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REVIEW

Antibiotic resistance crisis: challenges and imperatives

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Abstract

Antibiotic resistance is one of the greatest worldwide challenges to modern medicine, and society at large, and one of the least appreciated by practitioners and the lay community. Many strains of multi-drug resistant bacteria such as methicillin and vancomycin-resistant *Staphylococcus aureus* and multi-drug resistant *Mycobacterium tuberculosis* continue to plague both developed and underdeveloped countries alike. Collectively over 700,000 deaths occur annually as a consequence of infections from antibiotic-resistant bacteria. Presently, many scientists and clinicians seek to find solutions to this ever-growing crisis. Today, the most common causes of hospital-acquired and multi-drug resistant infections are the ESKAPE group of bacteria (*Enterococcus faecium, Staphylococcus aureus, Klebsiella pneumoniae, Acinetobacter baumannii, Pseudomonas aeruginosa*, and *Enterobacter* spp.), six genera and species with a host of mechanisms to survive against even extremely potent antibiotics. Limited research currently focuses on discovering or developing novel compounds that will hopefully turn the tide and tackle the problem of resistance, but significant changes in healthcare and consumer practices will be necessary as well, to successfully address this dilemma. This review provides a status report on a rather silent global crisis.

Keywords Antibiotic resistance · ESKAPE pathogens · Horizontal gene transfer · Antibiotic stewardship

Introduction

If no new antibiotics are isolated and made available by 2050, the Centers for Disease Control (CDC) estimates that 10 million annual deaths from multi-drug resistant (MDR) bacterial infections will occur globally as a result of this inaction, more than cancer and heart disease combined. Infectious disease is currently the 2nd leading killer in the world, and 4th in the U.S. Globally, with 17 million people dying annually from bacterial infections (Martens and Demain 2017). Yet, surprisingly few have even heard of antibiotic-resistant bacteria, or understand the implications for global health. In fact, no new classes of antibiotics have been developed to treat microbial infections in over 30 years, as pharmaceutical companies have instead pursued research and development of more lucrative drugs for non-infectious diseases Most large pharmaceutical companies have ceased novel product (NP) discovery, leaving academic labs and small start-up companies to explore antibiotic therapeutics (Hutchings et al. 2019). The backstory of this crisis is well-known among experts but rarely brought to the attention of the general public.

Alexander Fleming's discovery of penicillin in 1928 was met with his own prediction that bacterial resistance to this "miracle drug" would soon be documented (Khardori et al. 2020; Tand and Tatsumura Tan and Tatsumura 2015). In the 70+ years since penicillin was introduced, overuse and misuse of antibiotics have contributed to the problem of MDR bacterial infections, as has the widespread application of antibiotics in agriculture for prophylaxis and growth promotion (Santesmases and Gradmann 2011). In fact, the CDC has reported that over 70% of antibiotics used in the U.S. are in production animal environments (Abadi et al. 2019; Michael et al. 2014). Clinically, antibiotic stewardship and surveillance programs have shown limited success in addressing the MDR crisis (Romo and Quiroz 2019). In recent years, both the CDC and the White House have outlined clear goals and objectives for directly addressing antibiotic resistance in order to slow the spread of MDR bacteria, while offering a timeline on collaborative international efforts required to make this happen by 2020 (Centers for Disease Control, 2020; Obama White House Archives 2015). Unfortunately, this Executive Order signed by President Barack Obama (#13676) has not been addressed, during which time MDR bacterial infections have worsened and become more frequently diagnosed (CDC

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2020; Floris et al. 2020). If no new effective antibiotics are developed and approved for clinical use by 2050, MDR bacteria are predicted to kill more people globally than diabetes and cancer combined (Small World Initiative 2020), necessitating urgent immediate action to address this issue.

In today's world, the medical field is being challenged in a variety of ways that its many advances have struggled to keep up with adequately, which is a growing concern for medical science and society on the whole. Novel diseases of various types are crossing international lines and boundaries at alarming rates, bringing what were once geographically localized and isolated pathogens to distant places with new potential victims to infect. Old diseases once thought contained or extinct are cropping up again with new resistances to many conventional methods of treatment necessitating drastic or unprecedented measures in many cases. Diseases transmitted through infected living vectors such as insects and rodents are becoming a greater threat due to pesticide-resistant carrier organisms. These are just a few of problems modern medicine currently faces.

One of the greatest challenges to modern medicine is the currently increasing resistance of many common bacterial pathogens to antibiotics. According to the Centers for Disease Controls and Prevention (CDC), each year in the United States alone, close to 3 million people become infected with various species or strains of antibiotic-resistant bacteria or fungi, and more than 35,000 of those afflicted die from these infections (About Antibiotic Resistance 2020; Biggest Threats and Data 2020). Among some of the most concerning drug-resistant bacterial infections include such names as MRSA (methicillin-resistant *Staphylococcus aureus*), VRSA (vancomycin-resistant *S. aureus*), VRE (vancomycin-resistant *Enterococcus*) drug-resistant *Salmonella* and *Campylobacter* food-associated infections, drug-resistant *Clostridium difficile* enteric infections, and multi-drug-resistant *Mycobacterium tuberculosis* infections along with many others (Biggest Threats and Data 2020; World Health Organization 2020; Frieri et al. 2017; Zaman et al. 2017). Overuse of antibiotics against some of these infections, not to mention improper treatment with antibiotics prescribed for bacteria against organisms and microbial threats that these drugs are not designed to inhibit, such as viruses, have led to widespread resistance of numerous bacterial species to many common antibiotics and antibiotic classes. Abuse of antibiotics targeting bacteria has also resulted in many instances of opportunistic or nosocomial (hospital-acquired) infections in thousands of patients worldwide (D'Costa et al. 2011; Frieri et al. 2017; Davies and Davies 2010; Munita and Arias 2016; Chellat et al. 2016; Zaman et al. 2017). Many of these infections prove extremely difficult or even impossible to treat if the bacterial infection in question possesses a multitude of resistances (Frieri et al. 2017; Munita and Arias 2016). Resistance also spreads extremely quickly due to the fast cellular replication cycles of most bacteria and bacterial conjugation of genes from one cell to another thus passing on various forms of natural and plasmid-acquired resistances (Davies and Davies 2010; Chellat et al. 2016).

Davies and Davies (2010) have reported on bacterial resistance to antibiotics that there are likely well over 20,000 known resistance genes encompassing over 400 general types based on the vast library of known and available bacterial genome sequences, as well as potentially many more that will be discovered. From this, the authors also warn of the potential for the world to regress back into a pre-antibiotic age where these "miracle drugs" are all but useless, necessitating the need for effective solutions to the issue. Additionally, Munita and Arias (2016) report that bacterial antibiotic resistance is predicted to result in the global loss of 300 million lives prematurely and deal a crippling blow equivalent to 100 trillion U.S. dollars to the global economy by the year 2050. The current mission of many medical researchers and healthcare employees is to find solutions to this global problem of resistance. These include such measures as the curbing of excessive and unnecessary use of antibiotic medications, stifling the over-the-counter and irresponsible sale of antibiotic drugs, particularly in developing nations and economically poor regions of the world where such problems are all too frequent, and educating the general public on the responsible administration of antibiotics (Chellat et al. 2016; Santesmases and Gradmann 2011). Additionally, there are the more obvious tactics of developing or discovering novel antibiotic compounds and classes as well as chemically modifying existing compounds and classes.

Goal

The goal of this review is multifold. First, to provide some information on the history of antibiotic development and treatment, as well as current efforts to create or discover novel antibiotic compounds to combat various pathogens of interest. Secondly, to provide some key insights into the history and development of bacterial resistance to antibiotics, as well as discussing some of the key methods in which different species of bacteria resist the effects of antibiotics. Third, to describe some of the more common drug-resistant bacterial infections to provide context on several of the more sought-after targets of this medical challenge, primarily the bacterial species and genera that comprise the ESKAPE (*Enterococcus faecium*, *Staphylococcus aureus*, *Klebsiella pneumoniae*, *Acinetobacter baumannii, Pseudomonas aeruginosa*, and *Enterobacter* spp.) pathogens. Lastly, to provide a detailed description of the methods that will be utilized in the proposed thesis research project to potentially discover novel antibiotics and potential chemical variants of known compounds, as well as rediscover forgotten compounds while also testing the

effectiveness of these compounds against the ESKAPE group of pathogens, all of which are known to be drug-resistant to varying degrees.

