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Pseudomonas aeruginosa at the dawn of a post-antibiotic era: clinical significance, resistance mechanisms, novel antibiotics and alternative treatments

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Abstract. Since their discovery, antibiotics have helped treat diseases prior to which many were untreatable, saving millions of lives. However, due to the overuse of antibiotics in medicine and agriculture, the advent of resistant strains of bacteria followed shortly after. The current antibiotic resistance crisis is bringing humanity closer to a post-antibiotic era, when all the advancements made by modern medicine could easily be reversed. *Pseudomonas aeruginosa* is a Gram-negative, rod-shaped bacterium, ubiquitous owing to its minimal nutritional and growth requirements. *P. aeruginosa* is one of the pathogens included in the priority list of the WHO, being assessed as critical due to its high antimicrobial resistance, leaving only a few effective treatment options to combat it. As an opportunistic pathogen, *P. aeruginosa* establishes infection in immunocompromised patients, primarily in hospital settings. In order to initiate infection, it requires several virulence factors that mediate the invasion of the pathogen into host cells. Owing to the multiple resistance mechanisms of *P. aeruginosa*, it has developed resistance to most classes of antibiotics. Due to its increased resistance, treating *P. aeruginosa* infections is a great challenge for clinicians. Several β-lactam/ β-lactamase combinations have been approved and are available as treatment options, which overall show high efficacy against *P. aeruginosa*. Moreover, novel antibiotics are currently in development as possible antipseudomonal agents, including a *Pseudomonas*-specific formulation. In addition, new strategies such as bacteriophage therapy, pyocins or the inhibition of the quorum sensing system are being investigated for the treatment of *P. aeruginosa* infections.

Keywords: antibiotic resistance, *Pseudomonas aeruginosa*, *Pseudomonas* infections, resistance mechanisms, novel antibiotics.

The antibiotic resistance crisis

Antibiotic resistance is one of the biggest threats to global health. In 2013, the Centres for Disease Control and Prevention (CDC) published the first Antibiotic Resistance Threat Report which contains the following warning: "simply using antibiotics creates resistance" - sounding the alarm on the danger brought by the increasing number of antibiotic-resistant pathogens (CDC, 2013). According to the most recent report by the European Antimicrobial Resistance Surveillance Network (EARS-Net), more than 670,000 infections occur each year in the EU/EEA which result in approximately 33,000 deaths as a direct consequence of being infected by an antibiotic-resistant pathogen (ECDC, 2019). By 2050, an estimated of 10 million deaths per year would occur as a result of acquiring an infection by a resistant pathogen (Banin *et al.*, 2017).

The discovery of Penicillin

In the 20th century, the introduction of the first antibiotic into commercial use has changed the course of medicine. In 1928, Alexander Fleming discovered that a substance produced by the *Penicillium* mold inhibits the growth of *Staphylococcus aureus*. He later named this inhibitor substance penicillin. Norman Heatley, Ernst Chain and Howard Florey continued Fleming's research and in 1944, during wartime England, they started mass-producing penicillin to treat wounded soldiers (Landecker, 2016). By conducting researches on the effects of the antibiotic, it has been revealed that it can be used for the treatment of bacterial infections. At that time, penicillin was considered a 'miracle drug' for its lack of side effects and for treating diseases that were untreatable prior to its discovery (Landecker, 2016).

Before the mass production of penicillin, the death rate of *Staphylococcus aureus* infections was estimated at 80% (Landecker, 2016). With the introduction of antibiotics, the number of infections and death rates dropped significantly, saving the lives of millions of people (Landecker, 2016; Banin *et al*., 2017).

The life expectancy of the population has also increased significantly with the use of antibiotics. In 1920, the life span of the people living in the United States was estimated at 56.4 years, whereas nowadays is estimated at 80 years (Ventola, 2019). Furthermore, in countries with poor sanitation, antibiotics decreased the mortality and morbidity rate caused by poverty-related infections (Rossolini *et al*., 2014).

Despite the major advancement of medicine brought by the discovery of penicillin, shortly after its introduction penicillin-resistant strains of bacteria were detected (Landecker, 2016).

Causes of antibiotic resistance

Antibiotic resistance occurs when a drug fails to inhibit the growth of bacteria. Therefore, bacteria become resistant and are able to multiply even in the presence of antibiotics (Zaman *et al*., 2017).

The origin of antibiotic resistance is twofold. On one hand, sequencing the genome of ancient microorganisms revealed that antibiotics and antibiotic resistance were already present in soil bacteria long before their clinical use started in the 20th century (Landecker, 2016). Soil bacteria produce antibiotics in order to interact and compete with other microorganisms for space to live in. It has been found that *Vibrio cholera* from 19th century Philadelphia had shown signs of resistance by developing efflux mechanisms (Perry *et al.*, 2016). Moreover, the 30,000 years old permafrost in the Canadian High North preserved DNA samples that show beta-lactam, glycopeptide and tetracycline resistance encoded in the genes of microorganisms (Perry *et al*., 2016). On the other hand, even though antibiotic resistance has been present for a long time, the introduction of antibiotics into medicine and agriculture by humans significantly increased the spread of resistance, causing the crisis we are facing today (Landecker, 2016). Comparing clinical specimens from the beginning of the 20th century to samples collected recently, researchers have found that the frequency of resistance genes present on plasmids has increased over time (Landecker, 2016).

It has been found that the frequency and fast distribution of antibiotic resistance is strongly correlated with the scale of antibiotic consumption of the last 80 years (Landecker, 2016; Zaman *et al*., 2017). In 1945, Alexander Fleming predicted that humanity will abuse antibiotics leading to great consequences (Landecker, 2016). The overuse of antibiotics plays a major role in the advent of resistant strains of bacteria. Incorrectly prescribed antibiotics are one of the contributing factors to the misuse, which results in patients unnecessarily receiving a high dose of broad spectrum antibiotics, for a long period of time (Ventola, 2019). Treating viral infections with antibiotics has also exacerbated the problem, especially in countries where prescriptions are not required to obtain antibiotics (Fair and Tor, 2014; CDC, 2017). Even though antibiotic prescriptions for symptoms of sore throats, colds, sinusitis decreased over time, approximately 50% of antibiotic prescriptions in the US are still unnecessary (CDC, 2017; Ventola, 2019). Patients not respecting the full course of prescribed antibiotics can also aggravate the situation. By leaving bacteria intact these can acquire multiple resistance genes over time, becoming resistant to several classes of antibiotics (Zaman *et al*., 2017).

The extensive use of antibiotics in agriculture has also led to an increase in resistance. In 1949, researchers discovered that waste products of antibiotic production could be used to promote animal growth (Landecker, 2016). Shortly after, food supplements with antibiotics entered the market promising faster growth and less disease for the livestock. Food animals grew to a larger size in a shorter period of time, while receiving the same amount of food as before (Landecker, 2016). An estimated of 80% of antibiotics sold in the US are used in agriculture (Ventola, 2019). Researchers have demonstrated that by consuming animals treated with antibiotics, the drug could easily transfer to humans too, which in turn led to the development of antibiotic-resistant bacteria in their intestinal flora (Ventola, 2019). Moreover, up to 90% of antibiotics given to farm animals is excreted into their nearby environment, driving further the spread of antibiotic resistance (Ventola, 2019). In 2003, the Food and Drug Administration (FDA) banned the use of antibiotics as a means to promote animal growth in Europe, but in several countries this is still an ongoing problem (Fair and Tor, 2014).

