043, *C. ureicelerivorans* CCUG 53377^T, and *C. xerosis* ATCC 373^T (n=15) (American Type Culture Collection, Virginia, USA; Culture Collection, University of Göteborg, Sweden; Collection de l'Institut Pasteur, Paris, France). *C. afermentans* CIP 103499^T, as previously mentioned was provided by the NML to the CIP. *C. ammoniagenes* ATCC 6872 is now reclassified as *C. stationis* ATCC 6872 (5). A list of reference strains used as part of this study is shown in Table 2. All

strains were grown on Columbia Blood Agar plates (CBAB) and incubated for 24-48 hours in 5% CO_2.

Table 1: Complete list of clinical strains used in this project, including NML #, biological source (if known), province or country of origin, and final identification after 16S rDNA and *rpoB* sequencing (where possible).

a: “Closest to” refers to closest match within GenBank database to a known, published type strain of a particular species still falling beneath the required threshold to confirm it as that particular species.

*Distant refers to a 16S percentage match of less than 97%.

NML#	Source	Location	Final Identification
SB-00226	unknown	Ontario	*C. ulcerans*
SB-00303	unknown	Ontario	*C. ulcerans*
SB-00714	unknown	Alberta	*C. ulcerans*
73-0894	skin ulcer	Ontario	*C. striatum*
78-0150	valve	Quebec	*C. tuberculostearicum*
79-0331	CSF	Quebec	*C. tuberculostearicum*
79-0565	blood	Alberta	*C. jeikeium*
79-0882	ankle	British Columbia	*C. amycolatum*
79-0918	peritoneum	Quebec	*C. tuberculostearicum*
79-0969	stump	British Columbia	*C. jeikeium*
79-1040	unknown	Quebec	*C. striatum*
83-0626	unknown - gift	CDC - Atlanta, GA	*C. striatum*
83-0628	unknown - gift	CDC - Atlanta, GA	*C. striatum*
83-0629	unknown - gift	CDC - Atlanta, GA	*C. striatum*
83-0630	unknown - gift	CDC - Atlanta, GA	*C. striatum*
89-0174	chest burn	Alberta	*C. amycolatum*
89-0572	blood	Alberta	*C. imitans*
89-0826	back wound	British Columbia	*C. amycolatum*
91-0032	vulval ulcer	Nova Scotia	*C. aurimucosum*
91-0077	sternal fragments	Alberta	*C. amycolatum*
91-0169	blood	Alberta	*C. amycolatum*
92-0014	peritoneal dialysate bag	Alberta	*C. resistens*
92-0042	right shoulder aspirate	New Brunswick	*C. amycolatum*
92-0043	blood	New Brunswick	*C. amycolatum*
92-0130	leg	British Columbia	*C. tuberculostearicum*
92-0210	blood	New Brunswick	*C. tuberculostearicum*
92-0360	vaginal swab	Ontario	*C. aurimucosum*
92-0371	blood	Manitoba	*C. afermentans*
92-0395	blood	Alberta	*C. amycolatum*
92-0415	mid-stream urine	Ontario	closest to *C. imitans*[a]
92-0507	swab of CAPD exit site	Prince Edward Island	*C. amycolatum*
93-0012	CSF	Manitoba	*C. simulans*
93-0481	catheter	Massachusetts, USA	closest to *C. appendicis*
93-0569	blood	Alberta	*C. freneyi*
93-0607	blood	Alberta	closest to *C. lipophiloflavum*
93-0612	left wrist granuloma	Quebec	closest to *C. diphtheriae* (distant)*
93-0669	stock culture	Manitoba	*C. ulcerans*
93-0679	unknown	Spain	*C. coyleae*
93-0681	unknown	Spain	*C. jeikeium*
93-0686	unknown	Spain	*C. jeikeium*
93-0691	unknown	Spain	*C. jeikeium*
93-0696	unknown	Spain	*C. jeikeium*
93-0708	unknown	Spain	*C. jeikeium*

93-0717	unknown	Spain	*C. jeikeium*
93-0836	pleural fluid	Manitoba	*C. jeikeium*
94-0082	platelet pheresis	Saskatchewan	*C. appendicis*
94-0185	blood	New Brunswick	*C. jeikeium*
94-0264	right synovial fluid	Ontario	*C. pyruviciproducens*
94-0267	peritoneal fluid	Alberta	*C. jeikeium*
94-0278	blood	New Brunswick	*C. amycolatum*
94-0283	catheter skin site	Alberta	*C. amycolatum*
94-0358	triple line exit site	Alberta	*C. coyleae*
94-0380	blood	Manitoba	closest to *C. lipophiloflavum*
94-0398	right leg	British Columbia	*C. striatum*
94-0424	blood culture	Ontario	*C. stationis*
95-0021	bone right tibia	Alberta	*C. amycolatum*
95-0072	blood	Ontario	*C. amycolatum*
95-0266*	unknown	Switzerland	*C. xerosis*
95-0267	unknown	Switzerland	*C. xerosis*
95-0269	unknown	Switzerland	*C. amycolatum*
95-0270	unknown	Switzerland	*C. amycolatum*
95-0271	unknown	Switzerland	*C. amycolatum*
95-0280	unknown	Switzerland	*C. amycolatum*
95-0281	unknown	Switzerland	*C. amycolatum*
95-0358	blood	Ontario	*C. pyruviciproducens*
96-0034	draining foot wound	Alberta	*C. ulcerans*
96-0070	eye	Manitoba	*C. amycolatum*
96-0085	right scapular abscess	Ontario	*C. pyruviciproducens*
96-0103	C-section wound	British Columbia	*C. amycolatum*
96-0127	blood	British Columbia	*C. freneyi*
96-0244	blood	Ontario	closest to *C. simulans* (distant)*
96-0286-1	right lower leg ulcer	British Columbia	*C. ulcerans*
96-0286-2	right lower leg ulcer	British Columbia	*C. ulcerans*
97-0056	urine	Manitoba	*C. amycolatum*
97-0108	urine	Manitoba	*C. freneyi*
97-0143	blood	Newfoundland	*C. amycolatum*
97-0160	blood	Ontario	closest to *C. mucifaciens*
97-0186	blood	Ontario	closest to *C. mastitidis*
98-0058	blood	Ontario	closest to *C. lipophiloflavum*
98-0116	urine	Manitoba	closest to *C. ureicelerivorans*
98-0158	blood	Manitoba	*C. amycolatum*
99-0018	blood	Ontario	closest to *C. ammoniagenes*
99-0020	unknown	Ontario	closest to *C. imitans*
99-0021	blood	Manitoba	*C. amycolatum*
99-0071	bone	Manitoba	*C. amycolatum*
99-0145	breast abscess	Alberta	*C. confusum*
99-0162	sternum	Saskatchewan	*C. amycolatum*
99-0163	abdomen	Saskatchewan	*C. amycolatum*
99-0206	blood	Saskatchewan	*C. amycolatum*

99-0221	urine	Newfoundland	*C. urealyticum*
00-0042	urine	Manitoba	*C. urealyticum*
00-0105	blood	Manitoba	*C. aurimucosum*
00-0156	blood	Manitoba	closest to *C. imitans*
00-0163	blood	Manitoba	*C. afermentans*
00-0179	C-section incision fluid	Alberta	*C. pyruviciproducens*
00-0200	unknown	Alberta	*C. urealyticum*
00-0212	blood	Manitoba	*C. amycolatum*
00-0219	dialysis catheter tip	Saskatchewan	*C. amycolatum*
00-0237	jugular	Saskatchewan	*C. amycolatum*
01-0014	CSF	Manitoba	*C. aurimucosum*
01-0024	blood	Newfoundland	*C. afermentans*
01-0043	heel wound	Saskatchewan	*C. tuberculostearicum*
01-0048	blood	Alberta	*C. aurimucosum*
01-0064	blood	Saskatchewan	*C. amycolatum*
01-0105	eye	British Columbia	*C. ureicelerivorans*
01-0116	bypass incision site	Saskatchewan	*C. amycolatum*
01-0123	dialysis outflow	Ontario	*C. amycolatum*
02-0040	ruptured appendix	Manitoba	*C. afermentans*
02-0113	foot	British Columbia	*C. ulcerans*
02-0114	foot	British Columbia	*C. ulcerans*
02-0157	sputum	British Columbia	*C. ulcerans*
02-0162	blood	Saskatchewan	*C. ureicelerivorans*
02-0187	blood	Ontario	*C. argentoratense*
02-0190	blood	Manitoba	*C. tuberculostearicum*
02-0197	deep wound infection	New Brunswick	*C. striatum*
02-0204	CSF	Manitoba	*C. coyleae*
03-0027	blood	Ontario	closest to *C. afermentans*
03-0030	blood	British Columbia	*C. tuberculostearicum*
03-0033	nasal sinus	British Columbia	*C. diphtheriae*
03-0059	left ear	Saskatchewan	*C. amycolatum*
03-0173	blood	Ontario	*C. stationis*
03-0192	blood	Saskatchewan	*C. aurimucosum*
03-0193	blood	Saskatchewan	*C. tuscaniense*
03-0200	tissue	Manitoba	*C. accolens*
04-0018	blood	Saskatchewan	*C. pseudodiphtheriticum*
04-0095	blood	Manitoba	*C. confusum*
04-0185	blood	Ontario	*C. afermentans*
04-0264	blood	Quebec	*C. ulcerans*
04-0265	unknown	Newfoundland	*C. jeikeium*
05-0021	CAPD fluid	Manitoba	*C. amycolatum*
05-0356	blood	Manitoba	*C. accolens*
05-0368	peritoneal fluid	Manitoba	*C. jeikeium*
060127	blood	Saskatchewan	*C. aurimucosum*
060128	ear	Saskatchewan	*C. diphtheriae*
060357	blood	Saskatchewan	*C. jeikeium*

060367	atria & pacemaker	Manitoba	*C. striatum*
060661	bronchial aspiration	British Columbia	*C. durum*
060816	sputum	Alberta	*C. matruchotii*
070133	aspirate from hip	Saskatchewan	*C. tuberculostearicum*
070281	fluid culture	Alberta	*C. amycolatum*
070432	sputum	Manitoba	*C. durum*
070888	leg	British Columbia	*C. ulcerans*
070989	leg ulcer	British Columbia	*C. ulcerans*
070991	ankle wound	British Columbia	*C. ulcerans*
080024	blood	Manitoba	closest to *C. tuscaniense*
080188	unknown	London - UK	*C. ulcerans*
080212	eye	Manitoba	*C. macginleyi*
080668	left shoulder incision	British Columbia	*C. simulans*
080682	sputum	Saskatchewan	*C. diphtheriae*
080683	blood	Saskatchewan	*C. tuscaniense*
090066	left toe	British Columbia	*C. diphtheriae*
090132	left hip wound	British Columbia	*C. aurimucosum*
090182	leg wound	British Columbia	*C. resistens*
090322	blood	British Columbia	*C. canis*
090337	blood	Nova Scotia	*C. amycolatum*
090338	blood	Ontario	*C. jeikeium*
090339	blood	Nova Scotia	*C. afermentans*
090340	leg wound	British Columbia	*C. amycolatum*
090341	blood	Nova Scotia	closest to *C. lipophiloflavum*
090342	lower respiratory tract	Quebec	*C. striatum*
090343	lower respiratory tract	Quebec	*C. propinquum*
090344	blood	Manitoba	*C. jeikeium*
090358	abscess	British Columbia	*C. tuberculostearicum*
090379	bronchial wash	New Brunswick	*C. durum*
090384	Abdominal wall tissue	Manitoba	*C. amycolatum*
090385	blood	Manitoba	*C. jeikeium*
090386	CAPD fluid	Manitoba	*C. jeikeium*
090387	Osteomyelitis - left. foot	Manitoba	*C. striatum*
090388	Coccyx bone fragments	Manitoba	*C. striatum*
090389	right synovial fluid	Manitoba	*C. jeikeium*

Table 2. Complete list of reference strains used in this study, including culture collection accession number, source and final identification. Note: publication in press for ATCC 14403^T and ATCC 6872 (5). ATCC: American Type Culture Collection; CCUG: Culture Collection, University of Göteborg, Sweden; CIP: Collection de l'Institut Pasteur, Paris, France.

Collection Number	**Source**	**Identification**
ATCC 43734^T	blood culture	*C. jeikeium*
ATCC 43217	blood	*C. jeikeium*
ATCC 43042^T	bladder stone	*C. urealyticum*
ATCC 43043	urine	*C. urealyticum*
ATCC 14403^T	sea water	*C. stationis*
ATCC 49368^T	human skin	*C. amycolatum*
ATCC 373^T	ear discharge	*C. xerosis*
ATCC 6871^T	feces	*C. ammoniagenes*
ATCC 6872	feces	*C. stationis*
CCUG 50093^T	blood	*C. resistens*
CCUG 48298^T	abdominal abscess	*C. appendicis*
CCUG 38267^T	plantar abscess	*C. confusum*
CCUG 53377^T	blood	*C. ureicelerivorans*
CCUG 51321^T	blood	*C. tuscaniense*
CIP 103499^T	blood	*C. afermentans*

2.2 Species Identification

Species identification was done using a polyphasic approach utilizing metabolic and biochemical reactions, including colony morphology, Gram stain studies, biochemical tests done by conventional tube and various API strips, including the API Coryne, API ZYM, and API CH50 (Biomérieux, as described by the manufacturer) all have been described previously (3). Cellular fatty acid (CFA) analysis was performed to determine the types of long chain fatty acids present within the cellular membrane, as each *Corynebacterium* species possesses a certain cellular fatty acid profile, making it a useful tool in genus level and sometimes species level identification (2). CFA analysis is also useful to detect the presence of 10-methyloctadecanoic acid, or tuberculostearic acid (TBSA), a feature present in many *Corynebacterium* cell walls. CFA extractions were run on a gas chromatograph (Agilent). Results were analyzed using the MIDI operating system with Sherlock v. 4.5 (MIDI Inc., Newark, DE). The detection of the end products of glucose fermentation was also determined (4). Lactic acid, succinic acid and acetic acid are frequently seen as end products in a variety of Gram-positive pathogens; however propionic acid is frequently isolated as a fermentation end product of *Corynebacterium* species, particularly *C. amycolatum* (3). Amplification and sequencing of full 16S rDNA (approximately 1400-1500 bp) (4), and a partial fragment of the beta subunit of the RNA polymerase (*rpoB*, approximately 390-450 bp) were done when required (44). Strains were matched to the closest species utilizing a threshold cut-off percentage of greater than or equal to 98.7% for 16S PCR products (80), and greater than or equal to 95% for *rpoB* PCR products (44). Strains failing to meet the required

threshold marking were subsequently identified as *Corynebacterium* spp. The obtained sequences were compared against known reference sequences in the on-line database GenBank using the Basic Local Alignment Search Tool (BLAST). Rough phylogenetic trees were also generated to observe the relationship between *Corynebacterium* species already published and clinical strains undergoing characterization. This was accomplished using the Clustal W algorithm within the MegAlign program, version 7.2.1 (DNASTAR Lasergene 7).