A brief history of antibiotic development

The history of antibiotics can be traced all the way back to the late 1800s, when French physician, Ernest Duchesne, noted that certain fungal molds such as *Penicillium* were capable of inhibiting bacterial growth (Ramalingam 2015). Unfortunately, Dr. Duchesne would pass on in the early 1920s and was unable to discover why and how *Penicillium* and other fungal molds were able to combat bacteria. A few years later in 1928, Sir Alexander Fleming, a Scottish physician and researcher, discovered the existence of penicillin after noting the growth of *Penicillium* mold in a culture plate of *Staphylococcus* bacteria, observing that the mold colonies inhibited the growth of bacteria around them (Davies and Davies 2010; Zaman et al. 2017; Santesmases and Gradmann 2011; Ramalingam 2015). From his previous discovery of the enzyme lysozyme in 1923, an antibacterial substance present in human tears and an innate part of the immune system, Fleming surmised that the *Penicillium* mold utilized a similar chemical compound. Fleming was able to extract the substance, but was unable to purify it, thus bringing his work to a premature halt as the drug would not be purified and used in human clinical trials until roughly a decade later. In the early 1940s, two scientists, Howard Walter Florey and Ernst Boris Chain, managed to purify the substance of interest to Fleming and proceed with further laboratory experiments, demonstrating its effectiveness against bacterial infections (Santesmases and Gradmann 2011; Ramalingam 2015; Bjorkman and Phillips-Howard 1991). Shortly thereafter in 1943, penicillin G was put into mass production and subsequent medical application, proving to be extremely reliable and effective in treating bacterial infections of all kinds, particularly in the case of frontline soldiers during the Second World War (Davies and Davies 2010; Zaman et al. 2017; Santesmases and Gradmann 2011; Ramalingam 2015; Bjorkman and Phillips-Howard 1991).

During the downtime between Fleming's initial discovery of penicillin and Florey and Chain's purification of penicillin, in the year 1932, Gerhard Domagk, a German pathologist and bacteriologist, would discover and develop a group of synthetic compounds, sulfonamides, colloquially named "sulfa drugs" throughout the twentieth century (Ramalingam 2015; Bjorkman and Phillips-Howard 1991; Lesch 2007). However, these antibiotics not only were soon relegated to second-option drugs after the introduction of penicillin and other antibiotics in the 1940s, but it was eventually discovered they caused a number of dramatic side effects when used to treat human bacterial infections including blood dyscrasias, skin lesions, and liver and respiratory disorders, so they were discontinued. However, these drugs did serve to kickstart something of an antibiotic discovery and production revolution in the decades to follow.

During the mid-1940s to late 1950s, a multitude of new antibiotics derived from microbial sources followed the discoveries of penicillin and sulfonamides. These included such names as streptomycin, erythromycin, cephalosporins, bacitracin, chloramphenicol, polymyxin, tetracycline, aminoglycosides, macrolides, vancomycin, and neomycin (Zaman et al. 2017; Ramalingam 2015). These compounds were effective in the treatment of bacterial pneumonia (*Klebsiella pneumoniae* and others), syphilis (*Treponema pallidum*), and tuberculosis (*Mycobacterium tuberculosis*) among others (Ramalingam 2015). However, the downside of these antibiotic drugs is that many of them, such as neomycin, proved to be too toxic for treating bacterial infections in the human body, so their prevalence in medical treatment had to be severely decreased. This prompted the search for new semi-synthetic and fully synthetic compounds with modifications to increase their antibiotic activity. In the year 1960, the first semi-synthetic antibiotic, methicillin, was derived from penicillin. Two years later in 1962, the next synthetic drug to be produced was nalidixic acid. These antibiotics and others produced at the time all proved to be extremely effective against bacterial infections such as various species of *Staphylococcus*, *Escherichia coli*, *Haemophilus influenzae*, *Salmonella typhimurium*, and *Pseudomonas aeruginosa*. Further progression into the 1960s saw the development and production of the first-generation cephalosporins such as cephalothin and cephazolin. This in turn eventually led to the production of the second and third-generation cephalosporins (ceftriaxone, cefoxitin, ceftazidime, cefotaxime, etc.) in the 1970s and the carbapenems (thienamycin, imipenem, meropenem, doripenem, ertapenem, etc.) in the 1980s (Ramalingam 2015). The 1950s through the 1970s is considered to be the "golden age" of antibiotic discovery, but since roughly the late 1980s and early 1990s, there has been a time described as a discovery void where virtually no new antibiotic compounds were discovered or created, leading up to almost the present day (Davies and Davies 2010; Zaman et al. 2017). With this in mind, it is additionally important to note that increasingly drug-resistant infections started showing up even before the "golden age of discovery" around the 1960s and have continued to do so up to modern day.

Today, research into novel antibiotic compounds that can affect drug-resistant bacteria is more widespread than ever. The *British Medical Journal* warns of the current problem surrounding the lack of novel antibiotics being developed to combat drug-resistant bacterial infections, citing that antibiotic research regarding a number of extremely concerning drug-resistant bacteria is severely underfunded including for tuberculosis and a large number of Gram-negative infections

(Kmietowicz 2017). This being said, there are a large number of lines of research into treating various different infections using novel compounds as well as the discovery and development of said compounds.

Within the realm of fungi, He et al. have been performing studies on fungus-derived naphtho-γ-pyrones (He et al. 2016). These compounds are fungal polyketides that exhibit significant antimicrobial activity against bacteria and fungi such as *S. aureus, P aeruginosa, M. tuberculosis, E. coli,* pathogenic plant bacteria, and *Candida albicans*. In this study, the authors investigated the antimicrobial activity of eight of these compounds: flavasperone, fonsecinones A-C, rubrofusarin B, aurasperones A and E, and asperpyrone C. Results revealed that fonsecinones A and C, as well as aurasperones A and E possessed potential antimicrobial activity with minimum inhibitory concentrations in the micromolar range against MRSA, *E. coli, P. aeruginosa,* and *Enterococcus faecalis* with fonsecinone A possessing the highest antimicrobial activity of all the compounds investigated while asperpyrone C had the lowest activity due to possessing a C-6-C-7′ linkage (He et al. 2016).

As another example, Silber et al. note the enormous potential for novel antibiotic discovery and production from the cultivation of the vast number of known marine fungi (Silber et al. 2016). Their review on the biotechnological processes and discoveries surrounding marine fungi and cultivation of antibiotic products from them covers a sizeable list of potential candidate compounds, some of which demonstrate considerable antibiotic activity with low minimum inhibitory concentrations or even against some of the ESKAPE pathogens. For instance,ascosetin (derived from *Halichondria panicea*) being effective against *S. aureus* including MRSA strains, *Aspergillus chrysogenum* and *Cephalosporium chrysogenum*-derived cephalosporins being broad spectrum, corollosporin and its derivates from *Corollospora maritima* showing activity against *P. aeruginosa* and *S. aureus*, and enniatins derived from the genus *Halosarpheia* showing considerable activity against *Enterococcus faecium*, *S. aureus*, and *P. aeruginosa* among others (Silber et al. 2016). The study also goes into detail about various natural, semisynthetic, and fully synthetic biotechnological methods by which these compounds may be produced in mass quantities including full natural fermentation, precursor molecule fermentation, bioconversion of a synthetic product, or heterologous production within a genetically-modified host organism (Silber et al. 2016).