Another contributing factor has been the stalling of new antibiotic development by the pharmaceutical industry, leaving only a few effective options to treat resistant bacteria (Ventola, 2019). Half of the antibiotics that are in use today were discovered and developed up until the 1940's and 1960's. Several pharmaceutical companies have abandoned antibiotic development, others merged into one another reducing the number of research groups, moreover, academic research in this area has decreased significantly due to economic constraints (Ventola, 2019). Due to the fact that antibiotics are used only for a short period of time and result in fast recovery, it is not as profitable to pharmaceutical companies to invest in their development as it is in the case of drugs used in the treatment of chronic diseases (Ventola, 2019).

In recent months, the coronavirus pandemic has also lead to an increase in antibiotic use, accelerating the threat of global antibiotic resistance (Nature Editorial, 2020). Despite the fact that COVID-19 is caused by a virus, patients admitted in hospital units are given antibiotics to treat or prevent secondary bacterial infections (Khan, 2020).

On July 9, 2020 the AMR Action Fund has been launched in order to take action and respond to the pressing antibiotic resistance crisis (Bott and Holland, 2020). More than 20 leading biopharmaceutical companies have invested US$1 billion so far in the clinical development of new antibiotics to combat resistant pathogens (Jones and Holland, 2020). The AMR Action Fund aims to develop 2-4 new antibiotics by 2030 (Bott and Holland, 2020).

Important bacterial pathogens

Several Gram-positive and Gram-negative bacteria are responsible for causing serious infections in humans. In the 1990's, with the rise of methicillin- and vancomycin-resistant strains of *Staphylococcus aureus*, penicillin-resistant *Streptococcus pneumoniae* and multidrug resistant *Clostridium difficile*, Gram-positive bacteria represented a major public health threat (ECDC, 2019). Even though these bacteria are still widespread, recent EARS-Net (European Antimicrobial Resistance Surveillance Network) data show that their resistance has decreased or optimized over time (ECDC, 2019). During the last decade, Gram-positive bacteria were the focus of new antibiotic development leading to the appearance of several new strains of resistant tuberculosis and strains of resistant Gram-negative bacteria, which are harder to fight due to their complex outer membrane (Fair and Tor, 2014).

In 2017, the World Health Organization (WHO) published a global priority pathogen list (Fig. 1), with the purpose of promoting the research and development of new antibiotics (WHO, 2017). The list contains 12 species of bacteria that are classified based on their level of resistance (critical, high, medium). Ten criteria were chosen to group these pathogens in their respective category, assessed by 70 experts with different backgrounds (Tacconelli *et al*., 2018).

Pseudomonas aeruginosa is one of the bacteria among *Acinetobacter baumannii* and members of the Enterobacteriaceae family classified in the critical, priority 1 category. These bacteria pose a major threat to public health with the life-threatening infections they cause, due to the very few effective treatment options available. Novel antibiotics are in urgent demand to combat these pathogens.

Pseudomonas aeruginosa

Morphology

Pseudomonas aeruginosa is a Gram-negative, rod-shaped bacterium of the *Pseudomonadaceae* family, measuring 0.5-0.8 μm by 1.5-3.0 μm. (Neves *et al.*, 2014; Planet, 2017). Similarly to the other members of the *Pseudomonas* genus, *P. aeruginosa* can commonly be found in nature, soil, water, in humans, even on the surface of plants and sometimes of animals too (Todar, 2004). Almost all of its strains move by the means of one polar flagellum (Todar, 2004; Neves *et al*., 2014). By forming biofilms, *P. aeruginosa* is able to colonize various surfaces and is protected from the activity of several antimicrobial agents (Neves *et al*., 2014).

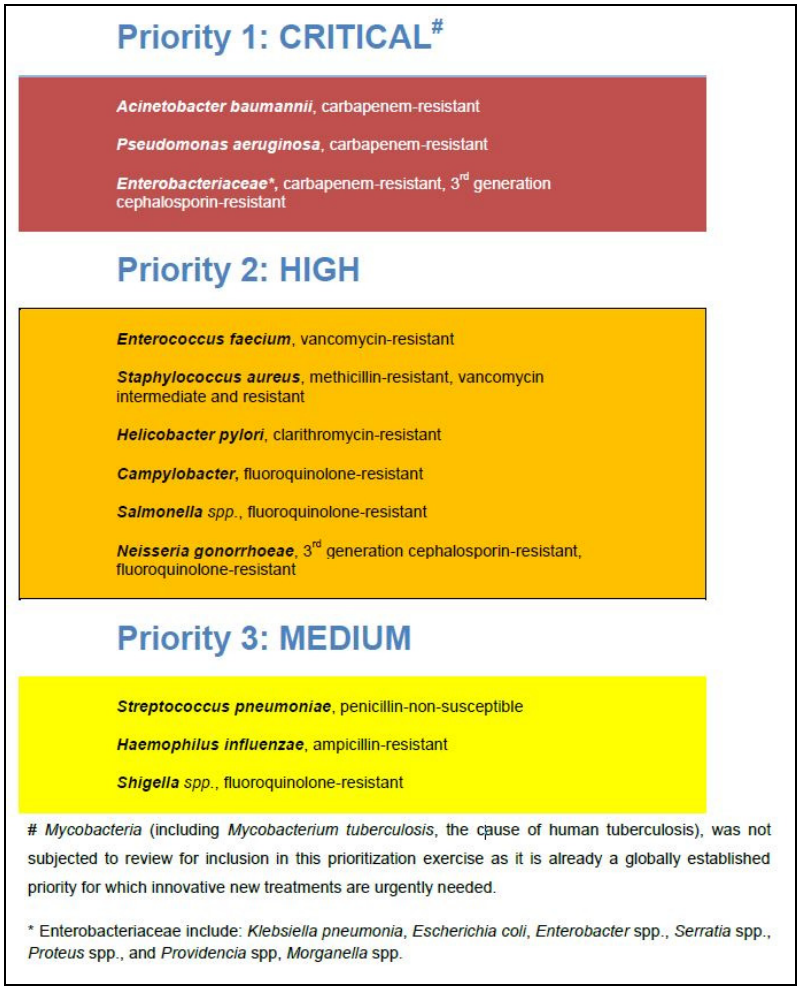

Figure 1. Global priority pathogen list (WHO, 2017)

From a clinical point of view, *P. aeruginosa* has a major significance due to its increasing antibiotic resistance, making the life-threatening conditions it causes very challenging to treat (Todar, 2004). *P. aeruginosa* is an opportunistic pathogen, exploiting the host's defense mechanisms in order to initiate infection, affecting especially immunocompromised patients in healthcare units (Todar, 2004).

P. aeruginosa is an obligate aerobe bacterium, but it can also thrive in environments that lack oxygen by using arginine and nitrate as a final electron

acceptor, allowing it to achieve anaerobic growth too (Neves *et al*., 2014). *P. aeruginosa* is able to metabolize a variety of substances, has basic nutritional requirements for growth and is able to multiply at as high as 42°C (Todar, 2004; Neves *et al*., 2014). All of these factors allow *P. aeruginosa* to adapt to environments hardly tolerated by other microorganisms.

Its name, *Pseudomonas aeruginosa*, comes from the Greek *pseudo* meaning "false" and from the Latin *monas* which means "single unit", meanwhile the name *aeruginosa* means "copper rust" in Latin (Neves *et al*., 2014). Throughout history, *P. aeruginosa* has received different names based on the blue-green color of its cultures. At first it was named *Bacillus pyocyaneus*, which encompasses the two main characteristics of *P. aeruginosa*: its rod-shape and the distinct color of the cultures which is due the pigments it produces (Lister *et al*., 2009).