2.3 Antimicrobial Susceptibility Testing

2.3.1 Microbroth Dilution using Sensititre® plates

Isolates were tested for antimicrobial susceptibilities using microbroth dilution according to Clinical Laboratory and Standards Institute (CLSI) methods using cation-adjusted Mueller-Hinton broth with 2.5% lysed horse blood, and Sensititre® GPN3F, STP3F and STP5F plates (Trek Diagnostics, distributed by Somagen, Edmonton, AB). Antimicrobials and the range tested (upon reconstitution with 50 or 100 µL broth, µg/mL) were as follows: ampicillin (0.12-16); cefepime (0.5-8); cefotaxime (0.12-4); ceftriaxone (0.5-2, 8-64); cefuroxime (0.5-2); chloramphenicol (1-32); ciprofloxacin (0.5-2); clindamycin (0.12-2); daptomycin (0.06-8); ertapenem (0.5-4); erythromycin (0.25-4); gatifloxacin (1-8); gentamicin (2-16, 500); levofloxacin (0.25-16); linezolid (0.25-8); meropenem (0.25-2); moxifloxacin (1-8); penicillin (0.03-8); quinupristin/dalfopristin (0.12-4); rifampin (0.5-4); streptomycin (1000); telithromycin (0.5-4); tetracycline (1-16); tigecycline (0.015-0.5); trimethoprim/sulfamethoxazole (0.5/9.5-4/76); and vancomycin (0.5-128). Plates were inoculated manually using a multichannel pipette, or automatically using the Sensititre® Automatic Inoculator (Autoinoculator). CLSI M45-A Table 5: *Corynebacterium* species (including *C. diphtheriae*) was used for interpretive criteria for the 16 antimicrobials represented in the table (16). Antimicrobials not represented in this table, including ampicillin and the newer fluoroquinolones, were not evaluated in terms of 'susceptible' and 'resistant.' MIC values were recorded for each strain and were compared to related antimicrobials represented in the M45-A

document (penicillin and ciprofloxacin). According to the CLSI, antimicrobial agents that should be considered for primary testing include erythromycin, gentamicin, penicillin and vancomycin, as these antimicrobials are frequently used in the treatment of suspected and/or confirmed *Corynebacterium* infections. The document was derived from the most current set of previously established breakpoints for *Enterococcus*, *Staphylococcus* and *Streptococcus* species. These organisms were chosen based on similarities between MIC distributions and types of infections caused by the organisms. Where possible, MIC_{50} and MIC_{90} values were determined for species whose total n value was greater than 10.

2.3.2 Macrobroth / Microbroth Dilution: Custom Set-up

2.3.2.1 Procedure

Extended concentration ranges of erythromycin and clindamycin were also tested to determine true MIC values using both macrobroth and microbroth dilution. In order to do this, antibiotics in powder form were obtained from Sigma-Aldrich Canada (Oakville, Ontario). Stock concentrations of erythromycin and clindamycin were prepared according to CLSI methods (17). Briefly, enough antimicrobial powder was dissolved in the minimum amount of solvent required to make a homogenous suspension (glacial acetic acid or 95% ethyl alcohol for erythromycin, water for clindamycin). The appropriate diluent (water for both antimicrobials) was then added to each suspension to reach a final concentration of 5,000 µg/ml for erythromycin and 1,024 µg/ml for clindamycin for macrobroth dilution experiments, or 8,192 µg/ml for

erythromycin, and 2,048 µg/ml for clindamycin for microbroth dilution tests. The antimicrobial solutions were filtered through a 0.2 µm syringe filter containing a cellulose acetate membrane (VWR International, Mississauga, Ontario) prior to being divided into 400 µl aliquots.

For macrobroth dilution, a starting concentration of 64 µg/ml was used for each antimicrobial. One millilitre (ml) of broth (cation-adjusted Mueller-Hinton broth; caMHB) was added to each 13mm test tube used in the experiment. One ml of the antimicrobial-containing broth was then added to the starting and second test tubes, giving a final starting concentration (within the tube) of 32 µg/ml. A 1:2 series dilution was then performed beginning with the second test tube to give an ending concentration of 0.03 µg/ml in the final tube. Approximately 2 ml of sterile saline (0.85%) was then inoculated with either the control or test organism to a 0.5 McFarland standard. One hundred µl was then added to approximately 15 ml of caMHB (an approximate 1:150 dilution). One ml of inoculum was added to each tube, for a final volume of 3 ml. One hundred µl of inoculum was then added to 900 µl of sterile saline (10^{-1}), and was diluted using a 1:10 series dilution to 10^{-3}. Ten µl of each dilution was then plated onto CBAB to determine final colony counts. A final colony count between $1x10^5$ and $1x10^6$ was considered appropriate.

For microbroth dilution, a starting concentration of 1,024 µg/ml was used for each antimicrobial. This was achieved by inoculating a 500 µl aliquot of cation-adjusted Mueller-Hinton broth with lysed horse blood (MHBwLHB: Somagen, Edmonton, Alberta, Canada) with 62.5 µl of erythromycin stock solution, or 1 ml of clindamycin stock

solution. Fifty microlitres of broth was added to each well of a 96-well microplate, except for the wells containing the highest concentration of antimicrobial. Three samples could be tested on each plate. Fifty microlitres of antimicrobial-containing broth was then added to the uninoculated wells and the well with the second highest concentration. A 1:2 series dilution was then carried out beginning with the well containing the second highest concentration so that the final well contained a concentration of 0.015 µg/ml. A diagram of plate set-up is shown in Figure 5. As was done for macrobroth dilution, 2 ml of sterile saline (0.85%) was inoculated with the control or test organism to a 0.5 McFarland standard. Fifty µl was then added to approximately 7 ml of MHBwLHB (approximate 1:150 dilution). Fifty µl of inoculum was added to each well of the microplate, for a final volume of 100 µl. A similar 1:10 series dilution followed by plating was then carried out to determine final colony counts.

2.3.2.2 Strain Selection

A total of 21 strains were chosen for extended MIC range experiments. Four reference strains (*C. jeikeium* ATCC 43734^T, *C. resistens* CCUG 50093^T, *C. urealyticum* CCUG 18158^T and *C. ureicelerivorans* CCUG 53377^T), and 17 clinical strains were tested. These strains were chosen to represent a random sampling of the wide variety of species tested in this study, particularly highlighting those strains that are frequently isolated clinically. A complete list of strains used, MIC data and interpretation results is shown in Table 7.

2.3.3 Double Disk Diffusion

Three *Corynebacterium ulcerans* strains were subjected to a double disk diffusion assay to test for inducible erythromycin resistance. Briefly, an appropriate agar plate (Mueller-Hinton agar with 5% sheep blood) was swabbed to generate a lawn of growth of the test or control organism using a standardized inoculum of approximately 1×10^8 cfu/ml, or a 0.5 McFarland standard. Using sterile forceps, a 15 µg erythromycin disk and a 2 µg clindamycin disk were placed within approximately 20 mm of each other (31). These plates were incubated for a total of 48 hours, as after 24 hours of incubation the amount of growth was insufficient to determine accurate inhibition zone diameters. Following 48 hours of incubation, bacterial growth was sufficient to visualize the zones of inhibition. The method for this particular procedure is standardized for *Staphylococcus* species, both *S. aureus* and coagulase-negative staphylococci (CoNS). This method of testing has not been standardized with *Corynebacterium* species, and was done merely for the sake of interest. A similar assay was attempted using E-Test strips (Biomérieux, St-Laurent, Quebec) placed within 20-25 mm of each other and incubated for a total of 48 hours, after which they were observed for blunting of the elliptical zone of inhibition for erythromycin.

Figure 6. Checkerboard diagram for erythromycin and clindamycin microbroth dilution custom set-up. E: erythromycin; C: clindamycin.

	1	2	3	4	5	6	7	8	9	10	11	12
A	E 0.015	E 0.03	E 0.06	E 0.12	E 0.25	E 0.5	E 1.0	E 2.0	E 4.0	E 8.0	E 16.0	E 32.0
B	E 64.0	E 128.0	E 256.0	E 512.0	C 0.015	C 0.03	C 0.06	C 0.12	C 0.25	C 0.5	C 1.0	C 2.0
C	C 4.0	C 8.0	C 16.0	C 32.0	C 64.0	C 128.0	C 256.0	C 512.0	E 0.015	E 0.03	E 0.06	E 0.12
D	E 0.25	E 0.5	E 1.0	E 2.0	E 4.0	E 8.0	E 16.0	E 32.0	E 64.0	E 128.0	E 256.0	E 512.0
E	C 0.015	C 0.03	C 0.06	C 0.12	C 0.25	C 0.5	C 1.0	C 2.0	C 4.0	C 8.0	C 16.0	C 32.0
F	C 64.0	C 128.0	C 256.0	C 512.0	E 0.015	E 0.03	E 0.06	E 0.12	E 0.25	E 0.5	E 1.0	E 2.0
G	E 4.0	E 8.0	E 16.0	E 32.0	E 64.0	E 128.0	E 256.0	E 512.0	C 0.015	C 0.03	C 0.06	C 0.12
H	C 0.25	C 0.5	C 1.0	C 2.0	C 4.0	C 8.0	C 16.0	C 32.0	C 64.0	C 128.0	C 256.0	C 512.0

2.3.4 Quality Control

The Clinical Laboratory Standards Institute recommends the use of quality control bacterial strains when conducting susceptibility experiments. *Streptococcus pneumoniae* ATCC 49619 is recommended for use as a quality control strain when testing fastidious bacteria (16, 17). *Escherichia coli* ATCC 25922 is used in conjunction with *S. pneumoniae* ATCC 49619 when gentamicin is being tested, as this particular strain of *S. pneumoniae* is tolerant of gentamicin. Both strains were acquired from the ATCC for this purpose, and used for quality control when testing one or more plates. For extended MIC range experiments, *Streptococcus pneumoniae* ATCC 49619 was used alone, as gentamicin was not part of the extended MIC range test.

2.4 PCR

2.4.1 Bacterial DNA Extraction

Bacterial cultures were grown for 24-48 hours on CBAB plates containing 5% sheep blood. Bacterial DNA was harvested using a crude boil extraction. Briefly, approximately 10 µL of pure bacterial culture was emulsified in 1 mL of nuclease-free water (Gibco, Burlington, Ontario, Canada) and boiled for 10 minutes. Once cooled, the bacterial lysate was centrifuged in a microcentrifuge at 10 000 x *g* for 10 minutes. The DNA-containing supernatant was then decanted off and stored at -20°C until required. Bacterial DNA extractions were also performed using the Qiagen Viral mRNA Mini Kit utilizing a modified protocol. This kit was used in part because it was in abundant supply

within the laboratory, and contains identical reagents to regular DNA extraction kits. This method was chosen in part because the components and protocol used do not have any steps involving DNA degradation (such as a step involving digestion of DNA with an endonuclease), which resulted in a total nucleic acid preparation upon completion of bacterial lysis. However, since the isolation and purification of RNA was not required in this study, this final product was treated as a DNA preparation.

2.4.2 *ermX*

Presence or absence of the *ermX* gene was determined using conventional PCR specific for an approximately 390 bp fragment, according to the methods of Rosato *et al* (68). The PCR reaction (50 µl/reaction) included 3-5 µL template DNA, 200 µM deoxynucleotide triphosphates (dNTPs) (Roche, Canada), 1X HiFi buffer (Invitrogen, Carlsbad, California, USA), 2 mM $MgSO_4$ (Invitrogen), 1 µM of each primer (forward and reverse), and 0.5-1 U Platinum Taq DNA polymerase (Invitrogen). Nuclease-free water (Gibco) was added to reach a final reaction volume of 50 µl. A complete list of primers used is shown in Table 3. Amplification was carried out using a Biorad Tetrad 2 DNA engine thermal cycler (Mississauga, Ontario, Canada) using the following program: initial denature at 94°C for 5 minutes, followed by 35 cycles of 94°C for 1 minute, 65°C for 1 minute, and 68°C for 2 minutes, followed by a final extension at 68°C for 7 minutes. *Corynebacterium jeikeium* ATCC 43734^T and *Corynebacterium urealyticum* CCUG 18158^T were used as positive controls and *Corynebacterium urealyticum* ATCC 43043 and *Corynebacterium xerosis* ATCC 373^T were used as negative controls. A negative control

containing no template was also included in each experiment to ensure that there was no contamination. An approximately 400 bp fragment of the housekeeping gene *rpoB* was amplified alongside *ermX* as a control for template DNA integrity and PCR inhibition. Attempts were also made to amplify the entire *ermX* gene (approximately 890 bp) utilizing the same protocol as *ermX* fragment amplification. Primers for this assay and expected amplicon size are shown in Table 3.