Sometimes, it is not just a single species of fungi that produce antibiotic, but a multi-species colony that produces a compound or series of compounds. This is the case in Stierle et al.'s study which discovered and described a new series of macrolide antibiotics (Stierle et al. 2017). These novel macrolides, called berkeleyactones, are a group of 16-membered-ring antibiotics derived from what the authors describe as a carefully-timed, coculture fermentation process involving *Penicillium fuscum* and *P. camembertii/clavigerum* (Stierle et al. 2017). Both of these species are extremophilic fungi isolated from surface water of Berkeley Pit Lake, an acidic lake that was formerly a copper mine in Butte, Montana, United States (Stierle et al. 2017). The researchers discovered that while no useful compounds were produced when the species were grown axenically, when cocultured they produced this series of compounds they dubbed the berkeleyactones, as well as a few other known antibiotics and secondary metabolites (Stierle et al. 2017). From testing all eight berkeleyactones, the researchers discovered that berkeleyactone A exhibits the greatest antibiotic activity of the series with a minimum inhibitory concentration (MIC) of 1–2 μg/mL when tested against four MRSA strains, *Bacillus anthracis, Streptococcus pyogenes, Candida albicans, and Candida glabrata* (Stierle et al. 2017). They also discovered that berkeleyactone A likely has a novel antibiotic mode of action compared to other macrolides which has yet to be described (Stierle et al. 2017).

Others may have found potential ways to bring older antibiotic classes back into service. Shang et al. in their study on biotransformation of tetracycline antibiotics via various fungi species note in particular the resiliency of viridicatumtoxins (Shang et al. 2016). Viridicatumtoxins, derived from fungi such as *Penicillium viridicatum* and *P. aethiopicum* among other species, were shown in the experiments of Shang et al. to be particularly resistant to fungal biotransformation (Shang et al. 2016). In fact, one of the compounds they studied, viridicatumtoxin B, was shown to have an MIC of 40 nanomoles (nM) when tested against vancomycin-resistant *Enterococci* (Shang et al. 2016). It was the conclusion of the researchers that such tetracyclines may have the potential to greatly resist enzymatic degradation, making them useful against various bacterial and fungal targets, as well as guiding the development of new tetracycline antibiotics that are similarly resistant to enzymatic degradation (Shang et al. 2016).Other sources and studies point to the potential for discovery of novel compounds from natural sources (Moloney 2016; Hug et al. 2018; Landwehr et al. 2016). This research notes a number of examples including teixobactin (a bacterially-produced compound with significant activity against a large range of organisms, particularly Gram-positive bacteria), ulleungamides, salinamide F (with significant activity against both Gram-positive and negative bacteria), copsin (a compound isolated from a co-cultivated fungal source), cystobactamids, hymenosetin, kibdelomycin, hunanamycin (isolated from the marine-derived bacterium *Bacillus hunanensis*), simocyclinones, and others. Much untapped potential for antibiotic discovery remains from the *Actinobacteria* phylum and *Myxobacteria* group of bacteria, particularly from various underexplored environments including marine, tropical, semi-arid, and polar regions (Hug et al.

2018; Landwehr et al. 2016). Serpi, Ferrari, and Pertusati's overview discusses the potential use of nucleosides and their analogs as antibiotic compounds to combat bacterial and fungal infections (Serpi et al. 2016). The article notes that nucelosides and nucleoside analogs have demonstrated moderate to good antibiotic activity in the past and are already key parts of antiviral and anticancer treatments with the potential for their antibiotic usage to be even greater pending further study into their mechanisms and chemical interactions (Serpi et al. 2016). Nucleoside-derived compounds have been shown to target a number of vital biochemical processes in bacteria and fungi including nucleoside metabolism, as well as cell wall, nucleic acid, and protein biosynthesis (Serpi et al. 2016). Nucleoside analogs are also noted in this study to target many other cellular processes within these organisms, but are less understood, opening up the potential to discover new chemical compounds and mechanisms that may prove to have antibiotic capabilities (Serpi et al. 2016).

Other studies have noted novel treatment possibilities for specific bacterial targets. For example, Bassères et al. (Basséres et al. 2016) cite a number of potential novel drugs that may prove useful in treating *Clostridioides difficile* infections including surotomycin (semisynthetic lipopeptide), ridinilazole (a narrow spectrum antibiotic with activity against *C. difficile* and other members of the genus), ramoplanin (a glycolipodepsipeptide), and cadazolid (a hybrid fluoroquinolone/oxazolidinone antibiotic). Additionally, Koulenti et al. (2019) note a number of novel compounds that could be utilized for the treatment of primarily Gram-positive bacterial infections such as *S. aureus* and *Streptococcus* pneumonia including novel cephalosporins (ceftaroline and ceftobiprole), glycopeptides (telavancin, dalbavancin, and oritavancin), the oxazolidinone tedizolid phosphate, quinolones (besifloxacin, delafloxacin, and ozenoxacin), and the tetracycline omadacycline.

Specifically relating to *S. aureus*, one the most common and troublesome drug-resistant bacterial agents of human disease, Mohammad et al. in their experiments were able to synthesize two novel thiazole compounds that demonstrated significant antimicrobial activity against multi-drug resistant *S. aureus*, including MRSA and VRSA strains with an MIC of 1.38 μgml^{-1} for the first compound and an MIC of 1.40 μgml^{-1} (Mohammad et al. 2015). Interestingly, the second compound produced, which was a derivative of the first compound, was able to re-sensitize VRSA to the effects of vancomycin. It was concluded that both compounds either alone or in combination with vancomycin could be effective against multi-drug resistant *Staphylococcus* species and are capable of disrupting mature biofilms produced by these bacteria.

In order to fully understand the problem of antibiotic resistance, the history of resistance and not just the history of development and medical use of antibiotics must be examined. As stated earlier, antibiotic resistance is not exactly a new phenomenon, having been report as early, if not earlier, than the so-called golden age of antibiotic discovery (Davies and Davies 2010; Zaman et al. 2017; Ramalingam 2015). There is even evidence to suggest that antibiotic resistance may have a far more extensive history than first thought (D'Costa et al. 2011). It is therefore important to know where this problem started for modern medicine in order to understand how to proceed with the development of future antibiotic compounds so as to minimize or eliminate the problem of resistance going forward. Moreover, this information is important so that the same mistakes are not repeated again.

The development of antibiotic resistance and how Bacteria resist the effects of antibiotics

As surprising as it may seem, there is evidence that antibiotic resistance in microorganisms is far more ancient than medical history would suggest, indicating that antibiotic resistance is a process with an extensive natural history unrestricted to contemporary human records. D'Costa et al. (2011), through a combination of paleogenetic studies mediated through polymerase chain reaction (PCR) and genome sequencing, suggest that antibiotic resistance may have a history as young as 40 million years or as ancient and primordial as 2 billion years. Focusing on mainly Pleistocene megafauna mammals and the Actinobacteria family, this same study revealed that antibiotic resistance genes are actually quite prevalent from far before contemporary history. The authors also discovered that antibiotic resistance has been a widespread phenomenon naturally occurring in the environment for all that time. They additionally suggest that new antibiotics select for pre-existing resistances and this must be one of the points that guides current and future antibiotic design and usage.

The modern record of antibiotic resistance begins roughly around the time of the introduction of sulfonamide antibiotics in the 1930s. As soon as the late 30s after the large-scale introduction of sulfonamides in 1937, resistant strains of bacteria began appearing clinically (Zaman et al. 2017). The widespread introduction of penicillin and other post-sulfonamide antibiotics in the early 1940s also saw developing resistance of several bacterial strains (Davies and Davies 2010). In fact, several years before the public introduction of penicillin into medical use, during the time of the purification and laboratory experiments of Florey and Chain, bacterial penicillinases were identified by two members of the discovery team. Once penicillin treatment became common, resistant bacterial strains able to deactivate the drug became more prevalent. This necessitated developing the means to modify penicillin chemically to prevent cleavage by penicillinases.