In 1882, French scientist Carle Gessard was the first to report this bacterium, after successfully isolating it from the wound infections of patients whose bandages had turned to a blueish-green color (Lister *et al*., 2009). He published his findings in a scientific study entitled "On the Blue and Green Coloration of Bandages". This study showed that *P. aeruginosa* produces water-soluble pigments which are fluorescing in a blue-green color under a specific light (Neves *et al*., 2014). Most colonies of *P. aeruginosa* are able to produce two pigments: pyocyanin, which is soluble in water and chloroform, giving colonies a blue color; and pyoverdin, soluble only in water, giving a yellow-green color to the cultures (Neves *et al*., 2014). In addition, by producing other pigments like pyorubrin or pyomelanin, *P. aeruginosa* colonies can turn to a red or brown color (Neves *et al*., 2014). During growth, the colonies produces a grape-like odor (Neves *et al*., 2014).

Epidemiology of Pseudomonas aeruginosa infections

P. aeruginosa can initiate infections on any part of the human body**.** The colonization rate varies and is specific to different sites: skin (0-12%), nasal mucosa (0-3.3%), throat (0-6.6%) and stool (2.6-24%) (Morrison and Wenzel, 1984). The prevalence of colonization in a hospital setting can be up to 50%, especially in the case of patients whose skin barrier and mucosa have been compromised due to severe burns, surgery, mechanical ventilation or catheters (Lister *et al*., 2009). Patients who are suffering from a significant underlying disease have a bigger chance of developing serious infections (Golemi-Kotra, 2008).

A recent report from the US classifies *P. aeruginosa* as the sixth most common nosocomial pathogen and the second most common cause of ventilator-associated pneumonia (VAP) *(*Nguyen *et al*., 2018). *P. aeruginosa* is accountable for 10-15% of nosocomial infections worldwide (Strateva and Yordanov, 2009),

17% of pneumonia cases, 7% of urinary tract infections and 8% of surgery site infections (Skariyachan *et al*., 2018). Due to its intrinsic resistance to antibiotics, treating infections caused by *P. aeruginosa* is a real challenge to health officials (Strateva and Yordanov, 2009).

Clinical features of Pseudomonas aeruginosa infections

When *P. aeruginosa* colonizes the endocardium of the host, it can lead to endocarditis, the infection of heart valves, which can result in heart failure and the destruction of the heart valves (Golemi-Kotra, 2008).

Respiratory infections caused by *P. aeruginosa* affect mostly patients who have compromised lower respiratory tracts and their immune system is weakened due to a recent transplant, HIV infection, neutropenia or cancer (Sadikot *et al*., 2005; Golemi-Kotra, 2008). Lung infections result in the highest mortality rates (Fujitani *et al*., 2011). Bacteremic *P. aeruginosa* pneumonia can be characterized by necrosis and the invasion of the blood vessels, nonbacteremic pneumonia on the other hand shows signs of hemorrhage, microabscesses and focal necrosis (Fujitani *et al*., 2011). When *P. aeruginosa* invades the upper respiratory tracts, it can cause community-acquired pneumonia (CAP), which is thought to be the cause of cystic fibrosis in children and chronic lung infection (Fujitani *et al*., 2011). A more common syndrome associated with *P. aeruginosa* lung infection is hospital-acquired pneumonia (HAP) (Fujitani *et al*., 2011).

Immunocompromised patients are also susceptible to bacteremia and sepsis. Nearly 25% of bacteremia occurring in hospitals is due to *P. aeruginosa* infections (Golemi-Kotra, 2008). Analyzing the wound infections of burn victims, it has been observed that during the first week of hospitalization the frequency of *P. aeruginosa* colonization increases significantly (Driscoll *et* al., 2007). Patients undergoing transplants are at greater risk of developing bacteremia compared with patients who are not requiring such a n intervention (Driscoll *et al*., 2007).

P. aeruginosa can also initiate infection in the central nervous system (Golemi-Kotra, 2008). These infections can lead to meningitis or brain abscesses. The pathogen invades the brain from the inner-ear or para-nasal sinus, but it can also get to the infection site as a consequence of head traumas, surgery or during invasive diagnostic procedures. Moreover, it can transfer to its final destination from a different infection site (Golemi-Kotra, 2008).

Infections of the skin caused by *P. aeruginosa*, such as folliculitis, dermatitis, otitis externa, are related to burn injuries or other types of infections (Pál, 2013). More severe forms of ear infections can occur in elderly patients who develop face paralysis, hearing deficiencies as a consequence of acquiring *P. aeruginosa* infections, which in some cases can be fatal (Golemi-Kotra, 2008).

P. aeruginosa can also lead to severe eye infections. *P. aeruginosa* is accountable for the majority of bacterial keratitis cases, which can be attributed to improper contact lens use (Golemi-Kotra, 2008). In severe cases, this can result in the degradation of the entire eye (Golemi-Kotra, 2008).

Urinary tract infections (UTIs) are the most prevalent hospital-acquired diseases that affect humans (Mittal *et al*., 2009). Catheterization of the urinary tract predisposes patients to infections by different pathogens, *P. aeruginosa* accounting for 7-10% of the cases (Mittal *et al*., 2009; Lamas Ferreiro *et al*., 2017).

Virulence factors produced by *Pseudomonas aeruginosa*

Several factors contribute to the success of *P. aeruginosa* to initiate infection: its versatile metabolism, the intrinsic and acquired antibiotic resistance mechanisms, its ability to form biofilms and the expression of virulence factors (Balasubramanian *et al*., 2013).

Adhesins

An important first step in establishing infection is the adhesion to the host tissues. *P. aeruginosa* can attach to a variety of surfaces which the pathogen uses as a means to colonize, multiply and exploit for resource acquisition (Wu *et al*., 2014). In the case of *P. aeruginosa*, adhesion takes place with the help of cell-surface components: type IV pili, flagella and the LPS (Wu *et al*., 2014).

Type IV Pili-Mediated Adhesion

The surface of *P. aeruginosa* is covered with type IV pili, which are flexible and retractable filaments having pilin components and measuring 5.2 nm in diameter and 2.5 μm in length (Wu *et al*., 2014). Type IV pili play an important role in biofilm formation, in the movement and signaling processes of the pathogen (Wu *et al*., 2014). It has been demonstrated that pili promote adhesion to host cells in 90% of the cases, the virulence of *P. aeruginosa* decreasing significantly in the event of pili malfunction (Wu *et al*., 2014).

Flagella-Mediated Adhesion

P. aeruginosa moves by the means of a polar flagellum having a FliC flagellin as its main component (Wu *et al*., 2014). The flagellum has a role in motility and biofilm formation, thus, in the event of its deterioration a decrease in pathogenicity can be observed (Wu *et al*., 2014).

The flagellum has several receptors. The main adhesion point of *P. aeruginosa* is represented by mucin, located on the host cell surface (Wu *et al*., 2014). In addition, it has been found that an asialo GM1 receptor on the host

epithelial cells could also play a role in binding the pathogen (De Bentzmann *et al*., 1996). Flagellin can also attach to a Toll-like receptor 5 (TLR5), which plays a role in activating the immune system (Hayashi *et al*., 2001; Wu *et al*., 2014).