2.4.3 *Tn*5432

Conventional PCR was also used to determine the presence of the transposon *Tn*5432 within the isolates tested. For *Tn*5432 amplification, the following program, adapted from van Hoek *et al* was used (91): initial denature at 94°C for 5 minutes, followed by 35 cycles of 94°C for 1.5 minutes, 60°C for 1.5 minutes, and 68.0°C for 3 minutes, followed by a final extension phase at 68°C for 7 minutes (91). Primers used for this assay and expected amplicon size are shown in Table 3. *C. jeikeium* ATCC 43734^T and *C. urealyticum* CCUG 18158^T were used as positive control strains, as it has been previously determined that these two strains are known to possess the transposon in question. *C. xerosis* ATCC 373^T was used as the negative control.

2.4.4 Gel Electrophoresis and PCR Product Purification

All PCR products from all assays were subjected to gel electrophoresis at 100 V for 35-40 minutes using 2 g of agarose (Invitrogen, Carlsbad, California, USA) with 100 mL 1X TAE buffer (Tris-Acetate-EDTA) and 2.5 µL of ethidium bromide (10 mg/mL,

Sigma-Aldrich, Canada). Products were visualized using a Biorad GelDoc (UV light), (Mississauga, Ontario, Canada), and sizes were estimated using a 100 bp ladder (Fermentas, Burlington, Ontario, Canada). Correct gene products were confirmed through DNA sequencing after purification using Millipore Microcon Centrifugal PCR filters, according to the manufacturer (Montage, Fisher Scientific, Ottawa, Canada).

2.4.5 DNA Sequencing

Successfully amplified fragments obtained from different *Corynebacterium* species were sequenced in-house at the DNA core facility within the NML. Once completed, these sequences were then analyzed using the computer program SeqMan (DNASTAR Lasergene 7). Good quality sequences were then compared against known reference sequences deposited in the on-line database GenBank utilizing the Basic Local Alignment Search Tool (BLAST). In-house sequences were compared to each other using the MegAlign software (DNASTAR Lasergene 7).

2.4.6 Plasmid Extraction

Plasmid extraction was attempted using the Qiagen Plasmid Mini kit using a protocol modified specifically for plasmid extractions from *Corynebacterium* species. Two *Corynebacterium* strains, NML #'s 090342 (*C. striatum*) and 090389 (*C. jeikeium*) were used as they displayed an antimicrobial resistance phenotype consistent with that of a plasmid previously isolated from *Corynebacterium* strains (85). These isolates

displayed high-level resistance to erythromycin, clindamycin, chloramphenicol, and tetracycline.

2.4.7 Quality Control

In order to ensure that no contamination or cross-reaction took place when carrying out PCR experiments, certain quality control measures were taken. All bacterial extractions took place in a designated 'clean' room, where no external DNA was brought in. All components were added in a biological safety cabinet, and care was taken to reduce the chances of aerosols forming. *rpoB* PCR was set up alongside *ermX* PCR to ensure DNA integrity of the bacterial lysate, and 16S rDNA PCR was set up alongside *Tn*5432 PCR for the same reason. These two particular housekeeping genes were chosen because they are similar in size to the expected products obtained with a successful *ermX* or *Tn*5432 PCR experiment.

Table 3. Complete list of primers, gene targets, fragment sizes and sequence information used in molecular assays.

[a]: Ref: (68)

[b]: Ref: (91)

[c]: Ref: (44)

[d]: W: weak bond (A or T); Y: pyrimidine (C or T); B: any base except A (C,G,T); R: purine (A or G).

Gene Target	Primer	Sequence (5'→ 3')	Size of fragment (bp) (approx.)
ermX (fragment)[a]	Cerm1	GACACGGCCGTCACGAGCAT	390
	Cerm2	GGCGGCGAGCGACTTCC	
ermX (full)[a]	erm3282	TGCCCGGCTCCCTTTCA	890
	erm4176	CTGGTGGATTTCGGTTTTGGTG	
*Tn*5432 (fragment[s])[b]	tnp1249-31F	ATGTCGAAGAACCAACCACG	470
	tnp1249-503R	CCTTGGCCGCCGTCGATG	
	tnp1249-486F	CATCGACGGCGGCCAAGG	720
	tnp1249-1194R	TTGAATGCCGATTGAGTGGG	
*IS*1249[a]	IS1249-1	CCGCTACACCACCACCAACC	450
	IS1249-2	GATCGTCAGGCAGTTCCGTTTTTA	
rpoB (fragment)[c]	C2700F	CGWATGAACATYGGBCAGGT[d]	420
	C3130R	TCCATYTCRCCRAARCGCTG[d]	
16S rDNA	pA	AGAGTTTGATCCTGGCTCAG	1400-1500
	pHr	AAGGAGGTGATCCAGCCGCA	

3.0 RESULTS

3.1 Interpretation of Antimicrobial Susceptibility Testing MIC Data

All antimicrobial susceptibility testing data was recorded on worksheets corresponding to the particular antimicrobials and concentrations within the testing plate. As previously mentioned, a particular range for each antimicrobial was tested. Each strain tested displayed a particular minimum inhibitory concentration, or MIC to each antimicrobial tested. For certain antimicrobials, the MIC was at times below or beyond the scope tested in the plate. Table 4 highlights the MIC_{50} and MIC_{90} values of four *Corynebacterium* species against twelve antimicrobials for which interpretation is possible using CLSI recommended guidelines (16). The MIC_{50} value corresponds to the concentration of antimicrobial required to inhibit 50% of the bacterial strains tested, while the MIC_{90} value is the concentration required to inhibit 90% of the bacterial strains tested (38). Figures 7, 8, and 9 highlight the overall spectrum of MIC values obtained among *Corynebacterium* species for seven antimicrobials: three representing the MLS_B phenotype, and others frequently used in clinical practice, including penicillin, ceftriaxone, tetracycline and trimethoprim/sulfamethoxazole.

3.2 Determination of Multidrug Resistant *Corynebacterium* Strains

A total of one hundred and eighty-six *Corynebacterium* strains were subjected to antimicrobial susceptibility testing via microbroth dilution. One or two panels were tested for each strain. Based on the data generated, a variety of resistance phenotypes

among *Corynebacterium* species exist, ranging from full susceptibility to resistance to more than nine antimicrobial classes. For this study, multidrug resistant strains were defined as those displaying a resistant phenotype to three or more antimicrobial classes. Significant multidrug resistant strains greater to four or more antimicrobial classes are highlighted in Table 6. A total of 75/186 strains corresponded to a multidrug resistance phenotype, or 40.3%.

3.3 Determination of MLS_B Phenotypes

Each strain corresponded to one of four susceptibility profiles determined in this study based on antimicrobial susceptibility data: erythromycin/clindamycin resistant (ERY^R/CLI^R); erythromycin resistant/clindamycin intermediate or sensitive ($ERY^R/CLI^{I/S}$); erythromycin sensitive/clindamycin resistant or intermediate ($ERY^S/CLI^{R/I}$); or erythromycin/clindamycin sensitive (ERY^S/CLI^S). Of the strains tested, 114 of the 186 displayed elevated MICs to both erythromycin and clindamycin, or 61.2%. Strains displaying resistance to the macrolides and lincosamides also showed resistance to the beta-lactams and sulfonamides at rates of 52.6% and 57.9%, respectively. Five strains of 186 showed resistance to erythromycin alone, and an intermediate or susceptible MIC to clindamycin (2.7%). Thirty-six strains displayed the $ERY^S/CLI^{R/I}$ phenotype (19.4%), and 31 strains displayed the ERY^S/CLI^S phenotype (16.7%).

3.4 *ermX* PCR

All *Corynebacterium* strains corresponding to a phenotype displaying resistance to both erythromycin and clindamycin were subjected to *ermX* PCR. Strains belonging to the other phenotypes were also tested; however not all strains belonging to the other susceptible phenotypes were subjected to *ermX* PCR. Of the 114 strains displaying this phenotype, 111, or 97.3% were positive for the *ermX* gene. The remaining three strains were negative for *ermX* after repeated attempts. Strains used as positive controls had their PCR product purified and sent for sequencing for confirmation of the presence of *ermX*. The *ermX* sequences of these two strains, *C. jeikeium* ATCC 43734^T and *C. urealyticum* CCUG 18158^T showed >99% similarity to *ermX* sequences already deposited into GenBank for these particular strains.

Two strains of the five displaying the ERY^R/CLI$^{I/S}$ phenotype tested positive for *ermX*, or 40%. Phenotypically these strains displayed low-level resistance to erythromycin with an MIC of 2 µg/ml and intermediate susceptibility to clindamycin with an MIC of 2 µg/ml. Repeat testing of these isolates confirmed the presence of *ermX.* Of the 36 strains belonging to the ERY^S/CLI$^{R/I}$ phenotype, 0% tested positive for *ermX*. There were a total of 31 strains corresponding to the ERY^S/CLI^S phenotype, however 0% tested positive for *ermX*. A complete list of *Corynebacterium* species belonging to the four main resistance phenotypes isolated are shown in Table 5.

Seventeen clinical strains subjected to *ermX* PCR were sent for sequence analysis to confirm the amplified product was indeed a fragment of *ermX*. These 17 strains represented 11 different species, including *C. afermentans, C. amycolatum, C.*

aurimucosum, C. diphtheriae, C. jeikeium, C. propinquum, C. pyruviciproducens, C. resistens, C. striatum, C. tuberculostearicum, and one strain currently unidentifiable to the species level. Five reference strains were also submitted for partial *ermX* sequencing; including *C. resistens* CCUG 50093^T, *C. jeikeium* ATCC 43217, *C. ureicelerivorans* CCUG 53377^T, *C. jeikeium* ATCC 43734^T and *C. urealyticum* CCUG 18158^T. Purified PCR products submitted for sequencing yielded an approximately 390 bp fragment that corresponded to the first half of the *ermX* coding sequence, once analyzed with the program SeqMan. These sequences were then aligned against known *ermX* reference sequences in GenBank using BLAST.

Sequencing of the full *ermX* gene was also attempted, using the same protocol as to amplify the *ermX* fragment. Amplification was only successful for 15 strains: three reference strains (*C. resistens* CCUG 50093^T, *C. urealyticum* CCUG 18158^T, *C. ureicelerivorans* CCUG 53377^T) and twelve clinical strains, including the species *C. amycolatum, C. aurimucosum, C. diphtheriae, C. jeikeium, C. propinquum, C. pyruviciproducens, C. resistens, C. striatum,* and *C. tuberculostearicum.* The *ermX* sequences of these strains were compared to each other using the program MegAlign. Analysis determined that these sequences, coming from many different species shared ≥99% homology with each other. One base within the approximately 900 bp sequence was a particular point of interest for polymorphism. Table 8 highlights these positive strains and the polymorphism associated with its full-length *ermX* sequence. Other differences in sequence were seen among the strains tested, yet those were inconsistent in location, unlike this one particular polymorphism.

3.5 *Tn*5432 PCR

Four strains positive for *ermX* were further tested for the presence of the transposon *Tn*5432 using a PCR assay modified from two different approaches (68, 91). These strains included *C. jeikeium* ATCC 43734^T, *C. xerosis* ATCC 373^T, *C. resistens* CCUG 50093^T, and *C. appendicis* CCUG 48298^T. These strains were chosen to represent a positive *ermX* control (*C. jeikeium* type strain), a negative *ermX* control that would likely not have the transposon (*C. xerosis* type strain), and two strains that were positive for *ermX*. A variety of primer combinations were used in order to attempt to amplify the entire transposon, including primers covering the areas including the transposase enzyme, known as *IS*1249 as well as the full *ermX* gene and a leader peptide sequence. Additional primers based on the coding sequence of *ermX* and *IS*1249 were also designed using the Primer-BLAST feature on the BLAST website in order to amplify smaller fragments. The transposase enzyme was amplified successfully and sequenced; however, it was not possible to amplify upstream or downstream from the transposase enzyme using the various primer concentrations.

3.6 *Corynebacterium ulcerans* Disk Diffusion Test

Three *C. ulcerans* strains displayed the erythromycin sensitive/clindamycin resistant phenotype. These strains were subjected to a double disk diffusion test where erythromycin and clindamycin-impregnated disks were placed in close proximity on a CBAB plate swabbed with a lawn of the test organism. Blunting of the erythromycin zone of inhibition was observed after 48h of incubation. A similar assay was attempted

using E-Test strips rather than antimicrobial disks; however the results obtained were more difficult to interpret. Attempts were made to determine the presence of the *ermX* gene, with the experiment yielding a PCR product larger in size than expected in certain strains, particularly those displaying true clindamycin resistance. Attempts were made to sequence this product, but were unsuccessful after numerous tries.

3.7 Plasmid Extraction

Attempts were made to determine whether the resistance determinant responsible for MLS_B resistance in *Corynebacterium* species was located on a plasmid or within the bacterial chromosome. The Qiagen Plasmid Mini kit was used, and the protocol modified specifically for the isolation of plasmids from *Corynebacterium* species, particularly *C. glutamicum*. The strains chosen for this assay were as follows: NML #'s 060367 (*C. striatum*), 090387 (*C. striatum*), and 090389 (*C. jeikeium*). These strains were chosen as they displayed a resistance phenotype identical to that of strains possessing a particular resistance plasmid that was previously isolated from *Corynebacterium* spp. Cultures of the strains being tested were grown up in the appropriate culture medium, and all steps were followed as per the required protocol, yet no positive results were obtained. Further study into whether the resistance gene was plasmid-borne was not attempted.