Ironically, Alexander Fleming himself was the first who warned that bacterial resistance to penicillin could arise if used

incompletely for treatment or in a less-than-effective dosage (Zaman et al. 2017). Soon after penicillin's introduction, in the year 1944, the microbially-produced streptomycin was being used to treat tuberculosis infections. However, problems started arising when resistant strains of the infection started appearing and surviving therapeutic concentrations of the antibiotic during treatment of patients. Many other antibiotics since produced to treat tuberculosis infections have followed a similar pattern.

In the 1950s, the genetic transfer of antibiotic resistance through bacterial conjugation was identified in Japan, a phenomenon initially greeted with skepticism in the Western world, but introduced the rather startling revelation that bacterial resistance genes could be rapidly and efficiently disseminated throughout an entire population of bacterial cells and even between bacterial cells of different, but closely related genera and species. Also in the 1950s, within six years of the production of aminoglycosides, resistant strains of *Staphylococcus aureus* began to appear (Zaman et al. 2017; Ramalingam 2015). At the beginning of the 1960s, methicillin was introduced as the first semisynthetic penicillinase-resistant antibiotic to combat *S. aureus* strains capable of producing penicillinases, but resistance against methicillin was unfortunately reported soon after it was approved for use. In the 1980s, fluoroquinolones were administered primarily for the treatment of Gram-negative infections, but resistance soon began to emerge with these drugs as well. Quinolone resistance was discovered to have come about from stepwise mutations, particularly in methicillin-resistant bacterial strains. In 2002, clinical isolates of VRSA (vancomycin-resistant *S. aureus*) were discovered, 44 years after the introduction of the antibiotic in 1958.

There are a large number of different biological and biochemical methodologies by which bacteria evade or resist the effects of antibiotics. Some of the more notable examples are discussed here. One way by which bacteria are able to resist or evade antibiotics is by chemically altering the drugs. This is often accomplished through enzymes that chemically change the antibiotic in some fundamental way. Steric hindrance through the processes of acetylation, adenylation, or phosphorylation is one method of rendering an antibiotic less effective against a bacterium (Munita and Arias 2016; Zaman et al. 2017). As one specific example, aminoglycosides can be modified by bacterial aminoglycoside modifying enzymes (AMEs) that covalently modify the hydroxyl or amino groups of the antibiotic molecule. Another example of enzymatic alteration involves chloramphenicol, which inhibits protein synthesis by targeting the peptidyl transfer center of the 50S ribosomal subunit in bacteria. Chemical modification of chloramphenicol usually happens due to acetyltransferases known as CATs (chloramphenicol acetyltransferases).

Another important mechanism of antibiotic resistance utilized by some bacteria is β-lactamases to destroy antibiotics, specifically as the name suggests, β-lactams. One common drug-resistant bacterium that utilizes β-lactamases is *S. aureus* (Munita and Arias 2016). One of the key problems regarding β-lactamases is that new ones regularly and quickly evolve, usually rendering new β-lactam antibiotics ineffective in a short time. In fact, the common estimate is that there are well over 1000 β-lactamases known and likely many more will be discovered as research continues. Extended spectrum β-lactamases (ESBL enzymes) are able to hydrolyze and destroy various antibiotics including penicillin and its derivatives, third-generation cephalosporins, and monobactams, but have modest to no activity against cephamycins and carbapenems.

Another way in which bacteria can resist or evade antibiotics comes down to the three mechanisms of decreased permeability, efflux, and target site changes. Decreased membrane permeability is particularly troublesome in the case of Gram-negative bacteria since many such bacteria have multilayered membranes and some antibiotics are chemically incapable of penetrating these membranes due to various factors including differential expression of membrane molecules such as porins, the main method of entry for hydrophilic antibiotics such as β-lactams. Efflux pumps are bacterial membrane machineries that are able to pump antibiotics back out of a bacterial cell before they can exert their effects. Often these efflux pumps are substrate-specific, meaning they are able to pump out specific types of antibiotics, including macrolides and β-lactams. Yet another method of antibiotic resistance utilized by bacteria involves changes to the target site for the antibiotic. This method can take on a number of different forms including but not limited to target protection, target site non-genetically induced modification, target site mutations, enzymatic alterations to the target sites, and complete replacement of or bypass of the target site (Munita and Arias 2016; Zaman et al. 2017).

One of the most persistent and problematic forms of bacterial resistance against antibiotics is the production of biofilms. Biofilms are groupings of microorganisms in close association with each other and often adhered to an abiotic surface that are embedded in a slimy and hard-to-remove extracellular matrix composed of polysaccharides, proteins, and nucleic acids (Frieri et al. 2017; Davies and Davies 2010). Several species of drug-resistant bacteria such as *Staphylococcus aureus* and *Pseudomonas aeruginosa* are capable of forming biofilms. This mechanism often affords them both physical and chemical protection from many conventional antibiotics making treatment of these infections even more complicated and troublesome, not to mention life-threatening for many patients who find themselves afflicted with these particularly resistant strains. Biofilms are also incredibly difficult to remove once they adhere to a surface. Biofilms allow for bacteria to attach and colonize medical instruments, surgical implants such as metal joint, hip, or bone replacements,

commonly-used surfaces such as surgical trays, countertops, and hospital bed frameworks, or human tissues such as the skin, near open wounds, or internal tissues.

Several of the ESKAPE pathogens are particularly difficult to treat because they can form biofilms, sheltering the bacteria from some of the most potent antibiotics to date. The ESKAPE pathogens, among others, are some of most high-priority targets when it comes to dealing with antibiotic resistance due to their resistance mechanisms against multiple antibiotics and sometimes multiple antibiotic classes. These bacteria and their resistance mechanisms will be addressed and described in the next section.

ESKAPE pathogens

There are a large number of high-profile bacterial targets when it comes to dealing with antibiotic resistance, some implicated in drug-resistant infections more commonly than others. This section discusses some of these more concerning pathogens, specifically those that belong to the ESKAPE group of bacteria. It is worth noting all of the bacterial species described here will be part of the experimental methods that will be detailed later in this paper. ESKAPE is an acronym formed from the first letters of the genus names of each bacterium that is part of the group. Specifically, they are in this order: *Enterococcus faecium*, *Staphylococcus aureus*, *Klebsiella pneumoniae*, *Acinetobacter baumannii, Pseudomonas aeruginosa*, and various species of the genus *Enterobacter*. Each is discussed in detail on the following pages.

The first member of the ESKAPE group of bacteria is *Enterococcus faecium* which is a Gram-positive sphere-shaped (coccus) bacterium. *E. faecium*, the closely-related *Enterococcus faecalis*, and some other members of the *Enterococcus* genus are normally present as a benign part of gastrointestinal microbiome of both humans and non-human animals, the female reproductive tract, and in water and soil (Pathogen Page 2020; Willems et al. 2005; O'Driscoll and Crank 2015; Vancomycin Resistant Enterococci 2020). However, as with other bacterial species inhabiting the human body, they can prove to be opportunistic pathogens under various conditions. Of particular concern is vancomycin-resistant strains of *Enterococcus*, especially in the case of the *E. faecium* and *E. faecalis* species. VRE infections were estimated by the CDC to be responsible for nearly 55,000 drug-resistant bacterial infections from 2017 alone and of those cases 5400 or roughly 10% of those afflicted died. The CDC also estimates that roughly 30% of all nosocomial enterococcal infections are vancomycin-resistant, nearly all instances of VRE infections are hospital-related, and that the resistance of various *Enterococcus* species to different antibiotic compounds is increasing which raises grave concerns for the future of treating such infections. Additionally, there are great concerns about VRE bacteria becoming a reservoir of resistance genes that can be transferred to other bacterial pathogens. In fact, in 2002 when the first case of VRSA was reported, it was discovered that it had occurred as the result of the transmission of vanA resistance genes from a vancomycin-resistant *Enterococcus* strain (Frieri et al. 2017; Willems et al. 2005; O'Driscoll and Crank 2015).