Lipopolysaccharide-Mediated Adhesion

The lipopolysaccharide (LPS) is a surface structure, a complex glycolipid, that comprises a major part of the outer membrane of Gram-negative bacteria, including *P. aeruginosa* (Al-Wrafy *et al*., 2017).

The lipopolysaccharide of *P. aeruginosa* is composed of three domains: lipid A anchored in the membrane, the core oligosaccharide and O-polysaccharide (Al-Wrafy *et al*., 2017). Factors such as susceptible patients, the structure of lipid A and the modifications in the O-specific polysaccharide all lead to an increase in the lipopolysaccharide mediated pathogenicity of the bacterium (Al-Wrafy *et al*., 2017).

The core oligosaccharide of the lipopolysaccharide promotes the attachment of *P. aeruginosa* to the CFTR receptor (Cystic Fibrosis Transmembrane Conductance Regulator) of epithelial cells (Wu *et al*., 2014). The CFTR protein assures that the epithelial cell membranes are covered with a moderate amount of mucus (Wu *et al*., 2014). In case of a malfunction of the CFTR, the overload of mucus creates an ideal environment for the pathogen to thrive (Wu *et al*., 2014).

Secretion systems of *Pseudomonas aeruginosa*

The secretion of different proteins allows *P. aeruginosa* to compete with other microorganisms, but it can also lead to the host manipulation and invasion (Green and Mecsas, 2016). Through secretion systems, pathogens are able to transfer their toxins and exoenzymes in the extracellular matrix or the cytosol of host cells (Green and Mecsas, 2016).

Seven secretion systems of Gram-negative bacteria have been described, from which five can be found in *P. aeruginosa* (Wu *et al*., 2014).

The type I secretion system (T1SS) allows small molecules, such as toxins or antibiotics, to be exported in a one-step process from the bacterial cell (Green and Mecsas, 2016). This systems has three structural components: the ABC transporter found in the inner membrane, a membrane fusion protein (MFP) situated in the membrane and the outer membrane factor (OMF) which forms pores on the surface (Green and Mecsas, 2016). In the case of *P. aeruginosa*, two types of T1SS have been found: a system that secrets AprA protease, an important virulence factor; and one that secrets HasAp which binds to the heme part of hemoglobin, which is thought to play a role in the initial infection and the survival of the pathogen (Wu *et al*., 2014).

The type II secretion system (T2SS) consists of a two-step process in which proteins are secreted (Wu *et al*., 2014). Because T2SS is situated in the outer membrane, another pathway is needed in order to transfer proteins first to the periplasm and only then it can be secreted to the extracellular matrix (Green and Mecsas, 2016). Two T2SS can be found in *P. aeruginosa:* Xcp (extracellular protein) and Hxc (Wu *et al*., 2014). Important virulence factors such as exotoxin A, phospholipase C, LasA, LasB and PrpL can be excreted through T2SS (Wu *et al*., 2014).

Virulence factors are directly injected into host cells through the type III secretion system (T3SS) (Green and Mecsas, 2016). T3SS has three domains: the base complex, the needle and the translocon (Green and Mecsas, 2016). In animal models, T3SS has shown a significant role in the initiation of burn infections, pneumonia and lung infections (Wu *et al*., 2014).

The type V secretion system (T5SS), similarly to the T2SS, uses an additional pathway in order to transfer proteins to the periplasm (Wu *et al*., 2014; Green and Mecsas, 2016). Unlike other secretion systems, T5SS doesn't require a special membrane channel, they secretes proteins to the extracellular matrix by themselves (Green and Mecsas, 2016). EstA, LepB and LepA are among the proteins that are secreted with T3SS (Wu *et al*., 2014).

The most recently discovered structure is the type VI secretion system (T6SS), which can inject proteins directly into host cells through its needle component (Green and Mecsas, 2016). Three T6SS can be found in *P. aeruginosa* (HIS-I, HIS-II, HIS-III), from which one (HIS-II) plays a significant role in initiating infection at the site of epithelial cells (Wu *et al*., 2014).

Toxins and exoenzymes produced by *Pseudomonas aeruginosa*

P. aeruginosa is able to invade tissues through the production of toxins and exoenzymes. By damaging host cell barriers, such as the exopolysaccharides, lipopolysaccharides and the components of the host defense mechanisms, the pathogen can initiate infection with the evasion of the innate immune system.

Exotoxin A

Exotoxin A is a member of the mono-ADP-ribosyltransferases class and is being excreted by the T2SS (Wu *et al*., 2014). As a result of its ADP-ribosyltransferase activity, it can modify and inactivate elongation factor 2 (Eef-2), which leads to the inhibition of protein synthesis, resulting in cell death (Wu *et al*., 2014). Exotoxin A has three domains, which all have different roles in the invasion process: the N terminal-domain (Ia) binds to the receptor, the second domain (II) transports the exotoxin through the membranes and the third domain (III) inactivates elongation factor 2 (Wu *et al*., 2014).

Exoenzymes S, T, U

Four enzymes are secreted by the T3SS: ExoS and ExoT, having ADP-rybosiltransferase and GTPase-activating activity; ExoU, exhibiting lipase activity and the adenylate cyclase ExoY (Wu *et al*., 2014). ExoS plays a role in the transport of vesicles, cell proliferation, differentiation and apoptosis (Wu *et al*., 2014). Its ADP-rybosiltransferase activity has been shown to be correlated with the pathogen's long term survival and dissemination at lung infection sites (Wu *et al*., 2014).

Proteases

The two metalloproteases (elastase and alkaline protease) produced by *P. aeruginosa*, have a role in evading the host immune system, allowing the pathogen to successfully establish infection (Kharazmi, 1991; Golemi-Kotra, 2008). These enzymes interfere with the activity of neutrophils, monocytes, natural killer cells and T cells, that defend the host against bacterial invasions (Kharazmi, 1991). Elastase and alkaline protease have been shown to cleave immunoglobulins G and A, moreover, cytokines such as interleukin-1, interleukin-2, tumor necrosis factor and interferon gamma (Kharazmi, 1991).

Elastases are the most abundant proteases produced by *P. aeruginosa* (Wu *et al*., 2014). They damage components of the extracellular matrix by cleaving elastin, an important element of the lung tissue and blood vessels (Wu *et al*., 2014; Planet, 2017).

Neuraminidases

Neuraminidases are glycoside hydrolase enzymes which cleave terminal neuraminic acids of glycoproteins, glycolipids and gangliosides expressed on the surface of epithelial cells (Ghazaei *et al*., 2010). This results in the increase of gangliosides that lack neuraminic acids, also known as asialo Gm1, which are prevalent in cystic fibrosis lung tissues and serve as receptors of the respiratory tract (Strateva and Mitov, 2011). This enzyme plays an important role in different processes involving colonization, such as attachment to host cells, especially epithelial cells, and invasion (Ghazaei *et al*., 2010; Planet, 2017). Moreover, in early phases of invasion, neuraminidase also promotes biofilm formation (Strateva and Mitov, 2011).

Cytotoxic exoproducts produced by *Pseudomonas aeruginosa*

P. aeruginosa produces three cytotoxic proteins: a cytotoxin and two hemolysins (Strateva and Mitov, 2011). The cytotoxin forms pores in the membranes of immune cells, resulting in cell inactivation (Strateva and Mitov, 2011).