Table 4. Complete list of MIC data for four *Corynebacterium* species against twelve different antimicrobials.

Species/Antimicrobial	**MIC (µg/ml)**		
	Range	MIC_{50}	MIC_{90}
***C. amycolatum* (n=41)**			
Erythromycin	0.25-4	>4	>4
Clindamycin	0.12-2	>2	>2
Quinupristin/Dalfopristin	0.12-4	≤0.12	0.5
Tetracycline	1-16	1	8
Penicillin	0.03-8	8	>8
Ceftriaxone	0.12-2; 8-64	2	>64
Trimethoprim/Sulfamethoxazole	0.5/9.5-4/76	>4/76	>4/76
Rifampin	0.5-4	≤0.5	>4
Gentamicin	2-16; 500	≤2	>16
Linezolid	0.25-8	0.5	0.5
Daptomycin	0.06-8	0.12	0.25
Vancomycin	0.5-128	≤0.5	1
***C. jeikeium* (n=21)**			
Erythromycin	0.25-4	>4	>4
Clindamycin	0.12-2	>2	>2
Quinupristin/Dalfopristin	0.12-4	0.25	1
Tetracycline	1-16	2	>16
Penicillin	0.03-8	>8	>8
Ceftriaxone	0.12-2; 8-64	>64	>64
Trimethoprim/Sulfamethoxazole	0.5/9.5-4/76	>4/76	>4/76
Rifampin	0.5-4	≤0.5	>4
Gentamicin	2-16; 500	>16	>500
Linezolid	0.25-8	1	1
Daptomycin	0.06-8	0.5	1
Vancomycin	0.5-128	≤0.5	1
***C. striatum* (n=12)**			
Erythromycin	0.25-4	≤0.25	>4
Clindamycin	0.12-2	2	>2
Quinupristin/Dalfopristin	0.12-4	0.25	0.5
Tetracycline	1-16	≤1	>16
Penicillin	0.03-8	0.5	8
Ceftriaxone	0.12-2; 8-64	2	32
Trimethoprim/Sulfamethoxazole	0.5/9.5-4/76	≤0.5/9.5	>4/76
Rifampin	0.5-4	≤0.5	>4
Gentamicin	2-16; 500	≤2	4
Linezolid	0.25-8	0.5	0.5
Daptomycin	0.06-8	0.25	0.5
Vancomycin	0.5-128	≤0.5	≤0.5

***C. tuberculostearicum* (n=10)**	Range	MIC_{50}	MIC_{90}
Erythromycin	0.25-4	>4	>4
Clindamycin	0.12-2	>2	>2
Quinupristin/Dalfopristin	0.12-4	0.25	1
Tetracycline	1-16	1	2
Penicillin	0.03-8	0.12	>8
Ceftriaxone	0.12-2; 8-64	>2	>64
Trimethoprim/Sulfamethoxazole	0.5/9.5-4/76	2/38	>4/76
Rifampin	0.5-4	≤0.5	>4
Gentamicin	2-16; 500	≤2	16
Linezolid	0.25-8	1	1
Daptomycin	0.06-8	0.25	0.25
Vancomycin	0.5-128	≤0.5	≤0.5

Table 5. Resistance phenotypes vs. *Corynebacterium* strains tested (n=186).

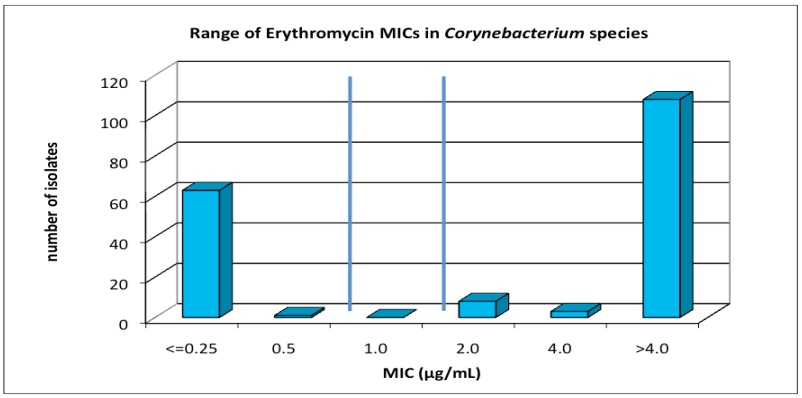
a: ERY^R/CLI^R: erythromycin/clindamycin resistant; $ERY^R/CLI^{I/S}$: erythromycin resistant/clindamycin intermediate or sensitive; $ERY^S/CLI^{R/I}$: erythromycin sensitive/clindamycin resistant or intermediate; ERY^S/CLI^S: erythromycin/clindamycin sensitive.

b: Reference strains corresponding to a certain phenotype are in brackets.

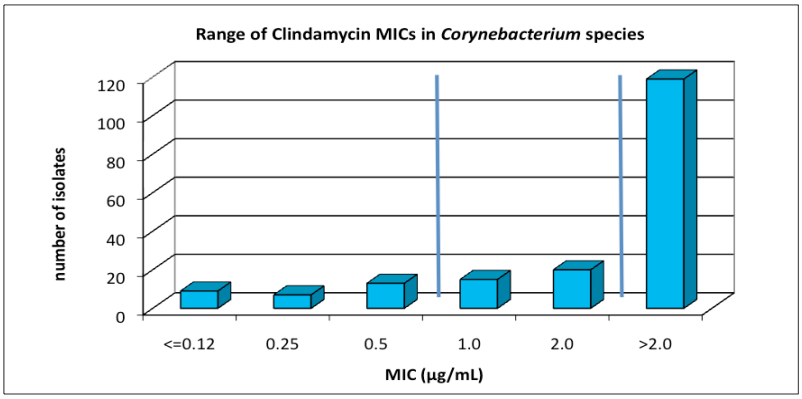
c: Strains unidentifiable to the species level based on 16S rDNA and *rpoB* sequencing.

Phenotype [a]	**# Strains Tested**	***ermX* PCR (% positive)**	**Species Represented** [b]
ERY^R/CLI^R	114	97.4%	*C. afermentans; C. amycolatum; C. appendicis; C. aurimucosum; C. confusum; C. coyleae; C. diphtheriae; C. durum; C. freneyi; C. jeikeium; C. macginleyi; C. propinquum; C. pseudodiphtheriticum; C. resistens; C. striatum; C. tuberculostearicum; C. tuscaniense* (CCUG 51321^T)*; C. urealyticum; C. ureicelerivorans; Corynebacterium* spp. [c]
$ERY^R/CLI^{I/S}$	5	40%	*C. afermentans; C. aurimucosum; C. stationis; C. xerosis*
$ERY^S/CLI^{R/I}$	36	0%	*C. accolens; C. amycolatum; C. aurimucosum; C. imitans; C. jeikeium; C. simulans; C. striatum; C. tuberculostearicum; C. ulcerans; Corynebacterium* spp. [c]
ERY^S/CLI^S	31	0%	*C. amycolatum; C. argentoratense; C. diphtheriae; C. durum; C. freneyi; C. stationis; C. striatum; C. tuscaniense; C. ulcerans; C. urealyticum* (ATCC 43043)*; C. xerosis; Corynebacterium* spp. [c]

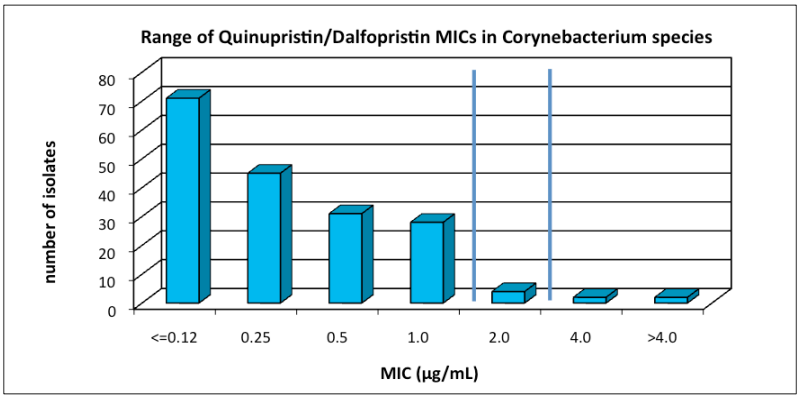
Figure 7. Erythromycin (A), clindamycin (B), and quinupristin/dalfopristin MICs (C) vs. total number of *Corynebacterium* species tested. Erythromycin breakpoints: ≤0.5 = S; 1 = I; ≥2 = R. Clindamycin breakpoints: ≤0.5 = S; 1-2 = I; ≥4 = R. Quinupristin/dalfopristin breakpoints: ≤1 = S; 2 = I; ≥4 = R. Blue lines designate range depicting susceptible, intermediate and resistant values. Total n=186.



A.

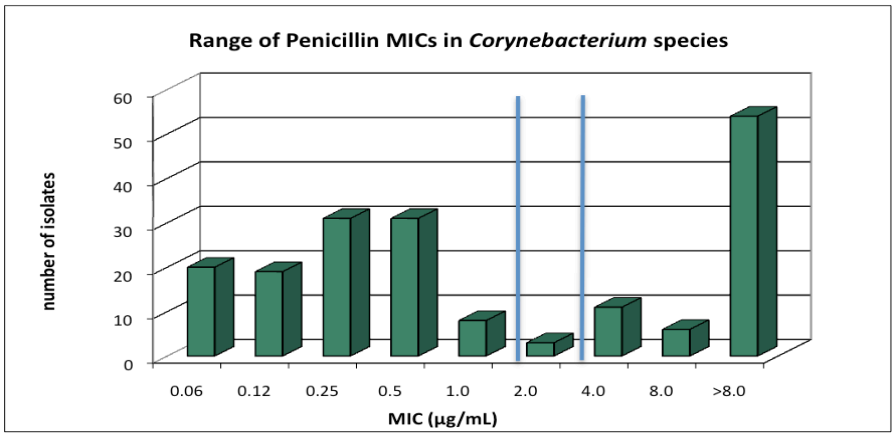


B.

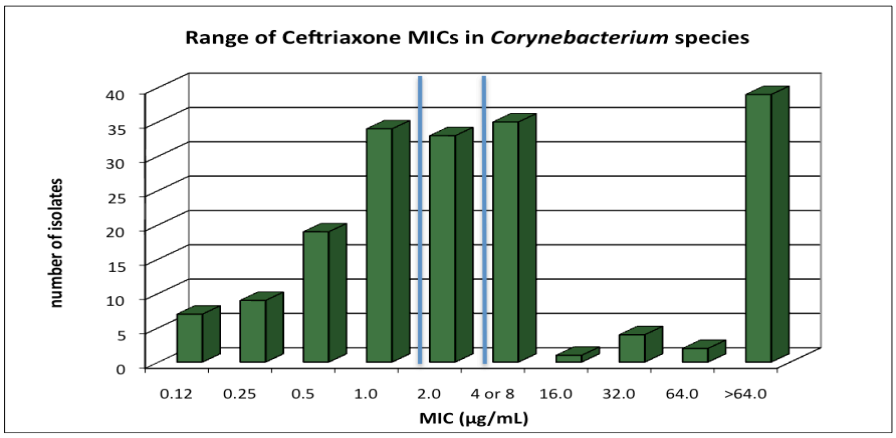


C.

Figure 8. Penicillin (A) and ceftriaxone (B) MICs vs. total number of *Corynebacterium* species tested. Penicillin breakpoints: ≤1 = S; 2 = I; ≥4 = R. Ceftriaxone breakpoints: ≤1 = S; 2 = I; ≥4 = R. Blue lines designate range depicting susceptible, intermediate and resistant MIC values. Total n=186.

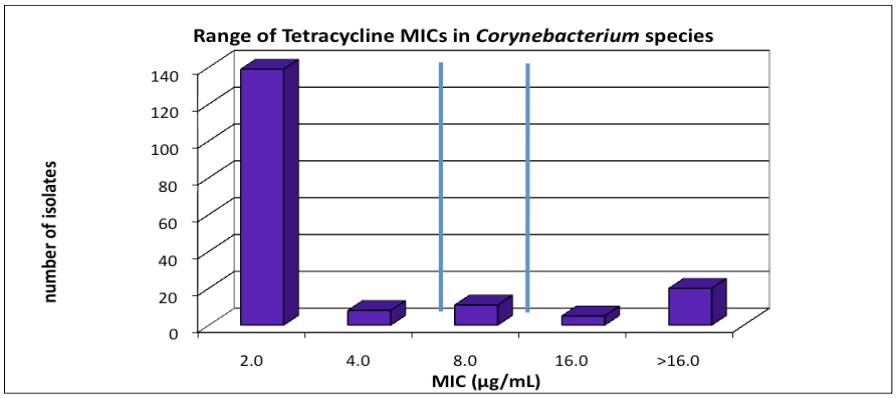


A.

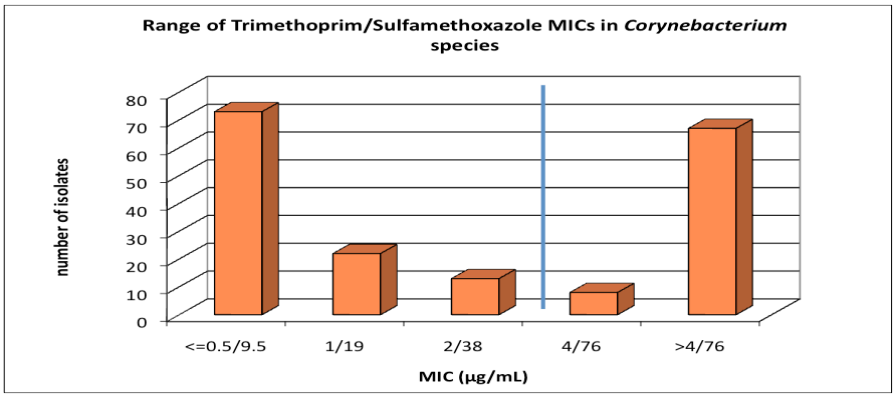


B.