Besides the utilization of resistance genes and the ability to transfer such genetic material to other bacteria, *Enterococcus faecium* and other members of its genus possess a number of mechanisms that allow them to evade or resist a number of conventional antibiotics (O'Driscoll and Crank 2015; Heikens et al. 2007). In addition to vancomycin, *E. faecium* has proven to be resistant to antibiotic classes including β-lactams, aminoglycosides, and glycoproteins due to factors such as penicillin binding proteins (PBPs), aminoglycoside modifying enzymes, and elimination of high-affinity D-alanine amino acid membrane precursors that glycoprotein antibiotics would normally exert their effects on. *E. faecium* also has the ability to survive on various surfaces for a significant stretch of time, up to one hour on human hands and up to four months on inorganic surfaces. *E. faecium* also possesses surface proteins that allow them to not only adhere to a variety of surfaces, but as a result form biofilms like some other members of the ESKAPE pathogens, making antibiotic treatment even more difficult than usual (Heikens et al. 2007).

Many of the previously discussed traits allow these bacteria to infest a variety of environments including inorganic healthcare-related surfaces such as countertops, surgical trays, and medical equipment, as well as numerous surgical implants such as joint and bone replacements, heart valves, cardiac stents, solid organ transplants, and catheters (Vancomycin Resistant Enterococci 2020; Willems et al. 2005; O'Driscoll and Crank 2015; VRE Pathogen Page 2020; Heikens et al. 2007). According to the CDC pathogen page for VRE, in solid organ transplant units, vancomycin-resistant *E. faecium* is the leading cause of nosocomial bloodstream infections.

Vancomycin-resistant *E. faecium* is also a common cause of several other diseases including but not limited to bacterial endocarditis, intraabdominal and pelvic infections, urinary tract infections (UTIs), very rare central nervous system infections including meningitis, and skin infections including abscesses. The most ideal way to prevent such infections is good hygiene and regular disinfection of surfaces that may potentially come in contact with material that contains VRE bacteria. There is evidence that using treatments that involve multiple antibiotics in synergy to compensate for certain resistance features of *E. faecium* may be a promising future method for dealing with infections. It is also possible for a healthy individual to have VRE living in their gastrointestinal and/or reproductive tract and not suffer from any infection because the bacteria are a natural part of the microbiota and thus are relatively harmless.

One of the more commonly known members of the ESKAPE group is of course *Staphylococcus aureus*. *S. aureus* normally is a common, Gram-positive, coccus bacterium that is benignly associated with the human body, estimated to be a commensal organism in the nasal passages of roughly 30% of the human population as well as being a common part of the skin microbiome. *S. aureus* is usually only associated with minor skin infections such as in boils or pimples, but the rise of antibiotic-resistant strains has only served to make it a microorganism of great concern (Frieri et al. 2017; Davies and Davies 2010; Zaman et al. 2017). Of particular concern are strains that show the strongest resistance to particular drugs including the all-too-familiar MRSA and VRSA strains, both of which have emerged as major nosocomial infections (MRSA 2020). Following the introduction of penicillin and its derivatives such as methicillin, *S. aureus* was quickly showing significant resistance to these antibiotics due to possessing penicillinases and various other defense mechanisms. In fact, in the early 1960s, when methicillin was first introduced, it only took 3 years for resistant *S. aureus* strains to develop.

According to the CDC pathogen page for MRSA, even though the number of reported MRSA cases are dropping gradually, there were over 300,000 cases of MRSA infections in 2017 alone and an estimated 10,600 deaths out of those cases (Dinges et al. 2000). *S. aureus* is an opportunistic pathogen with a number of different mechanisms to cause infection and perpetuate in a human host. *S aureus* is known to produce many enzymes, toxins, adhesins, and other molecules that aid it in its infection of a host (Frieri et al. 2017; Dinges et al. 2000; Chambers 2001). There is a large group of these toxin molecules called pyrogenic toxin superantigens (PTSAgs) which consists of a large number of exotoxins split between those produced by *S. aureus* and those produced by *Streptococcus pyogenes*. One such toxin is the TSST-1 exotoxin which is a superantigen that is produced by about a quarter of all *S. aureus* strains and is known to be a prime contributor to the symptoms of *S. aureus*-induced toxic shock syndrome.

It is also worth noting that *S. aureus* is capable of producing biofilms that make it extremely difficult to treat or remove in many cases, some of which are made worse if the strain in question happens to be a drug-resistant one such as MRSA or VRSA. Besides skin and soft tissue infections, *S. aureus*, including drug-resistant strains, are implicated in a variety of diseases including toxic shock syndrome, bacteremia (bacterial invasion of the bloodstream), sepsis, respiratory infections such as pneumonia, osteomyelitis, and endocarditis (Frieri et al. 2017; Davies and Davies 2010; Chambers 2001; MRSA Pathogen Page 2020). MRSA in particular most commonly spreads through skin-to-skin contact as well as sharing of personal hygiene items, and is able to spread quite rapidly throughout communities of people (MRSA 2020). This makes it vitally important for individuals to practice good hygiene habits for both the skin and other parts of the body in order to stop the bacterium from spreading, not to share personal items such as towels and razors, and to keep cuts, scrapes, and wounds clean and covered until healed.

Klebsiella pneumoniae is a bacterial species that many who have experienced pneumonia infections, particularly those contracted from hospital environments, are no doubt familiar with. *K. pneumoniae* is a Gram-negative, non-motile, lactose-fermenting, rod-shaped (bacillus) bacterium that is part of the *Enterobacteriaceae* family (Ashurst and Dawson 2020). Although the bacterium is sometimes found as a normal part of the microbial flora of the skin, nasopharynx, and gastrointestinal tract among others, it is an opportunistic pathogen and thus heavily implicated in instances of human bacterial infections, most commonly bacterial pneumonia (Vuotto et al. 2014). *K. pneumoniae* is also involved in a number of other diseases and infections such as UTIs, biliary tract infections, bacteremia, sepsis, septic shock, and other upper respiratory infections (Nordmann et al. 2009). *K. pneumoniae*-mediated pneumonia is distinguished from other forms of bacterial pneumonia such as that caused by *Streptococcus pneumoniae* by the kind of sputum a patient produces. *K. pneumoniae* causes a large amount of tissue inflammation and necrosis when it infects the lungs and surrounding tissues causing the patient to produce a thick, yellowish-to-brownish, jelly-like sputum. Even more concerning is some of the epidemiological statistics of *K. pneumoniae*. According to the CDC, it is estimated that 80% of all carbapenem-resistant *Enterobacteriaceae* infections in the year 2013 were caused by *K. pneumoniae* (Ashurst and Dawson 2020).

Additionally, approximately 12% of all hospital-acquired pneumonia infections worldwide are estimated to be caused by *K. pneumoniae*. Patients on ventilators are at slightly increased risk of contracting a pneumonia infection from this bacterium, and patients with chronic alcoholism and septicemia are at extremely increased risk for mortality from *K. pneumoniae* infections (50–100%). Some of the most concerning infections are drug-resistant strains that produce a form of antibiotic-destroying enzymes known as *Klebsiella pneumoniae* carbapenemases (KPCs). These antibiotic-hydrolyzing enzymes were first discovered in the state of North Carolina, United States in 1996. As their name suggests, they mostly target carbapenem antibiotics, but they are also capable of hydrolyzing penicillin and its derivatives, all of the cephalosporins, monobactams, and even β-lactamase inhibitors. In fact, they are a form of β-lactamases primarily produced by members of the *Enterobacteriaceae* family, but many Gram-negative bacteria can possess them, mostly as the result of conjugation or other genetic material transfer (Ashurst and Dawson 2020; Diancourt et al. 2005; Nordmann et al. 2009; Munoz-Price et al. 2009; Gasink et al. 2009). Though these enzymes confer significantly

reduced susceptibility to carbapenems and other antibiotics, they do not give complete resistance, which requires the additional measure of impaired outer membrane permeability to antibiotics, a trait that many Gram-negative bacteria possess naturally.