The two hemolysins of *P. aeruginosa,* phospholipase C and rhamnolipid, have a role in breaking down lipids and lecithin, respectively (Golemi-Kotra, 2008; Strateva and Mitov, 2011). Rhamnolipid is a glycolipid surfactant which is thought to solubilize the lung surfactant phospholipids, making it easier for the phospholipase C to cleave them (Strateva and Mitov, 2011). This synergistic activity has been found to have an important role in initiating acute or chronic lung infections (Strateva and Mitov, 2011). In addition, it has been observed that rhamnolipids have functions in mucociliary transport, in the formation of the complex structure of biofilms and in disrupting macrophage activity (Wu *et al*., 2014).

Phospholipase D is a common protein found in eukaryotic cells. By hydrolyzing phospholipids, phospholipase D it generates a signaling lipid, phosphatidic acid (PtdOH), which promotes several cellular processes (Spencer and Brown, 2015).

Iron chelation

Iron chelation is essential for *P. aeruginosa* to initiate infection. Being bound to hemoglobin or ferritin, iron is very limited in the host environment, therefore the pathogen is unable to access it directly (Pál, 2013). In order to sequester iron from the environment, *P. aeruginosa* produces iron chelator molecules, siderophores, to be able to grow under low-iron conditions (Golemi-Kotra, 2008; Al-Wrafy et al., 2017). These siderophores are pyochelin, a pyocyanin derivative, and pyoverdine (Strateva and Mitov, 2011).

Pyocyanin

Pyocyanin, belonging to the class of phenazines, is one of the pigments produced by *P. aeruginosa* and is secreted through the T2SS (Hall *et al*., 2016). The exact function of pyocyanin in the pathogenicity of *P. aeruginosa* is not well understood, but it was shown in high concentration in wounds, urine and sputum samples following *P. aeruginosa* infections (Hall *et al*., 2016). Pyocyanin increases levels of reactive oxygen species inside host cells, causing oxidative stress (Hall *et al*., 2016). Moreover, pyocyanin has shown pro-inflammatory properties by interfering with cytokines, inhibiting interleukin-2 release, decreasing the expression on interleukins on T-cells, decreasing immunoglobulin secretion, all resulting in *P. aeruginosa* evading the host immune system (Hall *et al*., 2016). The oxidative stress and inflammation caused by *P. aeruginosa* infections leads to the damage of the respiratory system (Hall *et al*., 2016; Al-Wrafy *et al*., 2017).

Lectins

The outer membrane of *P. aeruginosa* has two soluble lectins, LecA and LecB, which might contribute to the adhesion process of the pathogen to host cell surfaces (Al-Wrafy *et al*., 2017). In addition, lectins could also have a role in the dissemination of the bacterium at infection sites, affecting the survival and biofilm formation of *P. aeruginosa* (Al-Wrafy *et al*., 2017).

Biofilm formation

P. aeruginosa has been an important model organism in studying bacterial biofilm formation (De Kievit, 2009; Miller *et al*., 2012). This opportunistic pathogen is able to form biofilms on medical equipment, catheters, implants, resulting in severe infections of hospitalized patients (Driscoll *et al*., 2007).

Depending on the conditions of growth, *P. aeruginosa* biofilms can have either complex, 'mushroom-like' architectures or a flat appearance (Klausen *et al*., 2003; Miller *et al*., 2012). Type IV pili and the polar flagellum help the pathogen move in a variety of environments which is essential for generating biofilms (Klausen *et al*., 2003). In the first stage of biofilm formation, planktonic cells reversibly attach to the surface via their flagellum, showing surface associated motility, followed by irreversible attachment and matrix production, and finally the microcolony is formed (Miller *et al*., 2012). Two different types of subpopulations can be observed at this stage: one that keeps exploring the surface and one that has anchored, forming a cap-like structure (Miller *et al*., 2012). When the motile subpopulation moves atop of the attached cells, it creates a cap-like formation, resulting in a mushroom-like architecture (Miller *et al*., 2012). Undergoing maturation, the biofilm forms cavities filled with fluid on the surface of the cap, allowing planktonic cells to detach from the matrix and disperse, presumably forming new microcolonies in a new site (Miller *et al*., 2012).

By producing biofilms, *P. aeruginosa* demonstrates higher antibiotic resistance and is protected from the defense mechanisms of the host, in contrast with planktonic cells that remain susceptible to different antipseudomonal agents (Wu *et al*., 2014). It has been found that *P. aeruginosa* biofilms in cystic fibrosis lungs show higher level of resistance to antibiotic therapies, which has led to the longer survival of the pathogen in the lung tissue (De Kievit, 2009; Wu *et al*., 2014). Moreover, bacterial cells living in proximity to each promotes horizontal gene transfer (De Kievit, 2009).

Biofilms are composed of microcolonies (15%) and of matrix elements (85%) (Skariyachan *et al*., 2018). Extracellular polysaccharides produced by bacteria (exopolysaccharides) comprises of DNA, proteins and polysaccharides (Skariyachan *et al*., 2018).

Exopolysaccharides have an important role in the formation of microcolonies and the architecture of biofilms (De Kievit, 2009; Papp, 2015). *P. aeruginosa* can produce three types of polysaccharides: *pel*, *psl* and alginate (Skariyachan *et al*., 2018). It has been demonstrated that in the lungs of cystic fibrosis patients the mucoid *P. aeruginosa* strains promote oxidative stress and degradation of tissues by attracting immune cells to the site of infection (Skariyachan *et al*., 2018). Alginate overproduction leads to the appearance of mucoid phenotypes of *P. aeruginosa*, which are protected from the host defense mechanisms and show high level of antibiotic resistance (Skariyachan *et al*., 2018). Extracellular DNA also has an important role in generating the structure of biofilms (De Kievit, 2009).

When biofilms mature, cavities on their surface allow the acquisition of nutrients and the secretion of waste products (De Kievit, 2009). It has been elucidated that rhamnolipids are the components that maintain the open structure of these channels (De Kievit, 2009). Moreover, rhamnolipids are important in the development of complex biofilm structures, the detachment of planktonic cells from biofilm cavities and the formation of microcolonies (De Kievit, 2009).

Quorum sensing

Quorum sensing (QS), a cell-to-cell communication mechanism, plays an important role in generating biofilms and in regulating virulence factor production (De Kievit, 2009; Papp, 2015; Al-Wrafy *et al*., 2017). The signal molecules (autoinducers) of quorum sensing systems can be classified in three groups: acylhomoserine lactones (AHLs), oligopeptides and the autoinducer 2 (De Kievit, 2009). In the case of Gram-negative bacteria, autoinducers belong to the AHL family which activate a transcription factor (R protein), resulting in gene expression (De Kievit, 2009). *P. aeruginosa* has two primary AHL quorum sensing systems: Las and Rhl (De Kievit, 2009; Strateva and Mitov, 2011). These systems are generally composed of an acyl-homoserine lacton and a transcriptional activator (Strateva and Mitov, 2011). In addition, a quinolone-based system can also be found in *P. aeruginosa* (Al-Wrafy *et al*., 2017).