Figure 9. Tetracycline (A) and trimethoprim/sulfamethoxazole (B) MICs vs. total number of *Corynebacterium* species. Tetracycline breakpoints: ≤4 = S; 8 = I; ≥16 = R. Trimethoprim/sulfamethoxazole breakpoints: ≤2/38 = S; ≥4/76 = R. First column of tetracycline (2.0) represents all isolates with an MIC of less than or equal to 2. Blue lines designate range depicting susceptible, intermediate (if applicable) and resistant values. Total n=186.



A.



B.

Figure 10. Percentage of *Corynebacterium* strains displaying the ERY^R/CLI^R phenotype that are resistant to other antimicrobial classes (n=114). ERY: erythromycin; CLI: clindamycin; QDA: quinupristin/dalfopristin; FEP/CTX: cefepime/cefotaxime; AXO; ceftriaxone; PEN: penicillin; GEN: gentamicin; SXT: trimethoprim/sulfamethoxazole; CIP: ciprofloxacin; TET: tetracycline; RIF: rifampin; VAN: vancomycin; LZD: linezolid; DAP: daptomycin.

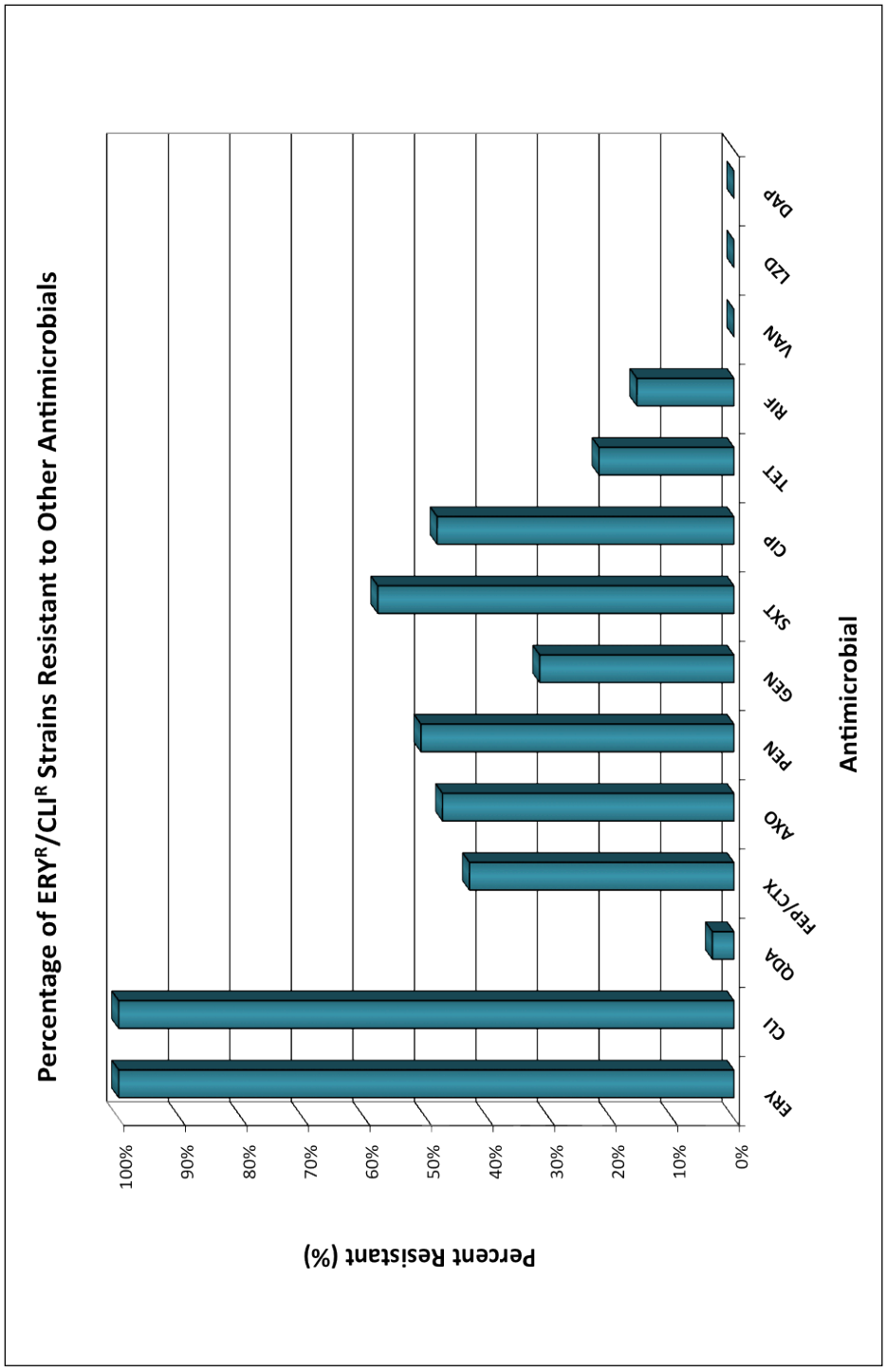

Percentage of ERY^R/CLI^R Strains Resistant to Other Antimicrobials
Percent Resistant (%)
100%
90%
80%
70%
60%
50%
40%
30%
20%
10%
0%
ERY
CLI
QDA
FEP/CTX
AXO
PEN
GEN
SXT
CIP
TET
RIF
VAN
LZD
DAP
Antimicrobial

Figure 11. Percentage of *Corynebacterium* strains displaying the $ERY^{S}/CLI^{R/I}$ phenotype that are resistant to other antimicrobial classes (n=36). ERY: erythromycin; CLI: clindamycin; QDA: quinupristin/dalfopristin; FEP/CTX: cefepime/cefotaxime; AXO; ceftriaxone; PEN: penicillin; GEN: gentamicin; SXT: trimethoprim/sulfamethoxazole; CIP: ciprofloxacin; TET: tetracycline; RIF: rifampin; VAN: vancomycin; LZD: linezolid; DAP: daptomycin.

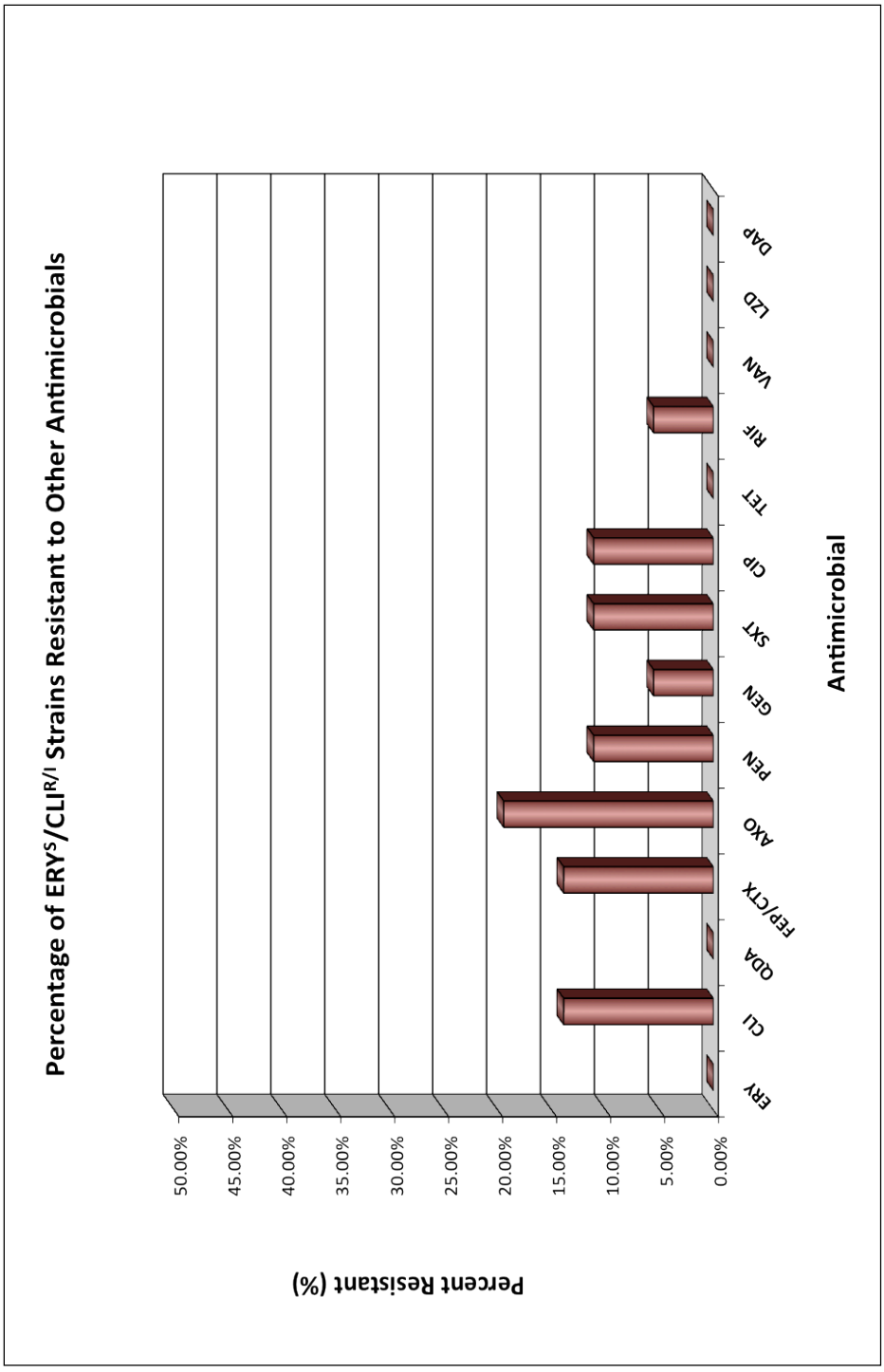

Percentage of ERY^{S}/CLI$^{R/I}$ Strains Resistant to Other Antimicrobials
Percent Resistant (%)
50.00%
45.00%
40.00%
35.00%
30.00%
25.00%
20.00%
15.00%
10.00%
5.00%
0.00%
ERY
CLI
ODA
FEP/CTX
AXO
PEN
GEN
SXT
CIP
TET
RIF
VAN
LZD
DAP
Antimicrobial

Figure 12. Percentage of *Corynebacterium* strains displaying the ERY^S/CLI^S phenotype that are resistant to other antimicrobial classes (n=31). ERY: erythromycin; CLI: clindamycin; QDA: quinupristin/dalfopristin; FEP/CTX: cefepime/cefotaxime/cefuroxime; AXO; ceftriaxone; PEN: penicillin; GEN: gentamicin; SXT: trimethoprim/sulfamethoxazole; CIP: ciprofloxacin; TET: tetracycline; RIF: rifampin; VAN: vancomycin; LZD: linezolid; DAP: daptomycin.

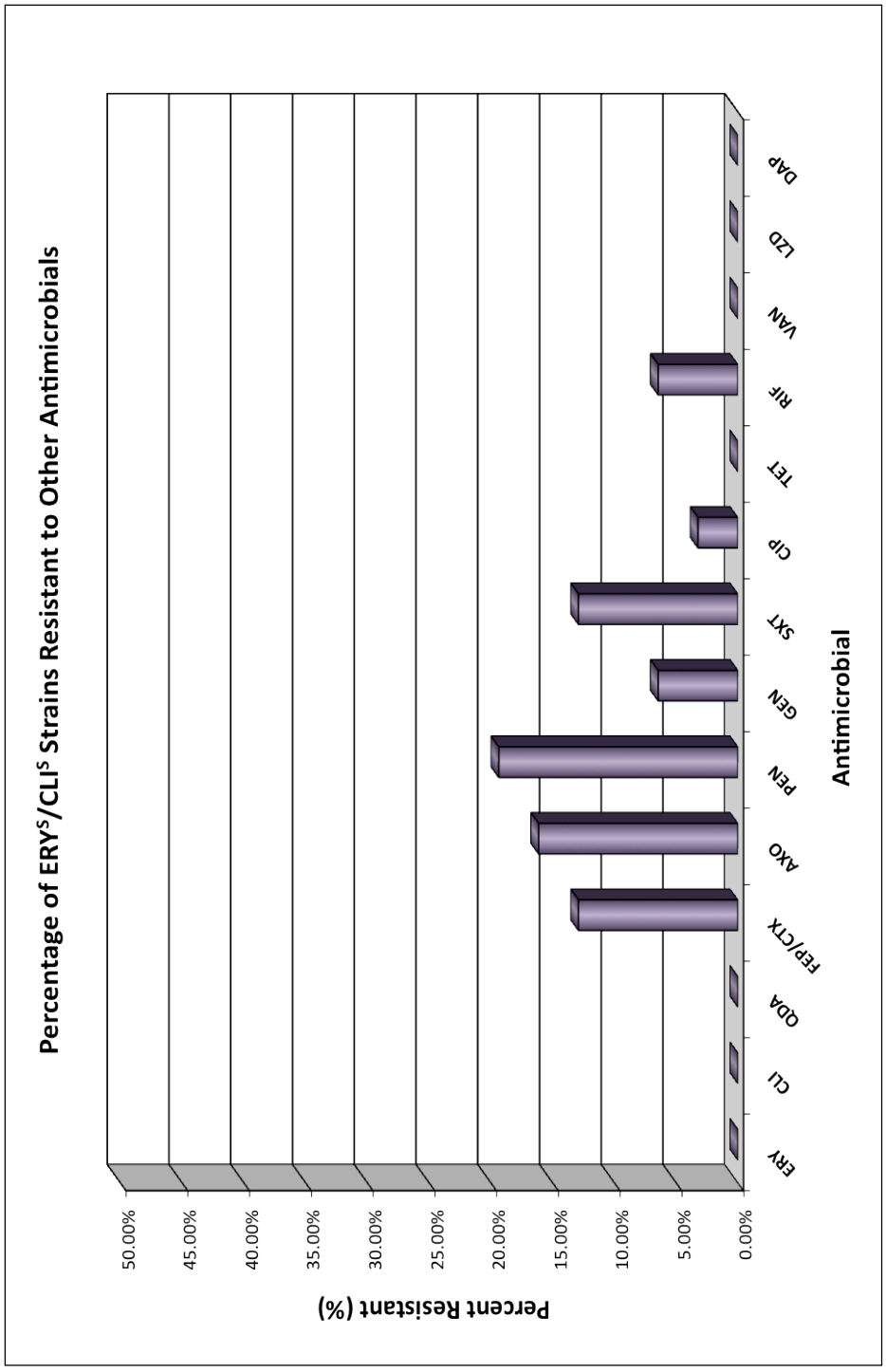

Percentage of ERY^S/CLI^S Strains Resistant to Other Antimicrobials
Percent Resistant (%)
50.00%
45.00%
40.00%
35.00%
30.00%
25.00%
20.00%
15.00%
10.00%
5.00%
0.00%
ERY
CLI
QDA
FEP/CTX
AXO
PEN
GEN
SXT
CIP
TET
RIF
VAN
LZD
DAP
Antimicrobial