Among those antibiotics that can still treat KPC-producing bacterial infections are colistin, tigecycline, and aminoglycosides, but some strains are resistant even to these treatments. As Munoz-Price et al. (2009) point out, even under the best of conditions, these treatments are limited in their effectiveness. What makes the situation even worse is that there are few new drugs that are being developed currently to combat KPC-producing infections. However, these authors do suggest that taking the remaining antibiotic treatment options available for KPC-producing *K. pneumoniae* and combining them may be a viable option for the time being until new and more effective compounds can be found or developed. This method may potentially improve the survival chances of affected patients, particularly those suffering from *K. pneumoniae*-mediated bacteremia. One other method by which *K. pneumoniae* can avoid the effects of antibiotics is the production of biofilms, which in the specific case of *K. pneumoniae* can be particularly thick making antibiotic treatment of infections even more difficult (Anderl et al. 2000; Vuotto et al. 2014). Due to the strong adhesiveness of *K. pneumoniae*, this also can play a role in recurrent infections which can be hard to effectively eliminate. In order to prevent the spread of the bacterium particularly in hospitals, strict infection control protocols including good hygiene practice, proper antibiotic administration, regular cleaning of medical equipment including ventilators, and other infection control strategies are the ideal and necessary methods.

Acinetobacter baumannii is one the most dangerous and concerning members of the ESKAPE pathogen group. It is a Gram-negative, coccobacillus, non-motile bacterium possessing a large number of antibiotic resistance mechanisms that make its treatment almost impossible in some cases (Davies and Davies 2010; Acinetobacter Pathogen Page 2020; Acinetobacter CDC 2020; Peleg et al. 2008; Dijkshoorn et al. 2007; Maragakis and Perl 2008;). The CDC lists this pathogen as one of urgent concern, having infected an estimated 8500 patients in 2017 alone with a calculated total of 700 deaths (Acinetobacter Pathogen Page 2020; Acinetobacter CDC 2021). *A. baumannii* is the cause of a variety of different human diseases including some instances of hospital-acquired pneumonia, UTIs, bacteremia, meningitis, skin and soft tissue infections, and burn and open wound infections. *A. baumannii* is also involved, particularly in the case of soft tissue, burn, and open wound infections, with necrotizing fasciitis, a severe form of tissue necrosis often accompanied by septicemia and bacteremia (Howard et al. 2012). Though this type of bacterial infection is quite rare, especially compared to other members of the ESKAPE group, what makes it so dangerous are its multitude of drug resistance mechanisms .

Being an opportunistic pathogen and frequently found in hospital or other healthcare-related environments, *A. baumannii* has developed into one of the most drug-resistant microorganisms on the planet, with even pan-drug resistant strains having been reported and having a reputation for being resistant to most known antimicrobials. *A. baumannii* is also incredibly ubiquitous in the environment, being present quite commonly in soil and water samples and has the ability to survive in a large range of pH levels, temperatures, moisture conditions, and nutrient availabilities (Peleg et al. 2008; Howard et al. 2012). *A. baumannii* as a pathogen comes equipped with the complete toolkit of bacterial antibiotic resistance methods including β-lactamases (including ESBLs), the ability to change the expression of outer membrane proteins such as porins, efflux pumps, the ability to decrease antibiotic influx, modification of target sites using both genetic and non-genetic methods, rapid assimilation of resistance genes, antibiotic modifying enzymes, and biofilms. In the particular case of β-lactamases and other antibiotic-hydrolyzing or modifying enzymes, these biomolecules afford *A. baumannii* a high degree of natural and acquired resistances to a large number of antibiotics and antibiotic classes including β-lactams, aminoglycosides, fluoroquinolones, tetracyclines, glycylcyclines, cephalosporins, penicillin and its derivatives, chloramphenicol, and carbapenems (Peleg et al. 2008; Dijkshoorn et al. 2007; Maragakis and Perl 2008; Howard et al. 2012). The resistance against carbapenems has been labeled as particularly concerning to the CDC as such resistances pose dire consequences for the current state of treatment for these types of infections. With all these resistances having been identified among some strains of *A, baumannii*, Dijkshoorn et al. (2007) have concluded that the bacterium effectively possesses enough of a molecular arsenal to match most antimicrobial drugs it could possibly encounter. More worrisome still is that the CDC has discovered that carbapenem-resistant strains in particular are a repository for resistance genes that can be easily shared among different bacteria and few novel drugs to combat *A. baumannii* infections are currently in development.

Due to its many resistance mechanisms, *A. baumannii* is capable of colonizing and inhabiting many different surfaces within healthcare-related environments and can be transmitted in a number of different ways. Transmission methods and colonizable surfaces include healthcare worker and visitor hands, contaminated medical equipment, and airborne transmission such as through sneezing or coughing. Good healthcare worker hygiene practices and regular vigorous disinfection and cleaning procedures are really the only ways to prevent the spread of these bacteria. These are especially important due to the limited treatment options available that can effectively breach the bacterial defenses.

Second to last in the ESKAPE group is *Pseudomonas aeruginosa*, another Gram-negative bacillus bacterium. *P. aeruginosa* is normally quite a benign microorganism, frequently and naturally occurring in soil and water (Pseudomonas CDC 2020). As one of the ESKAPE pathogens, it is implicated in roughly 10 to 15% of all nosocomial infections worldwide (Aloush et al. 2006; Strateva and Yordanov 2009). According to the CDC, the bacterium normally is not as concerning for healthy individuals, but can be more dangerous in the cases of immunocompromised patients or in cases of patients with chronic lung diseases (MDR Pseudomonas 2020; Pseudomonas CDC 2020). The CDC estimates that in the year 2017, 32,600 patients were diagnosed with *P. aeruginosa* infections with 2700 total deaths as a result. Most frequently, *P. aeruginosa* is implicated in cases of ventilator-associated pneumonia, bacteremia, UTIs, and surgical site infections (Sadikot et al. 2005). One particular group of patients that *P. aeruginosa* often affects is those suffering from cystic fibrosis, a condition which makes these individuals extremely susceptible to recurrent and persistent *P. aeruginosa* respiratory infections. *P. aeruginosa* has earned its place as a member of the ESKAPE pathogens due to the emergence of a number of strains that have proven to be multi-drug resistant including ones that are carbapenem-resistant. A small percentage of these carbapenem-resistant strains are carriers for a mobile genetic element that produces carbapenemase enzymes and is easily transmissible between different bacteria.

These bacteria normally acquire multiple drug resistances through a combination of chromosomal mutations due to antimicrobial exposure and acquisition of extraneous resistance genes such as those responsible for hydrolyzing or modifying various antibiotics with the former process being more common (Poole 2011; Strateva and Yordanov 2009). One of the various resistances of *P. aeruginosa* involves β-lactam antibiotics. B-lactamases possessed by *P. aeruginosa* are either endogenous or acquired with the bacterium naturally possessing two chromosomal genetically-derived B-lactamases in most cases, AmpC cephalosporinase and PoxB oxacillinase. Extended-spectrum β-lactamases are also capable of being acquired by this bacterium. *P. aeruginosa* can also possess or acquire various carbapenemases as previously stated, a frequent feature of multi-drug resistant strains of the bacterium. Aminoglycoside-modifying enzymes and resistance to fluoroquinolones are also not uncommon mechanisms. Like *A. baumannii*, *P. aeruginosa* also possesses such resistance methods as altered expression of outer membrane proteins, efflux pumps to remove antibiotics before they can exert their effects, target site modification either genetically or non-genetically, and the formation of biofilms (Banin et al. 2005; Poole 2011; Strateva and Yordanov 2009; Sadikot et al. 2005). Interestingly, in the case of biofilms, Banin, Vasil, and Greenberg suggest that denying the bacteria access to iron ions, which are a key signaling molecule in the formation of *P. aeruginosa* biofilms, may help to combat this aspect of antibiotic resistance, thus eliminating or reducing the effect of at least this particular mechanism (Aloush et al. 2006). As with all the ESKAPE pathogens, particularly in healthcare settings, the best way to prevent the spread of the bacterium is through strict hygiene and disinfection/cleaning protocols. This not only helps to protect everyone within the hospital, but especially patients with chronic respiratory conditions like cystic fibrosis and patients who have recently undergone surgery (Pseudomonas CDC 2020).