Antimicrobial resistance of *Pseudomonas aeruginosa*

Data of EARS-Net collected from 2015 to 2018 shows that in the EU/EEA 32.1% of *P. aeruginosa* isolates were resistant to at least one class of antibiotics, from which the highest population-weighted mean resistance in 2018 was found to be for fluoroquinolones (19.7%), followed by 18.3% of the isolates being resistant to piperacillin ± tazobactam, 14.1% to ceftazidime, 17.2% to carbapenems, and 11.8% of the isolates showing resistance to aminoglycosides (ECDC, 2019). Of all the *P. aeruginosa* isolates tested, 19.2% were resistant to

at least two or more classes of antibiotics (ECDC, 2019). The mean resistance observed to different classes of antibiotics has decreased from 2015 to 2018 (ECDC, 2019). EARS-Net data reported between 2015 and 2018 from Romania show a mean resistance of 49.4% for *P. aeruginosa* isolates, which is an alarming value compared to other EU countries, but this high percentage might be due to Romanian laboratories failing to provide necessary data, misrepresenting the actual state of *P. aeruginosa* resistance in the country (ECDC, 2019).

Extensively drug-resistant (XDR) *P. aeruginosa* strains are susceptible only to two classes of antibiotics, whereas pan-drug resistant strains show resistance to all classes of antibiotics (Horcajada *et al*., 2019). In a large-scale study conducted in Spain, researchers analyzed the resistance of *P. aeruginosa* isolates collected from 51 hospitals. It has been found that 26% of the tested isolates were multidrug-resistant and of those, 65% were extensively drug-resistant strains (Horcajada *et al*., 2019).

Infections caused by *P. aeruginosa* have a high mortality rate due to the fact that the pathogen has an increased resistance to antibiotics, leaving only a few effective options for treatment (Poole, 2011).

Resistance mechanisms of *Pseudomonas aeruginosa*

P. aeruginosa shows intrinsic resistance to most classes of antibiotics due to its less permeable outer membrane, prohibiting antibiotic molecules to enter the bacterial cell (Poole, 2011; Pál, 2013; ECDC, 2019). The acquired resistance mechanisms are a result of mutational changes and the harboring of resistance genes (Poole, 2011). Acquired resistance mechanisms of *P. aeruginosa* include: upregulation of efflux pumps which enable the removal of antimicrobials from bacterial cells, inactivation of antimicrobials by enzymes, such as β-lactamases, and the alteration of the target of antibiotics (Poole, 2011).

Mechanisms of resistance to β-lactam antibiotics

β-lactam antibiotics are one of the most widely used group of antibiotics in the fight against several Gram-negative and Gram-positive bacteria. Penicillins, cephalosporins, carbapenems and monobactams are all part of the β-lactam family, having in common the four-membered β-lactam ring as a core structural feature (Poole, 2004; Pál, 2013).

The mechanism of action of β-lactam antibiotics involves the obstruction of bacterial cell wall synthesis (Donowitz and Mandell, 1988). β-lactams bind to enzymes called penicillin-binding proteins (PBPs), located in the inner bacterial membrane, consequently inhibiting their activity (Cascella, 2019). PBPs facilitate the last steps of biosynthesis of the peptidoglycan layer, an important layer of

the cell wall. β-lactams share a similar structure with a substrate of PBPs, D-alanyl-D-alanine, thus allowing it to bind easily to the mentioned bacterial protein (Zhanel *et al*., 2014). Peptidoglycan cross-linking is prohibited with the binding and inactivation of PBPs, subsequently causing cell lysis. (Donowitz and Mandell, 1988). In the case of *P. aeruginosa*, β-lactams target a variety of PBPs: PBP1b, PBP1c, PBP2, PBP3 and the PBP4 (Zhanel *et al*., 2014).

β-lactamases

A major resistance mechanism of *P. aeruginosa* to β-lactams is the production of β-lactamases, which are hydrolytic enzymes that cleave the amide bond of β-lactam antibiotics (Poole, 2004; Poole, 2011).

P. aeruginosa carries genes for two different β-lactamases: AmpC (class D cephalosporinase) and PoxB (class D oxacillinase) (Poole, 2011). AmpC β-lactamase is strongly correlated with the β-lactam resistance of *P. aeruginosa* clinical isolates (Poole, 2011). The production of AmpC is induced by the presence of certain β-lactams and β-lactamase inhibitors, which in turn contributes to the intrinsic resistance of *P. aeruginosa* to these antibiotics (Poole, 2011; Nguyen *et al*., 2018). The presence of β-lactams result in the increased intracellular level of peptidoglycan components (Nguyen *et al*., 2018). These components bind to the AmpR repressor which subsequently deactivates the repression of the *ampc* gene, leading to the hyper-production of AmpC β-lactamases (Nguyen *et al*., 2018). This mutational derepression of the *ampc* gene is the most important mechanism of resistance to β-lactams (Poole, 2011). AmpC β-lactamases have effect against a few penicillins, cephalosporins and other agents such as ceftazidime and piperacillin-tazobactam (Nguyen *et al*., 2018).

In contrast with the above mentioned endogenous β-lactamases that only confer resistance to narrow-spectrum antipseudomonal agents, acquired β-lactamases contribute to a wider range of resistance to different antimicrobials (Poole, 2011). Acquired β-lactamases include: extended-spectrum β-lactamases (ESBL), which hydrolyze broad-spectrum cephalosporins and monobactams; and carbapenemases which are able to inactivate most β-lactams (Poole, 2011).

Efflux pump systems

From the five efflux pump families described in *P. aeruginosa*, the RND (resistance nodulation division) family plays an important role in the expulsion of antimicrobial agents from the bacterial cell (Poole, 2011). Three RND-type efflux pump systems contribute to the resistance of *P. aeruginosa*: MexAB-OprM, MexCD-OprJ and MexXY-OprM (Poole, 2011; Nguyen *et al*., 2018). These pumps confer resistance to carbapenems, primarily due to the MexAB-OprM pump, and to

non-β-lactam antibiotics (Poole, 2011; Nguyen *et al*., 2018). The MexXY-OprM pump was associated with resistance to cefepime, a fourth generation cephalosporin (Poole, 2011).

Lower permeability

Porin channels are responsible for the acquisition of nutrients, but also represent a way for antipseudomonal agents to enter and damage bacterial cells (Nguyen *et al*., 2018). Compared with other Gram-negative bacteria such as *Escherichia coli,* the outer membrane of *P. aeruginosa* is 92% less permeable (Nguyen *et al*., 2018). In the case of *P. aeruginosa*, OprD is the porin channel responsible for the transport of antibiotics (Nguyen *et al*., 2018). In addition to AmpC β-lactamases, the downregulation of OprD has primary role in the resistance of the pathogen to carbapenems (Poole, 2011; Nguyen *et al*., 2018).

Mechanisms of resistance to fluoroquinolones

Fluoroquinolones are a class of antibiotics used for the treatment of *P. aeruginosa* infections (Poole, 2011). Fluoroquinolones inhibit DNA synthesis by targeting the bacterial enzymes topoisomerase II (gyrase) and topoisomerase IV (Poole, 2011).

Alteration of fluoroquinolones

P. aeruginosa primarily modifies topoisomerase II, one of the main target sites of fluoroquinolones. In the case of highly resistant isolates, an additional mutation can be observed in topoisomerase IV (Poole, 2011; Pál, 2013). Mutational changes occur in the GyrA domain of DNA gyrase and the ParC domain of topoisomerase (Poole, 2011).

Efflux pump systems

Four representatives of the RND efflux pump family contribute to the fluoroquinolone resistance of *P. aeruginosa*: MexAB-OprM, MexCD-OprJ, MexEF-oprN and MexXY-OprM (Poole, 2011). The expression of *mexAB-OprM* is regulated by three repressors (MexR, NalD, NalC) which all have been shown to contain mutations in fluoroquinolone-resistant *P. aeruginosa* isolates (Poole, 2011). In addition, a mutation in *NfxB* can be observed in the case of MexCD-OprJ, an inactivator mutation occurs in the *mexT* activator of MexEF-oprN and mutations of *mexz* occur in MexXY-OprM (Poole, 2011).