Figure 13. Gel electrophoresis image of *ermX* and *rpoB* PCR. Lane 1: 100 bp ladder (Fermentas); Lane 2: *C. jeikeium* ATCC 43734^{T} (positive control); Lane 3: *C. xerosis* ATCC 373^{T} (negative control); Lane 4: *C. resistens* CCUG 50093^{T}; Lane 5: *C. appendicis* CCUG 48298^{T}; Lane 6: *C. tuscaniense* CCUG 51321^{T}; Lane 7: SB-00226 (*C. ulcerans*); Lane 8: NML# 070989 (*C. ulcerans*); Lane 9: NML# 92-0042 (*C. amycolatum*); Lane 10: NML# 96-0070 (*C. amycolatum*); Lane 11: NML# 070133 (*C. tuberculostearicum*); Lane 12: NML# 04-0185 (*C. afermentans*); Lane 13: NML# 92-0014 (*C. resistens*); Lane 14: NML# 93-0607 (*Corynebacterium* sp.); Lane 15: NTC (no template control).

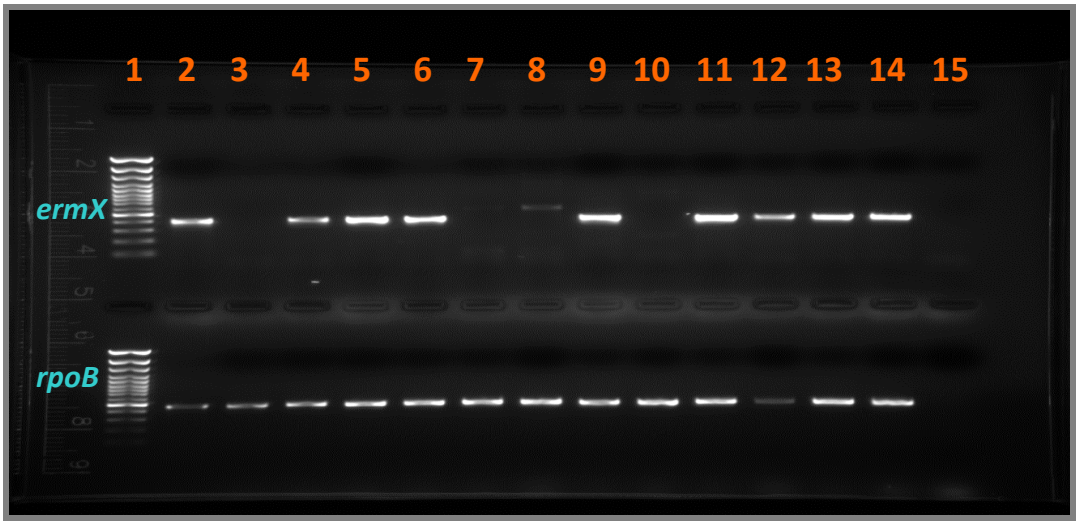


Table 6. Data summary of *Corynebacterium* strains displaying multidrug resistance to four or more antimicrobial classes. a: TMP/SMX: trimethoprim/sulfamethoxazole; b: QDA: quinupristin/dalfopristin; c: Identification to the species level not possible by 16S rDNA or *rpoB* sequencing. A total of 11 drug classes were tested against each strain, including the following: aminoglycosides; beta-lactams; carbapenems; cephalosporins; fluoroquinolones; folate inhibitors (sulfonamides); lincosamides; macrolides; RNA polymerase inhibitors; streptogramin B; and tetracyclines. *: varying resistance among strains.

# Drug Classes Resistant To	Antimicrobials Resistant To	# Strains	NML #'s Represented	Species Included	MLS_B Phenotype
9	cefepime; cefotaxime; ceftriaxone; ciprofloxacin; clindamycin; erythromycin; gentamicin; penicillin; rifampin; TMP/SMX[a]; meropenem*; QDA[b]*; tetracycline*	3	95-0270; 95-0280; 070133	*C. amycolatum; C. tuberculostearicum*	ERY^R/CLI^R
8	cefepime; cefotaxime; ceftriaxone; clindamycin; erythromycin; penicillin; TMP/SMX; ciprofloxacin*; gentamicin*; QDA*; rifampin*; tetracycline*	17	91-0169; 92-0042; 92-0043; 93-0708; 93-0717; 94-0185; 94-0267; 95-0269; 95-0271; 95-0281; 01-0043; 04-0265; 060367; 090387; 090389; *C. urealyticum* CCUG 18158^T; *C. resistens* CCUG 50093^T	*C. amycolatum; C. jeikeium; C. resistens; C. striatum; C. tuberculostearicum; C. urealyticum*	ERY^R/CLI^R
7	clindamycin; erythromycin; penicillin; TMP/SMX; cefepime*; cefotaxime*; ceftriaxone*; ciprofloxacin*; gentamicin*; QDA*; rifampin*; tetracycline*	23	79-0882; 79-0969; 91-0077; 92-0014; 92-0371; 92-0395; 93-0681; 93-0836; 97-0056; 99-0021; 99-0071; 99-0221; 00-0042; 01-0064; 01-0116; 04-0185; 090338; 090342; 090344; *C. jeikeium* ATCC 43734^T; *C. jeikeium* ATCC 43217	*C. afermentans; C. amycolatum; C. jeikeium; C. resistens; C. striatum; C. urealyticum; Corynebacterium* spp.[c]	ERY^R/CLI^R
6	ciprofloxacin; TMP/SMX; cefepime*; cefotaxime*; ceftriaxone*; clindamycin*; erythromycin*; gentamicin*; penicillin*; QDA*; tetracycline*	9	93-0691; 95-0072; 00-0200; 00-0212; 00-0219; 03-0027; 05-0368; 060127; 090339	*C. afermentans; C. amycolatum; C. aurimucosum; C. jeikeium; C. urealyticum; Corynebacterium* spp.	ERY^R/CLI^R; $ERY^S/CLI^{R/I}$
5	TMP/SMX; cefepime*; cefotaxime*; ceftriaxone*; ciprofloxacin*; clindamycin*; erythromycin*; gentamicin*; penicillin*; rifampin*	10	79-0565; 89-0826; 96-0070; 98-0158; 99-0162; 01-0123; 02-0190; 03-0192; 070281; 090182	*C. amycolatum; C. aurimucosum; C. jeikeium; C. resistens; C. tuberculostearicum*	ERY^R/CLI^R; $ERY^S/CLI^{R/I}$; ERY^S/CLI^S
4	cefepime*; cefotaxime*; ceftriaxone*; ciprofloxacin*; clindamycin*; erythromycin*; gentamicin*; penicillin*; tetracycline*; TMP/SMX*	13	89-0174; 93-0686; 94-0082; 97-0160; 99-0206; 01-0105; 00-0237; 03-0030; 060357; 090066; 090337; 090340; *C. urealyticum* ATCC 43043	*C. amycolatum; C. appendicis; C. aurimucosum; C. diphtheriae; C. jeikeium; C. tuberculostearicum; C. urealytcium; Corynebacterium* spp.	ERY^R/CLI^R; ERY^S/CLI^S

Table 7. MIC and interpretation data for extended test ranges of erythromycin and clindamycin concentrations. Isolates are considered resistant to erythromycin when the MIC is ≥ 2 μg/mL, and resistant to clindamycin when the MIC is ≥ 4 μg/ml.

INT: interpretation

R: resistant

S: susceptible

NML#	ID	Erythromycin		Clindamycin	
		MIC (μg/mL)	INT.	MIC (μg/mL)	INT.
ATCC 43734^T	*C. jeikeium*	4	R	>512.0	R
CCUG 18158^T	*C. urealyticum*	512	R	>512.0	R
CCUG 50093^T	*C. resistens*	32	R	256	R
CCUG 53377^T	*C. ureicelerivorans*	>512.0	R	512	R
79-0565	*C. jeikeium*	0.12	S	8	R
92-0014	*C. resistens*	>512.0	R	>512.0	R
93-0691	*C. jeikeium*	0.03	S	4	R
93-0696	*C. jeikeium*	0.03	S	0.25	S
00-0105	*C. aurimucosum*	512	R	>512.0	R
00-0163	*C. afermentans*	2	R	2	I
02-0162	*C. ureicelerivorans*	32	R	512	R
060357	*C. jeikeium*	>512.0	R	>512.0	R
070133	*C. tuberculostearicum*	>512.0	R	>512.0	R
090066	*C. diphtheriae*	2	R	>512.0	R
090132	*C. aurimucosum*	2	R	2	I
090182	*C. resistens*	512	R	512	R
090341	*Corynebacterium* sp.	4	R	512	R
090342	*C. striatum*	>512.0	R	>512.0	R
090343	*C. propinquum*	8	R	>512.0	R
090344	*C. jeikeium*	>512.0	R	>512.0	R
090387	*C. striatum*	>512.0	R	>512.0	R

Table 8. Comparison of *Corynebacterium* strains for which full-length *ermX* amplification and sequencing was successful. Base at position 511 of *ermX* sequence and MIC to erythromycin and clindamycin also shown. Sequences had >99% identity to each other.

NML or strain #	ID	Base at position 511	Erythromycin MIC (µg/ml)	Clindamycin MIC (µg/ml)
CCUG 50093^T	*C. resistens*	T	32	256
CCUG 18158^T	*C. urealyticum*	T	512	>512.0
CCUG 53377^T	*C. ureicelerivorans*	T	>512.0	512
89-0826	*C. amycolatum*	T	>4.0	>2.0
92-0014	*C. resistens*	A	>512.0	>512.0
96-0085	*C. pyruviciproducens*	C	>4.0	>2.0
00-0105	*C. aurimucosum*	T	512	>512.0
070133	*C. tuberculostearicum*	A	>512.0	>512.0
090066	*C. diphtheriae*	T	2	>512.0
090132	*C. aurimucosum*	T	2	2
090182	*C. resistens*	C	512	512
090342	*C. striatum*	A	>512.0	>512.0
090343	*C. propinquum*	C	8	>512.0
090344	*C. jeikeium*	C	>512.0	>512.0
090387	*C. striatum*	A	>512.0	>512.0

4.0 DISCUSSION

Corynebacterium species have formed a reputable niche in the history of infectious diseases, particularly with the study and eventual treatment of diphtheria due to the development of the antitoxin vaccine in the early 20th century. In the past two decades, there has been a renewed interest in the characterization of *Corynebacterium* species after many years of being understudied, underreported and dismissed as contaminants. This renewed interest lead to the discovery of over 50 novel species, the majority of them being clinically relevant. Many of these pathogens are being discovered as multidrug resistant, possibly due in part to the development of several antimicrobials during the latter half of the 20th century and their subsequent introduction into routine clinical practice. Many different resistance phenotypes exist among corynebacteria, yet one of the most prevalent phenotypes seen is resistance to erythromycin and clindamycin, also known as the MLS_B phenotype. Macrolide and lincosamide resistance in *Corynebacterium* species can fall into one of four categories: resistant to both macrolides and lincosamides, resistant to macrolides/susceptible or intermediate to lincosamides, sensitive to macrolides/resistant or intermediate to lincosamides, and sensitive to both macrolides and lincosamides. The MLS_B phenotype is expressed as a result of acquisition of the *ermX* gene on either a plasmid or transposon.