The last of the ESKAPE pathogens is *Enterobacter*, a genus of bacteria with a number of species that have proven to be resistant to several antibiotics. *Enterobacter* spp. are Gram-negative, facultatively anaerobic, bacillus bacteria commonly found throughout the environment, particularly in soil and sewage, but are also commonly a benign part of the microbial flora of the human gastrointestinal tract (Davin-Regli and Pagès 2015; Mezzatesta et al. 2012). Like *Klebsiella*, *Enterobacter* is a member of the *Enterobacteriaceae* family, several members of which are implicated in a number of healthcare-acquired infections with some being multi-drug resistant like *E. cloacae* (Enterobacteriaceae Pathogen Page 2020; ESBL Pathogen Page; CRE CDC 2020; ESBL CDC 2020; Falagas et al. 2010; Castanheira et al. 2017; Kanj and Kanafani 2011; Schultsz and Geerlings 2012; Davin-Regli and Pagès 2015; Mezzatesta et al. 2012). Among some of the most concerning of these multi-drug resistant *Enterobacter* strains are those that exhibit resistance towards β-lactams and carbapenems. According to the CDC, extended-spectrum β-lactamase-producing *Enterobacter* and other members of the *Enterobacteriaceae* family were responsible for over 197,000 drug-resistant bacterial infections in 2017 with 9100 estimated deaths from these infections (ESBL Pathogen Page 2020; ESBL CDC 2020; Castanheira et al. 2017; Kanj and Kanafani 2011; Schultsz and Geerlings 2012). ESBL-producing *Enterobacter* are capable of breaking down penicillin and its derivatives, cephalosporins, and β-lactams and can infect otherwise healthy people with no underlying health conditions.

In cases where these bacteria are involved, the usual recommended course of treatment is either oral or intravenous regiments of carbapenem antibiotics. Among drug-resistant members of *Enterobacteriaceae*, two enzymes, ST131 and CTX-M, are the main sources of antibiotic resistance, able to spread rapidly across related bacterial species very quickly, especially when in combination. The problem of resistance becomes even worse when infections involve carbapenem-resistant strains of *Enterobacteriaceae* (Enterobacteriaceae Pathogen Page 2020; CRE CDC 2020; Castanheira et al. 2017; Kanj and Kanafani 2011; Schultsz and Geerlings 2012). While less common that ESBL-producing strains, they are no less dangerous as the CDC estimates that in 2017, 13,100 cases of carbapenem-resistant *Enterobacteriaceae* (CRE) infection occurred with an estimated 1100 deaths.

CRE infections mostly target patients with implanted medical devices such as catheters and patients who have had to have long treatment courses of antibiotics for other infections. 30% of these bacteria possess highly mobile genetic elements that are easily shared between different species of bacteria and encode for carbapenemases. Because carbapenems are the typical first-line course of treatment for many instances of bacterial infections today, resistance to them is a major concern regarding not just the ESKAPE pathogens, but many other bacterial pathogens as well. *Enterobacter* spp. have also proven in some instances to be resistant to other classes of antibiotics including aminoglycosides and fluoroquinolones (Kanj and Kanafani 2011; Davin-Regli and Pagès 2015; Mezzatesta et al. 2012). Other resistance mechanisms of the genus include methods utilized by other ESKAPE bacteria such as changing expression of outer membrane proteins, efflux pumps, target site mutations, and acquired resistance genes usually through plasmids.

There are still some treatment options for drug-resistant *Enterobacter* such as colistin, tigecycline, and others, but even these treatments can sometimes prove to be less than ideal. However, at least two studies may have found potential future options for the treatment of not only *Enterobacter* infections, but also infections from other *Enterobacteriaceae* members. Falagas et al. cite that fosfomycin, an antibiotic normally used in lower doses for mild urinary system infections, has the potential of being an effective antibiotic against such bacteria, showing significant activity against various *Enterobacteriaceae* members in their experiments (Falagas et al. 2010). Other studies and reviews also cite the possibility of fosfomycin as an effective treatment (Kanj and Kanafani 2011; Schultsz and Geerlings 2012). Castanheira et al. (2017) propose the potential of the antibiotics meropenem and vaborbactam as a combination treatment against these bacteria, particularly in the case of CRE infections, which are currently the most dangerous and concerning *Enterobacteriaceae* infections with their experiments showing significant activity against the CRE bacteria.

Proposed research methods for future research

Fungal organisms found in soil, livestock, and raw milk (for example) contain untapped reservoirs of novel antibiotic compounds that if isolated and identified, offer great potential to be eventually purified and refined for future use in medical treatment of bacterial infections including multi-drug resistant strains. The goal of a proposed research project would not only genetically and phenotypically identify the fungal isolates (or bacterial isolates) producing antibiotic compounds, but to quantify their effectiveness against the members of the ESKAPE pathogen group.

The first step of this process would involve sample collection. Samples derived from dairy and other livestock-related sources may be sourced from local farms in the area. These samples will include raw milk, water and feed troughs, manure, soil, and silage. The intention would to obtain samples multiple times during the calendar year to determine if there are any differences in microbial communities during various seasons and how this might affect fungi or bacteria which may produce antibiotics. The samples would be placed in sterile containers for transport back to the laboratory, where immediate culturing of the samples onto appropriate culture media – for example Sabouraud dextrose agar (SDA) for isolation of yeast and molds, or tryptic soy agar (TSA) for chemoheterotrophic bacteria. Following ambient temperature incubation for 24-48 h, unique fungal colonies would then be subcultured onto fresh 50% SDA plates, and bacterial colonies would be picked onto new TSA overlaid with a lawn of an ESKAPE pathogen. This approach is designed to stress the fungi or bacteria enough to enhance secondary metabolite production and generate visible zones of inhibition.

Genus and species identification would be performed on confirmed pure culture fungal or bacterial isolates producing reproducible zones of inhibition for at least one of the ESKAPE pathogen. Proper identification of the antibiotic-producing fungi samples to the genus and species level would necessitate both phenotypic assessment (Larone 2011), and 18S rDNA or 16S rDNA (for bacteria) and/or ITS sequence analyses using NCBI BLAST.

In order to prepare for the isolation of biologically active metabolites for chemical analysis, it would be necessary to extract the metabolome generated by zone-producing isolates grown on both solid agar and in liquid media. Each zone-producing isolate would be grown in suspension culture and spread (in triplicate) onto new 50% TSA or SDA plates for a ≥ 48 h ambient temperature incubation. One of these plates would be harvested to inoculate 25 mL tryptic soy broth or SD broth fermentation cultures grown at ambient temperature for ≥48 h prior to extraction. The metabolome produced by liquid cultures should be extracted using synthetic adsorbent resin (Diaion® HP20), which would be washed with methanol to liberate the secondary metabolites. The resultant metabolome should be dried under reduced pressure. The agar and lawn of the remaining plates should be sliced into small rectangles and placed in a Falcon tube for ethyl acetate extraction. A methanol extraction of the solid-grown cultures should also be performed *in lieu* of ethyl acetate. After agitation overnight at ambient temperature, the ethyl acetate/methanol fractions would be removed to a new tube and dried under reduced pressure. Thus, multiple means of culturing fungal or bacterial isolates, as well as multiple means of performing metabolite extraction, would be implemented.