Mechanisms of resistance to aminoglycosides

Since their discovery in the 1940's, aminoglycosides have been one of the most commonly used antibiotics (Forge and Schacht, 2000). Aminoglycoside antibiotics belong to the aminocyclitols family, having amino groups attached to their structural rings (Forge and Schacht, 2000; Pál, 2013). Their mechanism of action consists in binding to the aminoacyl-tRNA recognition site of the 16S rRNA, component of the 30S ribosomal subunit, resulting in the inhibition of polypeptide synthesis and subsequently cell death (Doi *et al*., 2016). Aminoglycosides have a broad-spectrum antibacterial effect, showing bactericidal properties by inhibiting protein synthesis (Forge and Schacht, 2000). Aminoglycoside antibiotics have primarily been used in the treatment of pulmonary infections and cystic fibrosis (Poole, 2005).

Enzymatic alteration of aminoglycosides

Aminoglycosides can be modified and inactivated through different processes, mediated by enzymes such as acetyltransferases, nucleotidyltransferases, phosphoryltransferases (Poole, 2011). Acetyltransferases modify one of the four amino groups of aminoglycosides, resulting in a decreased affinity towards the tRNA binding site of the 30S ribosomal domain (Poole, 2011; Spohn, 2018). By acetylation, *P. aeruginosa* isolates exhibit resistance to gentamicin, tobramycin, amikacin, netilmicin (Poole, 2005). Nucleotidyltransferases are able to inactivate gentamycin and tobramycin and have been found in gentamicin-resistant and tobramycin-resistant *P. aeruginosa* isolates (Poole, 2011). Phosphoryltransferases contribute to the resistance of antipseudomonal agents such as neomycin, kanamycin and streptomycin, that are rarely used for the treatment of *P. aeruginosa* infections (Poole, 2005, 2011).

Efflux pump system

Aminoglycoside resistance of *P. aeruginosa* clinical isolates is conferred by the MexXY-OprM efflux pump, especially in cystic fibrosis isolates (Poole, 2011). The MexXY-OprM is encoded by *mexXY* (regulated by MexZ) and the *oprM* gene (Poole, 2011). It has been shown that mutations occur in *mexZ* in the case of aminoglycoside-resistant *P. aeruginosa*, isolated primarily from cystic fibrosis lungs (Poole, 2005). In addition, other genes might be contributing to the resistance mediated by the efflux system. The mutation of the *parR* gene has been correlated with the expression of important resistance components such as OprD and *mexXY* (Poole, 2011).

Enzymatic alteration of 16S rRNA

Modifying 16S rRNA, the binding site of aminoglycosides, represents an important resistance mechanism of *P. aeruginosa* against aminoglycosides

(Doi *et al*., 2016). Methylation of the 16 rRNA interferes with the binding of aminoglycosides, leading to aminoglycoside-resistant *P. aeruginosa* strains (Poole, 2011). This process in *P. aeruginosa* is mediated by different methylases: RmtA, RmtD, ArmA (Poole, 2011). By altering the binding site of aminoglycosides, *P. aeruginosa* has demonstrated resistance to gentamicin, tobramycin and amikacin (Poole, 2011).

Mechanisms of resistance to polymyxins

Polymyxins (polymyxin B and colistin) are polycationic polypeptides which show strong bactericidal properties (Pál, 2013). They are being used as a last-line treatment for *P. aeruginosa* infections (Al-Wrafy *et al*., 2017). Their bactericidal effect consist in charged-based interaction with the lipopolysaccharide found in the outer membrane of the pathogen (Moffat *et al*., 2019). Lipid A, the endotoxin component of the lipopolysaccharide, is negatively charged owing to the free phosphate groups in its structure, which allows polymyxins to bind to them (Moffat *et al*., 2019). This interaction leads to the destabilization of the lipopolysaccharide and to increased permeability, resulting in more polymyxin uptake (Moffat *et al*., 2019).

P. aeruginosa achieves resistance to polymyxins by altering the initial target of these antibiotics (Olaitan *et al*., 2014; Baron *et al*., 2016; Moffat *et al*., 2019). This process consists in covalently modifying the lipid A moiety of the lipopolysaccharide by adding 4-amino-4-deoxy-L-arabinose (L-Ara4N) to it (Poole, 2011; Olaitan *et al*., 2014). This process is stimulated by the mutations harbored in the two-component regulatory systems (TCSs), the following TCSs being described in *P. aeruginosa*: PmrA/PmrB, PhoP/PhoQ, ParR/ParS, ColR/ColS and CprR/CprS (Olaitan *et al*., 2014).

Biofilm-mediated resistance

P. aeruginosa cells aggregated in microcolonies produce an exopolysaccharide matrix that encapsulates them (Azam and Khan, 2019). This matrix has been found to confer protection to the bacterial cells from the activity of different antipseudomonal agents.

The biofilm-mediated resistance of *P. aeruginosa* involves different mechanisms. First of all, the exopolysaccharide matrix prevents antimicrobials to reach their target by acting as a barrier and limiting their penetration into bacterial cells (Al-Wrafy *et al*., 2017; Azam and Khan, 2019). Not all antibiotics are prohibited in the same way: penicillins are inactivated by the production of β-lactams, prohibiting it from entering the biofilm, whereas aminoglycosides bind to the negatively-charged alginate or interact with the extracellular DNA of the biofilm, resulting in a slow diffusion through the biofilm matrix (Al-Wrafy *et al*., 2017).

Another biofilm-mediated resistance consists in the alteration of the microenvironment of *P. aeruginosa* (Al-Wrafy *et al*., 2017; Azam and Khan, 2019). In contrast with the surface, deeper layers of the biofilms show anaerobic conditions (Al-Wrafy *et al*., 2017). This prohibits aminoglycoside antibiotics, which are proven to be less effective against *P. aeruginosa* biofilms (Al-Wrafy *et al*., 2017).

Bacterial cells growing inside biofilms are metabolically less active due to the limited nutrients and oxygen supply, as a consequence conferring resistance against antibiotics such as β-lactams and aminoglycosides that only target metabolically active cells (Poole, 2011; Al-Wrafy *et al*., 2017).

Another resistance mechanism of the biofilm is related to the presence of persister cell populations, which are highly resistant to antipseudomonal agents (Azam and Khan, 2019). Researchers concluded that whereas the majority of bacterial cells can easily be damaged with antibiotics, certain cells are not susceptible to the effect of these agents, concluding that this persister cell population is responsible for the resistance of the biofilm (Azam and Khan, 2019).

Hypermutator *Pseudomonas aeruginosa* strains

Mutations occurring in the DNA repair mechanism, primarily in the mismatch repair (MMR), have been found to result in highly resistant *P. aeruginosa* isolates, which can be observed in the case of isolates prelavated from chronic diseases (Poole, 2011). It has been found that strains harboring mutations in *mutS, mutL* and *mutU* have a higher resistance than strains lacking these types of mutations (Poole, 2011).

Treatment of *Pseudomonas aeruginosa* infections

Infections caused by multidrug-resistant *P. aeruginosa* strains pose a challenge to health-care officials due to the few available and effective options left for treatment (Hirsch and Tam, 2010). Studies have shown, that the delayed treatment is correlated with higher mortality and morbidity rates and a longer hospital stay for patients suffering from these type of infections (Hirsch and Tam, 2010).