The *ermX* gene was first determined to cause resistance to erythromycin and clindamycin in a strain of *C. diphtheriae*, and was initially designated *ermCd*. The gene was discovered on a plasmid that came to be known as pNG2 (73). It was also discovered at this time that resistance could be lost spontaneously due to the excision

of the resistance determinant, forming the plasmid pNG3. Some years later a larger resistance plasmid, pTP10 was found in a strain of *C. striatum*, formerly known as *C. xerosis* designated as M82B (46). At this point no further work was done to further delineate resistance. Approximately ten years later, it was determined that this strain possessed an erythromycin resistance gene nearly identical to one discovered in *C. diphtheriae* (84, 85). It was also shown that this strain carried genes conferring tetracycline, chloramphenicol and kanamycin resistance as well. The gene responsible for erythromycin and clindamycin resistance was given the title *ermCx*, stemming from the presumed identity of the strain. A massive overhaul of resistance determinant nomenclature took place at the end of the 20th century, and simplified the process as to how novel resistance determinants are named (67). At this time it was determined that *ermCd* and *ermCx* shared a high level of genetic similarity (>95%), leading to the two genes being reclassified as *ermX*. During the next ten years, *ermX* was discovered in some of the most frequently seen *Corynebacterium* isolates clinically, including *C. amycolatum, C. jeikeium,* and *C. urealyticum*. *C. coyleae*, a recently described species is also known to possess *ermX*.

Among non-diphtherial *Corynebacterium* species tested in this study, the most prevalent phenotype was resistance to both macrolides and lincosamides with 114 strains displaying this phenotype, while the least common phenotype was resistance to macrolides alone paired with either an intermediate or susceptible phenotype to lincosamides, with only five strains displaying that particular phenotype. Two of these strains, however, were positive for the *ermX* gene: NML# 00-0163 (*Corynebacterium*

afermentans) and NML# 090132 (*C. aurimucosum*) showed low-level resistance to erythromycin (2 μg/ml), while the MIC for clindamycin was also 2 μg/ml, considered an intermediate value according to CLSI guidelines. As possession of the *ermX* gene is usually associated with high-level resistance to the macrolides and lincosamides, these two strains are unusual in that the MICs to these antimicrobials were quite low in comparison to other *ermX* positive strains. Based on these findings, it can be determined that high-level macrolide and lincosamide resistance among *Corynebacterium* species is predominantly attributed to the acquisition of *ermX*.

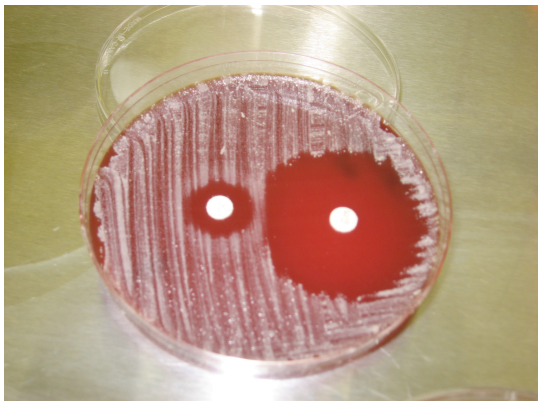
The antimicrobial concentration ranges for erythromycin and clindamycin are limited on the commercial panels (0.25-2 or 4 μg/ml for erythromycin; 0.12-1 or 2 μg/ml for clindamycin). CLSI guidelines for these particular antimicrobials state that resistance is observed when the MIC is equal to or greater than 2 μg/ml for erythromycin, or equal to or greater than 4 μg/ml for clindamycin. Selected strains were subjected to microbroth dilution using extended concentration ranges of erythromycin and clindamycin. Strains from varying phenotypes were selected, yet the majority of them displayed resistance to both erythromycin and clindamycin based on the commercial panels. The findings for these strains are reported in Table 7. Of the 21 strains subjected to extended testing, 12 displayed an MIC >32 μg/ml to erythromycin, while 16 of the 21 strains displayed an MIC >256 μg/ml to clindamycin. One strain in particular, NML# 090066 (*C. diphtheriae*) displayed low-level resistance to erythromycin (2 μg/ml, repeated 3x), yet the MIC for clindamycin was repeatedly >512 μg/ml. Previous antimicrobial susceptibility studies show elevated MICs for both erythromycin and

clindamycin, or a higher erythromycin MIC than clindamycin. One strain chosen for extended antimicrobial susceptibility analysis, NML# 090132 (*C. aurimucosum*) repeatedly showed low-level resistance to erythromycin (MIC 2 μg/ml) and an intermediate MIC of 2 μg/ml for clindamycin on repeat testing with both commercial panels and extended range testing, yet was *ermX* positive as previously stated.

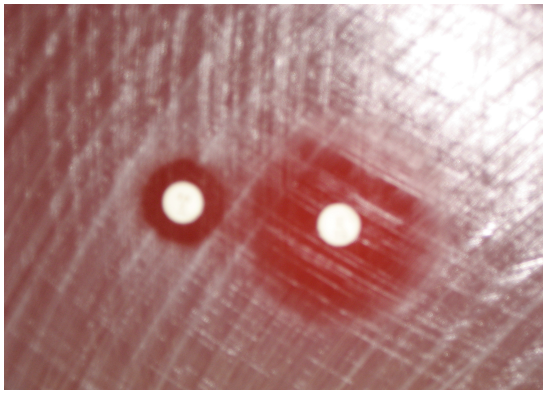
Fifteen *Corynebacterium ulcerans* strains all displayed susceptibility to erythromycin (MICs ≤0.25 μg/ml), yet showed intermediate or resistant MICs to clindamycin (MICs = 2 or >2 μg/ml). Testing for the *ermX* resistance determinant was negative in all strains, meaning this phenomenon is caused by some other resistance gene, or perhaps a mutation within the ribosome preventing the binding of clindamycin while remaining susceptible to erythromycin. For the sake of interest, a modified D-zone test was carried out to determine if resistance to erythromycin was inducible by clindamycin. The D-zone test was attempted with three strains: SB-00226, NML# 070888 and NML# 070989 because these displayed MICs greater than 2.0 μg/ml for clindamycin and susceptible MICs for erythromycin (≤0.25 μg/ml). Initial results seemed to indicate that some blunting did occur, as can be seen in the photographs that are part of Figure 14. Further molecular characterization of this phenomenon was not attempted.

Figure 14. Results of D-Zone test attempts with *C. ulcerans* strains SB-00226 (A), NML# 070888 (B), and NML# 070989 (C). A clindamycin disk (2 μg) placed approximately 20 mm apart from an erythromycin disk (15 μg) results in moderate blunting of the erythromycin zone of inhibition. For all images, the 2 μg clindamycin disk is on the left, while the 15 μg erythromycin disk is on the right.

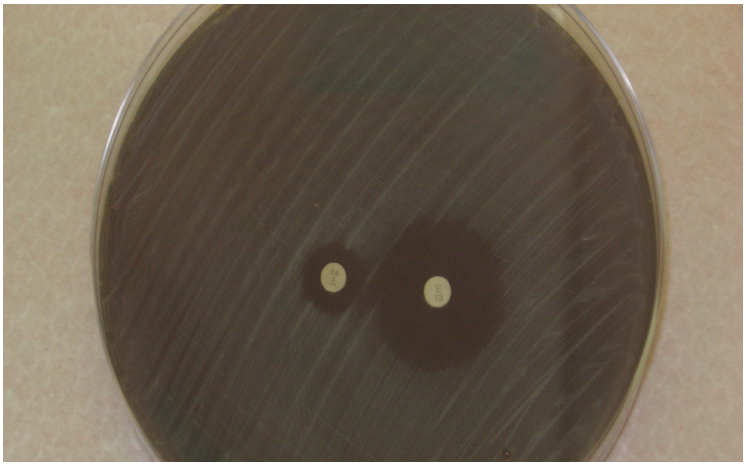
Images taken by C. Singh.



A.



B.



C.

Three strains (NML# 99-0018: *Corynebacterium* sp., NML# 090379: *Corynebacterium durum*, *Corynebacterium ammoniagenes* ATCC 6871^T) displayed resistance to both erythromycin and clindamycin (MIC >4.0 µg/ml and >2.0 µg/ml, respectively). However, testing for the *ermX* gene yielded a negative result. Repeat testing confirmed the absence of the *ermX* gene. Based on the results of this study, resistance to the macrolides and lincosamides in the absence of *ermX* is uncommon. The likely mechanism for the MLS_B phenotype in these bacterial strains is a ribosomal mutation within the 50S subunit that alters the binding site, effectively inhibiting the binding of the antimicrobial to the ribosome. Another possible explanation is that both macrolide efflux and nucleotidyltransferase genes (*mef* and *lnu*) have been acquired, yet this would be an unlikely phenomenon. Further testing of this hypothesis was not done.

C. accolens (NML #'s 03-0200, 05-0356) and *C. imitans* (NML# 89-0572) samples were isolated from human clinical samples, either blood or tissue cultures. Antimicrobial susceptibility testing showed that these species were fully sensitive to all antimicrobials tested, and were negative for the presence of *ermX*. This seems to indicate that the spread of multidrug resistance to these particular species has not occurred yet, but this finding does not rule out that it will not occur at some point in the future if care is not taken in the prescribing of antimicrobials.

C. argentoratense (NML # 02-0187)*, C. freneyi* (NML #'s 93-0569, 96-0127, 97-0108)*, C. stationis* (NML #'s 94-0424, 03-0173)*, C. ulcerans* and *C. xerosis* strains isolated from clinical samples were, for the most part, fully sensitive to all antimicrobials tested. However, certain strains within these species displayed resistance to one or two

antimicrobials. For example, the one strain of *C. argentoratense* (NML# 02-0187) displayed resistance to cefotaxime and ceftriaxone. Also, one *C. freneyi* isolate (NML# 96-0127) was resistant to erythromycin and clindamycin, one strain (NML# 93-0569) was resistant to only penicillin, and one other strain was fully sensitive to all antimicrobials tested (NML# 97-0108). All isolates currently identified as *C. stationis* were fully sensitive except for NML# 03-0173 which was resistant to erythromycin alone. Two *C. xerosis* isolates, NML #'s 95-0266 and 95-0267 were both resistant to erythromycin alone.

The majority of *C. diphtheriae* isolates seen within the laboratory setting were fully sensitive to all antimicrobials tested, including erythromycin and clindamycin. As a result, many of them were not incorporated into this study. An elevated MIC to ceftriaxone (2 μg/ml) was seen in several strains of *C. diphtheriae.* The reason for this phenomenon was not explored during this study and remains unknown. Two *C. diphtheriae* isolates referred to the laboratory within the last two years (NML #'s 080682, 090066) both displayed high MICs to erythromycin and clindamycin, suggesting the possible presence of *ermX*. The presence of the gene was confirmed in these two isolates. The other two *C. diphtheriae* strains incorporated into this study were fully sensitive to all antimicrobials tested (NML #'s 03-0033, 060128). The incidence of resistance in these two strains could possibly indicate the spread of the *ermX* resistance determinant to *C. diphtheriae*, yet these cases could also be isolated instances and nothing more.

C. appendicis isolates tested (CCUG 48298^T, 94-0082) were all resistant to erythromycin and clindamycin, including the type strain. The *ermX* gene was also detected in these isolates, which had not been previously described. Resistance to other antimicrobial classes was not observed in this species.

A total of five *C. urealyticum* strains were included in this study, including two reference strains acquired from two different culture collections, CCUG 18158^T and ATCC 43043. The number of *C. urealyticum* strains within the culture collection acquired by the Special Bacteriology section of the NML is far greater than five, however. With the exception of ATCC 43043, each strain was *ermX* PCR positive and resistant to at least 6 or more antimicrobial classes, including trimethoprim/sulfamethoxazole, which is frequently prescribed for the treatment of urinary tract infections. Prolonged antimicrobial use may be a factor in the probability of a *C. urealyticum* infection (78). This pathogen is also capable of forming biofilms in cases of prolonged catheter usage in patients, further impeding the success of antimicrobial treatment, as organisms within a biofilm display extremely high MIC values to antimicrobials (79).

Ten *C. tuberculostearicum* isolates were included as part of this study, isolated between 1978 and 2009. Three isolates were *ermX* PCR negative, and two of these (78-0150, 79-0331) were fully susceptible to all antimicrobials. Four isolates were resistant to erythromycin and clindamycin alone, and subsequently were *ermX* PCR positive, and the remaining three strains were both *ermX* PCR positive and resistant to at least five antimicrobial classes. One strain, NML# 070133, highlighted in Table 6 showed resistance to nine antimicrobial classes.

Twelve *C. striatum* isolates were isolated in total. Three strains (NML #'s 060367, 090342, 090387) were classified as being multidrug resistant, displaying resistance to more than 7 antimicrobial classes, including macrolides, lincosamides, cephalosporins, beta-lactams, fluoroquinolones, tetracycline, trimethoprim/sulfamethoxazole, and gentamicin. These three strains were also positive for *ermX*. The remaining nine strains were not multidrug resistant, or positive for *ermX*, but rather were either fully sensitive to all antimicrobials tested, or had elevated MICs to ceftriaxone only.

A total of 21 *C. jeikeium* isolates were included as part of this study, isolated from as long ago as 1979 to as recently as 2009. Each strain studied displayed a multidrug resistance phenotype that varied among strains. For example, two strains isolated in 1979 (NML #'s 79-0565, 79-0969) displayed resistance to antimicrobials that were in common use during that time, such as penicillin, gentamicin, sulfonamides, and macrolides and lincosamides. Of interest, these strains were fully susceptible to the fluoroquinolones, which is expected as these drugs are fairly new and would not have been in clinical practice at that time. The two reference strains acquired from the ATCC (ATCC 43734^{T}, ATCC 43217) also were susceptible to the fluoroquinolones. *C. jeikeium* strain 93-0696, however was fully susceptible to both erythromycin and clindamycin, while strains 79-0565 and 93-0691 were susceptible to erythromycin and resistant to clindamycin. These strains however were not fully susceptible to all antimicrobials, and still showed resistance to the cephalosporins, beta-lactams, and sulfonamides. Incidentally, these three strains also were *ermX* PCR negative. The remaining 18 strains,

including the two reference strains were all *ermX* PCR positive, and were resistant to several antimicrobials.