Following resuspension of the metabolome produced by both the solid-grown and liquid-grown cultures in a small volume of methanol, these fractions should be spotted onto

new spread plates of the relevant ESKAPE tester strain to replicate the production zones of inhibition. Each extract resulting in a reproducible zone of inhibition on the new spread-plated ESKAPE tester strain(s) should be subjected to bioautography analysis using thin layer chromatography (TLC) overlaid with 50% SDA or TSA containing ESKAPE pathogens of interest. This analysis through bioautography would allow investigators to identify the biologically active metabolite within the complex extracted metabolomes. The biologically active metabolites of interest may then be purified using bioactivity-guided fractionation. Following the identification of biologically active metabolites, researchers should perform medium-scale fermentation of the zone-producing organisms (0.5 L scale) to facilitate the production of the metabolites in sufficient quantities for isolation and identification. Medium-scale fermentations should be grown in 50% SD broth or TSB media at ambient temperature for ≥48 h prior to metabolome extraction, all completed under the direction of a trained chemist with experience in new product isolation.

The whole metabolomes should be purified using multiple chromatography methods (size-exclusion chromatography followed by silica gel chromatography). Following initial purification through size-exclusion chromatography (using Sephadex® LH20 resin), the resultant fractions may be analyzed through bioautography, allowing investigators to identify fractions containing the biologically active metabolite of interest. Fractions demonstrating biological activity should be be pooled and subjected to silica gel chromatography for further purification. These fractions may similarly be subjected to analysis through bioautography to identify the biologically active metabolite of interest.

To assess the compound's purity, additional TLC analysis should be performed, and a variety of stains may be used for visualization. Investigators will select from anisaldehyde, ninhydrin, and/or potassium permanganate after consulting with a natural product chemist. Following purification of biologically active metabolites, the structure of these compounds should be determined through nuclear magnetic spectroscopy (NMR) and mass spectrometry (MS). Researchers should perform one dimensional (^{1}H and ^{13}C) and two-dimensional NMR spectroscopic analysis (Correlation spectroscopy (COSY), Heteronuclear Single Quantum Coherence spectroscopy (HSQC) and Heterononuclear Multiple Bond Correlation spectroscopy (HMBC)). Spectroscopic data, along with molecular mass data provided by mass spectrometric analyses, will facilitate the final determination of the structure of the biologically active metabolites.

Imperatives

To address and rectify the global antibiotic resistance crisis worldwide, a paradigm shift will be necessary within and beyond clinical settings. Nothing less than a complete change in societal outlook (beginning with patient care personnel) will be required to alleviate this unspoken threat to human culture. Outlooks on how prescription antibiotics should be administered, to whom, and when, need to be completely reexamined, as does implementation of an effective education plan for patients and families to enhance antibiotic stewardship. Involvement of pharmaceutical companies to reinvest capital into new product development once again needs to be incentivized. In short, changing the entire culture of how to rewrite best practices when treating infectious diseases, production animal health and well-being, and prophylaxis will be required to thoroughly and appropriately deal with this alarming global health threat.

Recently, Romo and Quiroz (2019) published a perspective on the unmet needs for appropriate antibiotic use. Their conclusions are reflected in a myriad of other published reports as well, and include the overuse of antibiotics in both human applications, as well as large-scale production animal rearing, consuming nearly half of the antibiotics in use currently in North America. The large-scale use of prophylactic antibiotics in the North American poultry industry, swine, and dairy/beef cattle production sectors is meant to promote growth of the animals in high-density feeding operations while minimizing the chances of infections (mastitis in cattle, for example) and lost revenue. Likewise, the apiculture and aquaculture industries liberally use antibiotics as prophylaxis as well. For example, oxytetracycline, erythromycin, chloramphenicol, and fluoroquinolones have all been used in the beekeeping industry to control hive infections with *Paenibacillus larvae,* one of several pathogens that lead to collapse of the entire bee colony. Bees do not metabolize any of these antibiotics however (just as fish in aquaculture settings do not catabolize antibiotics), allowing for the drugs to make their way into the honey itself, soil and surrounding groundwater systems (Bowater 2017).The primary drawback to this type of antibiotic use is that many animals will receive sub-standard antibiotic dosages, giving rise to resistant pathogens that could easily make their way into the human food chain.

In late 2018, Europe placed new restrictions on antibiotics in agricultural settings. These drastic actions are in response to fact that in the early 2010s, the European Economic Area (EEA) countries sold nearly twice the total tonnage of antimicrobials for agriculture compared to human clinical treatments (Bowater 2017). These new mandates set by the European Parliament are expected to become law in 2022, and will ban antibiotics for animals that are important for human medicine and prohibits the administration of any antimicrobials in livestock without a prescription from a veterinarian (More 2020). The general consensus among the international scientific community is that the rest of the developed world needs to follow suit.

Specifically in human clinical settings, abuse is one of the principal driving forces for MDR bacterial infections. A

survey published in 2016 reported that nearly 50% of prescription antibiotics in the U.S. during 2010–2011 were incorrect in regard to dosage and appropriate time course of administration (Fleming-Dutra et al. 2016). Spread and transmission of MDR bacteria is amplified by improper or inadequate sanitization of hospital equipment, including patient beds, healthcare equipment, fixtures, improper glove wearing and handwashing practices by healthcare personnel, and relaxed infection control protocols in general (Lopez-Romero and Quiroz 2019). Increased and permanently implemented stringencies to document and comply with proper infection control practices is vital to abrogating the spread of MDR bacterial pathogens in healthcare settings. Other related factors involving modified medical practices include obtaining and taking antibiotics without a prescription (i.e., "borrowing" antibiotics from a friend or family member's prescription), often leading to suboptimal dosage. This exposes bacteria to levels of antibiotics which allow for resistance traits to develop quickly through HGT, as previously described. (Holmes et al. 2016).

A comprehensive strategy to minimize the development and transmission of MDR pathogens involves a multifaceted approach. Firstly, clearly documenting and enforcing a rigid surveillance program in clinical environments, along with a well-accepted hand hygiene program represent important elements. Next, a well-understood isolation policy needs to be in place for cases of MDR infections when they do arise, complemented by a rigorous environmental cleanliness policy during those cases. Most fundamentally, implementation of a thorough antibiotic stewardship program needs to be prioritized; this could include educational programs and initiatives that would target all healthcare personnel, patients, family members, as well as the agricultural sector – swine, cattle/dairy, and poultry producers, for example (Quirós and Valerio 2015).

Counterfeit and substandard antibiotics are an increasing problem in many developing nations, primarily aimed at tourists from North America and Western Europe (Bowater 2017). This, combined with a poor system of regulation and documentation in these countries, and taking advantage of needy visitors in need of an antibiotic without a prescription, and a recipe for disaster occurs. Tightening or eliminating this practice is the only obvious solution to this growing issue.

The antibiotic stewardship program would have three clearly communicated goals. First, to increase the likelihood to realize the best clinical outcome in patients receiving antibiotic chemotherapy in a cost-effective way. Secondly, to minimize the risk of adverse events associated with antibiotic misuse. Third, to prolong the life span of currently available antibiotics by reducing the selective pressure that drives the development of resistant pathogens, such as the aforementioned ESKAPE bacteria (Dellit et al. 2007). Multiple studies (randomized control trials) have conclusively shown that stewardship programs result in a more appropriate administration of antibiotics and increased cure rates among affected individuals compared to existing approaches in various hospitals (Fishman 2006; Bond and Raehl 2005; Bantar et al. 2003). These data offer assurance that if implemented properly, a multi-faceted approach will effectively address and perhaps even reduce the diagnosed MDR-related infections and deaths that now increase annually throughout the world.

Conclusion

Antibiotic resistance in bacteria is one of the most pressing concerns of the medical field today and continues to present many challenges to the efficient and safe treatment of patients suffering with bacterial infections. The ESKAPE group of pathogens are among some of the most high-profile targets of this problem. Through the discovery and development of novel antibiotic compounds, the hope is to find new ways of treating patients who suffer from these infections and to learn from the mistakes of the past regarding carelessness with antibiotic use. Through the proposed thesis research project, we hope to aid in the achievement of that goal. We also hope to expand the understanding of fungal antimicrobial products and fungal ITS genetics. Overall, it is important to continue to improve the treatment of bacterial infections to not only encourage and develop a healthier society, but a healthier environment as well.

Code availability Not applicable.

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