Forty-one *C. amycolatum* strains were studied as a part of this project, isolated from 1979 to 2009. Like *C. jeikeium*, the isolate from 1979 (79-0882) displayed a multidrug resistance phenotype to those antimicrobials currently in use, which excluded the fluoroquinolone antimicrobials. A variety of isolates were included, including the type strain ATCC 49368^{T}, and each isolate showed varying degrees of antimicrobial susceptibility. Two fully susceptible clinical isolates were observed, including NML #'s 97-0143 and 090384, as well as the reference strain (ATCC 49368^{T}). All three strains were *ermX* PCR negative. Other *ermX* PCR negative strains included 89-0174 and 01-0123, yet these strains showed intermediate susceptibility to clindamycin. The remaining 36 strains were all *ermX* PCR positive and resistant to both erythromycin and clindamycin. Five strains were resistant to erythromycin and clindamycin alone (92-0507, 94-0278, 96-0103, 03-0059, 05-0021), while the other strains displayed resistance to at least one other antimicrobial class. Two strains characterized had the most significant resistance phenotype which included resistance to nine antimicrobial classes (95-0270; 95-0280). These strains are highlighted in Table 6.

All isolates subjected to antimicrobial susceptibility testing were consistently sensitive to three antimicrobials: daptomycin, linezolid and vancomycin. Intermediate and resistant breakpoints are not available from the CLSI for these antimicrobials, yet it is suggested that susceptible *Corynebacterium* isolates should display an MIC no greater than 1 μg/ml for daptomycin, 2 μg/ml for linezolid, and 4 μg/ml for vancomycin (16). All

strains tested displayed results within these guidelines. There is one recently published case report highlighting a daptomycin-resistant strain of *Corynebacterium jeikeium* causing infection in a neutropenic patient (74). The mechanism responsible for conferring resistance in this strain is unknown, yet there was speculation as to whether the phenotype was caused by one genetic event, or if it was the accumulation of several genetic events. These data suggest that perhaps the course of treatment required for infections caused by *Corynebacterium* species is shifting from the more common, broad-spectrum drugs towards utilizing narrow-spectrum antimicrobials designed to target Gram-positive pathogens.

As previously stated, *ermX* was found in European isolates of *C. coyleae, C. diphtheriae, C. jeikeium, C. striatum,* and *C. urealyticum*. In this study, the *ermX* gene was found in 100 % of *C. coyleae* isolates tested, 50% of *C. diphtheriae* isolates tested, 86% of *C. jeikeium* isolates, 25% of *C. striatum* isolates and 80% of *C. urealyticum* isolates. The actual distribution of *ermX* among *C. diphtheriae* strains is likely far less than 50%, since the majority of strains received in the laboratory were fully sensitive to both erythromycin and clindamycin, indicating that the *ermX* gene or another resistance determinant was likely not present within the bacterial genome.

Several studies conducted indicated that the *ermX* gene is part of a larger transposon designated *Tn*5432 (68, 69, 84, 91). Attempts were made to amplify this transposon and sequence it in order to determine the genetic layout of the *ermX* gene; however repeated attempts to do this were unsuccessful. Several different primer combinations were tested from the primers mentioned in Table 3. The transposase gene

was successfully amplified and sequenced; however nothing downstream or upstream of this particular genetic element could be amplified or sequenced. During these attempts different concentrations of PCR components were utilized in order to determine if the problem was a problem with concentrations of particular elements, yet that did not seem to be the case, as manipulating concentrations of primers and/or components had no effect on the success of the experiment. Experimental trials with cycling temperatures and times also had no effect on success. Increasing annealing and extension times had no effect on a successful outcome.

Table 8 highlights an interesting phenomenon within the *ermX* gene: a localized base within the coding sequence that is prone to mutation. Located at approximately base 511 of the coding sequence, this base was compared against MIC data for the strains in which amplification of the entire gene was successful, however, no real correlation can be drawn from the results obtained, meaning that the reason for fluctuation in MIC values for certain strains such as NML # 090132 remains unknown. Other differences in sequence were present among the 21 strains, yet they were far less localized than the one polymorphism highlighted. When compared to each other, these sequences shared at least 96% homology with each other, with the majority displaying ≥99% homology to each other and to reference sequences in GenBank.

In total, 114 *Corynebacterium* strains displayed resistance to both erythromycin and clindamycin. Of these 114, 60 displayed resistance to penicillin, and 66 showed resistance to trimethoprim/sulfamethoxazole (52.6% and 57.9%, respectively). Fifty-seven strains showed resistance to all four antimicrobials, or 50%. It can be conjectured

from these findings that resistance to erythromycin and clindamycin seems to indicate a likelihood of being resistant to another antimicrobial such as penicillin; however the statistical significance of this was unable to be determined.

Multidrug resistance has not been reported in Canadian strains of *C. afermentans* or *C. aurimucosum*, yet European isolates of these two species showed reduced susceptibility to certain antimicrobials, including erythromycin and clindamycin (29). A previous study looking at the susceptibility profile of *C. afermentans* strains last took place in the mid 1990s (64), while *C. aurimucosum* had not been part of an antimicrobial susceptibility study prior to 2009. One Canadian isolate of *C. aurimucosum* (090132) was resistant to penicillin and ciprofloxacin in addition to displaying resistance to erythromycin, and displayed high MICs to other fluoroquinolones, including levofloxacin, moxifloxacin and gatifloxacin. Another strain of *C. aurimucosum* (03-0192) was also resistant to penicillin, as well as ceftriaxone and trimethoprim/sulfamethoxazole. *C. aurimucosum* strain 00-0105 was also resistant to penicillin, erythromycin, clindamycin, and trimethoprim/sulfamethoxazole. *C. aurimucosum* strain 060127 was resistant to 6 antimicrobial classes, including the cephalosporins, beta-lactams, fluoroquinolones and sulfonamides in addition to the MLS_B group, including quinupristin/dalfopristin. Five of the six *C. afermentans* strains (NML #'s 00-0163, 01-0024, 02-0040, 04-0185, 090339) isolated showed varying resistance patterns to the different antimicrobials tested. For example, NML# 00-0163 showed resistance to only erythromycin, while NML# 01-0024 showed resistance to erythromycin and clindamycin, as well as trimethoprim/sulfamethoxazole. NML# 02-

0040 showed resistance only to erythromycin and clindamycin. Strains 04-0185 and 090339 were resistant to several antimicrobials, including 2nd and 3rd generation cephalosporins, penicillin, ciprofloxacin and other fluoroquinolones, trimethoprim/sulfamethoxazole, and 04-0185 was also resistant to tetracycline. Based on the results of this study, *C. afermentans* and *C. aurimucosum* are two species capable of displaying multidrug resistant phenotypes due to the acquisition of one or more resistance genes.

In addition to discovering the widespread prevalence of the *ermX* gene throughout *Corynebacterium* species, this study was able to correctly identify *Corynebacterium* strains previously misidentified as belonging to other species or groups. As previously stated, prior to the advent of useful molecular genetic technologies, species identification for *Corynebacterium* species was done using a biochemical approach. A guide published in the early 1980s provided a way for dividing these *Corynebacterium* species into groups designated with letters, numbers, or both (39). Species exhibiting similarities to one another were placed in the same group, or were defined to be like a species if one or two biochemical tests differed. As molecular technologies improved, these groups were further delineated into species as tools such as PCR and 16S rDNA sequencing became less cumbersome. In spite of this, however, identification of *Corynebacterium* species is still a process plagued by inaccuracies. For example, one strain received at the NML from the HSC was provisionally identified as either *C. striatum* or *C. amycolatum*, which is commonly seen when the API Coryne strip is used as this is the matching record in the on-line database. However, after analysis of

16S and *rpoB* sequences, it was determined that this was in fact a strain of *C. propinquum*, a rare pathogen. Many strains over the years had been described as being *Corynebacterium jeikeium*-like, as they were lipophilic and reactive in the same biochemical tests, yet were sensitive to penicillin when a disk diffusion test was carried out. Strains that were part of this study and fit this profile ended up being reclassified as either *C. appendicis, C. tuberculostearicum*, or not belonging to any currently identified species, including NML #'s 92-0130, 92-0210, 93-0481 and 94-0082.

In spite of numerous studies being published concerning *Corynebacterium* species and antimicrobial susceptibility testing, minimal research has been published concerning antimicrobial resistance mechanisms. The first indication of a molecular basis for resistance came in the 1990s with the discovery of what would become *ermX*. From that point, other molecular resistance mechanisms have been discovered also, including mechanisms for chloramphenicol, aminoglycoside and tetracycline (83, 85-87). Macrolide efflux or *mef* genes were also discovered in *Corynebacterium* species (49). Until very recently, insight into the mechanism concerning beta-lactam resistance had not been deduced (47). It was discovered that *C. jeikeium* is capable of producing a modified penicillin binding protein (PBP) in order to avoid the bactericidal effect of the beta-lactam antimicrobials. This finding however was only noted in *C. jeikeium*, and no other *Corynebacterium* species. It was also determined that mutations within the target sites of the fluoroquinolone antimicrobials were responsible for resistance developing in *C. amycolatum, C. striatum* and *C. macginleyi* (25, 77). In spite of this, certain antimicrobial resistance mechanisms remain unknown, including resistance to rifampin,

sulfonamides, cephalosporins and carbapenems. Studies designed to determine whether resistance mechanisms are universal across all *Corynebacterium* species have also not been attempted.

Conclusions

Based on the results obtained in this study, *Corynebacterium* species should no longer be considered as emerging opportunistic pathogens, based on the wide variety of clinical sources of infection, the problem of multidrug resistance, and the severity of infection for each patient. The results of this study, along with others show that these bacterial pathogens are well established as nosocomial pathogens, and are capable of causing the same severe outcomes as other nosocomial or community acquired pathogens. While there are four *Corynebacterium* species responsible for the majority of infections, many other species, including several newly described in the last decade are also capable of causing disease and acquiring a multidrug resistant phenotype. The most frequently isolated multidrug resistant phenotype among corynebacteria is the MLS_B phenotype. This phenotype is seen in nearly all *Corynebacterium* species isolated from clinical human specimens, including *C. diphtheriae.* Care should be taken when prescribing treatment for cutaneous diphtheria, as strains responsible for infection may now be resistant to the most common antimicrobials used for this disease, which include erythromycin.

Among *Corynebacterium* strains in Canada, the most frequently isolated multidrug resistance phenotype is the MLS_B phenotype. Resistance to

trimethoprim/sulfamethoxazole or penicillin in addition to being resistant to the MLS_B class of antimicrobials was also frequently seen.

Based on the results of this study, it can be attributed that high-level macrolide and lincosamide resistance in Canadian *Corynebacterium* strains is likely due to the acquisition of *ermX*. There were three strains that did not carry *ermX* out of the 114 displaying the correct resistance phenotype (2.6%). It is likely that a ribosomal mutation confers resistance in these strains, or another unknown mechanism.

The results of this study show that *ermX* can be isolated from far more than the six species originally identified as being capable of possessing *ermX*. These include *C. afermentans, C. appendicis, C. aurimucosum, C. confusum, C. durum, C. freneyi, C. macginleyi, C. propinquum, C. pseudodiphtheriticum, C. pyruviciproducens, C. resistens, C. simulans, C. tuberculostearicum, C. tuscaniense,* and *C. ureicelerivorans*, bringing the total number of species capable of harboring *ermX* to 21. Over forty novel *Corynebacterium* species have been isolated from human clinical samples, including the species mentioned here that are capable of acquiring and expressing the *ermX* gene.

The results of this study were unable to decidedly confirm that *ermX* is always associated with *Tn*5432 and is not plasmid-borne. Further insight into this area is required.

The *ermX* resistance determinant is frequently isolated from *Corynebacterium* species displaying the MLS_B phenotype; however it is not responsible for 100% of all resistance to these antimicrobials. Other resistance mechanisms responsible for this

phenotype would likely include ribosomal mutations, or the acquisition of one or more other resistance genes.

C. afermentans and *C. aurimucosum* are two species that are capable of developing multidrug resistance, as was seen first in European isolates, and presently in Canadian isolates as a result of this study.

Antimicrobial resistance is a problem increasing in prevalence and severity, and options to combat this problem are decreasing. New antimicrobials are being developed, but the process is not rapid, and newer drugs present an increased risk of experiencing potentially severe side effects while only being effective against a limited number of pathogens. Also, because the field of novel antimicrobial development is not as robust as it once was, the consumer cost for more esoteric drugs is much higher than for other antimicrobials already developed.

As a result of this study, a database containing the antimicrobial susceptibilities and resistance mechanisms for a large collection of Canadian *Corynebacterium* species will be augmented. Further insight is required into additional antimicrobial resistance mechanisms, as several remain unknown to this day. The distribution of resistance determinants is on the rise globally and in particular the *ermX* gene in Canadian *Corynebacterium* species, indicating that antimicrobials once considered commonplace and effective may no longer be so. Future work can be considered in determining whether linkages between antimicrobial resistance determinants within *Corynebacterium* species exist, and whether resistance to certain antimicrobials such as

the beta-lactams is due to the same mechanism among all *Corynebacterium* species, or if each mechanism is species-specific.

As previously stated, antimicrobials suggested for primary use against suspected and/or confirmed *Corynebacterium* infections include penicillin and erythromycin. The results of this study show that this list of first-line antimicrobials may need to be re-evaluated in that two of the four drugs recommended for treatment regimes are highly likely to be ineffective. Newer antimicrobials, such as tigecycline and a small amount of others are the most likely candidates for treatment when dealing with a suspected or confirmed multidrug resistant *Corynebacterium* infection.

5.0 REFERENCES

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