An empirical therapy is defined as treatment initiation in the absence or prior to having laboratory results assessing the profile of the pathogen. Treating *P. aeruginosa* bloodstream infections with the appropriate empirical therapy requires the administration of antipseudomonal agents in a 24 hour period after bacterial isolates have exhibited susceptibility to the antimicrobials in blood cultures (Hirsch and Tam, 2010). Four different studies have found that the inappropriate empirical therapies result in a higher mortality rate, compared

with the group of patients receiving appropriate medication in case of acquiring *P. aeruginosa* bloodstream infections (Hirsch and Tam, 2010). A study has shown that delaying treatment for over 52 hours has increased the mortality rate greater than twofold compared with patients whose antibiotic therapies were initiated in 52 hours (Hirsch and Tam, 2010). These studies have shed light on the importance of the importance of promptly initiating appropriate empirical therapy in case of *P. aeruginosa* bloodstream infections. In addition, *P. aeruginosa* biofilms in early stages of development exhibit an increased susceptibility to antimicrobials, which also supports the importance of timely treatment (Ciofu and Tolker-Nielsen, 2019).

Due to the fact that *P. aeruginosa* acquires resistance to more classes of antibiotics in a short period of time, the infections caused by it result in higher mortality rates leading to poorer patient outcomes (Hirsch and Tam, 2010).

A potential treatment option could represent the combined administration of antibiotics, which could have several benefits compared with the administration of only one pseudomonal agent. First of all, it would lead to an increased bactericidal effect, slowing the progression of infections (Hirsch and Tam, 2010). Moreover, owing to the extended antibacterial spectrum of the combined antibiotics, it could lead to a better outcome in targeting and combating the pathogen (Hirsch and Tam, 2010). Studies comparing monotherapies with combined therapies have shown results in favor of the latter. The appropriate and inappropriate empirical therapies both had higher mortality rates compared with combined therapies (Hirsch and Tam, 2010). In addition, it has been found that monotherapies had a bigger chance of leading to inappropriate empirical therapies (Hirsch and Tam, 2010). A conflict of results has also been found when a study has demonstrated that patients suffering from sepsis showed the same outcomes both in the case of combined therapy and monotherapy (Hirsch and Tam, 2010).

Approved novel antipseudomonal antibiotics

Ceftazidime/avibactam

Ceftazidime is an older, third-generation antipseudomonal cephalosporin re-issued in 2015, targeting primarily the penicillin-binding proteins (PBPs) of *P. aeruginosa* (Nguyen *et al*., 2018; O'Neall *et al*., 2020). Avibactam is a non-β-lactam β-lactamase inhibitor, which binds reversibly to the active serine site of β-lactamases (Nguyen *et al*., 2018; Stone *et al*., 2018). Avibactam has an extended spectrum activity inhibiting class A, class C and few representatives of the class D β-lactamases, but has no activity against metallo-β-lactamases (Nguyen *et al*., 2018). In addition, avibactam protects ceftazidime from being degraded by β-lactamases, thus enhancing its activity (Stone *et al*., 2018).

Several *in vitro* studies have demonstrated the efficacy of ceftazidime/avibactam in the treatment of MDR and XDR *P. aeruginosa* infections, the inhibition rate varying between 66.1% and 86.5% (Horcajada *et al*., 2019). A different study has shown that ceftazidime/avibactam inhibited 92.4% of the 5,716 *P. aeruginosa* isolates tested (Horcajada *et al*., 2019). A study conducted between 2012 and 2014 has found that from the 7,062 *P. aeruginosa* strains analyzed, 562 (8%) has shown resistance to the antibiotic, mainly owing to the production of metallo-β-lactamases (Horcajada *et al*., 2019). Moreover, it has been found that the main mechanism of resistance of *P. aeruginosa* is due to the expulsion of the antibiotic through efflux pumps (Horcajada *et al*., 2019). Further clinical experiments are needed in order to assess the exact efficacy of ceftazidime/avibactam in treating *P. aeruginosa* infections.

Ceftolozane/Tazobactam

Ceftolozane/tazobactam is a new β-lactam/β-lactamase inhibitor, approved by the FDA in 2014 and subsequently approved by the European Medicines Agency (EMA) in 2015 as a treatment option for complicated urinary tract infections (cUTIs) and complicated intra-abdominal infections (cIAIs) (Haidar *et al*., 2017; Maraolo *et al*., 2020). Since then, it has been proposed for the treatment of *P. aeruginosa* related hospital-acquired pneumonia and ventilator-associated pneumonia, approved by the FDA in 2019 (Maraolo *et al*., 2020). Ceftolozane/tazobactam showed promising effects against MDR and XDR *P. aeruginosa* strains.

Ceftolozane is a fifth-generation cephalosporin, belonging to the class of β-lactams (O'Neall *et al*., 2020). It binds and inhibits the PBPs, interfering with cell wall synthesis which leads to cell lysis and death (Zhanel *et al*., 2014). Ceftolozane has an increased stability to AmpC β-lactamases and a high penetration rate compared with older generation cephalosporins (O'Neall *et al*., 2020). Tazobactam, a sulfone derivative of penicillanic acid, acts as a β-lactamase inhibitor (Zhanel *et al*., 2014). In contrast with the complex formed by the β-lactam, the complex created by tazobactam at the active site of β-lactamases results in a slower hydrolysis (Zhanel *et al*., 2014). Tazobactam inhibits class A β-lactamases, extended spectrum β-lactamases and a few class C β-lactamases (Zhanel *et al*., 2014).

Several studies have found that *P. aeruginosa* exhibited between 55% and 96.6% susceptibility to ceftolozane/tazobactam (Horcajada *et al*., 2019). It is considered the most active antipseudomonal agent, proven to be up to 20-25% more effective than the other available antibiotics of the class of β-lactams (Horcajada *et al*., 2019; Maraolo *et al*., 2020). Some studies have shown 4-14% of resistance to ceftolozane/avibactam in *P. aeruginosa* isolates (Horcajada *et al*.,

2019). More *in vivo* studies are required to evaluate the efficacy of ceftazidime/avibactam in treating *P. aeruginosa* infections.

Imipenem-Cilastatin-Relebactam (IMI-REL)

Imipenem-cilastatin-relebactam, sold under the name Recarbio, has been approved by the FDA in July 2019 for the treatment of complicated urinary tract infections and intra-abdominal infection (Smith *et al*., 2020). Imipenem is a carbapenem, and similarly to the other representatives of the β-lactams, it inhibits bacterial cell wall synthesis by binding to PBPs (Smith *et al*., 2020). Imipenem is a derivative of thienamycin, a substrate for renal dehydropeptidase-1 (DHP-1), thus it is necessary to combine it with the DHP-1 inhibitor cilastatin in order to prevent its inactivation (Smith *et al*., 2020). Relebactam is a new β-lactamase inhibitor that potentiates the activity of the β-lactam component and shows similarity to avibactam (Smith *et al*., 2020).

In vitro studies have found that 94.4% of *P. aeruginosa* isolates exhibited susceptibility to IMI-REL (Horcajada *et al*., 2019). However, *P. aeruginosa* strains resistant to IMI-REL demonstrated an overall of 80% susceptibility (Smith *et al*., 2020).

Cefiderocol

Cefiderocol is a novel siderophore cephalosporin, proved to be effective against multidrug-resistant Gram-negative bacteria (Horcajada *et al*., 2019).

Owing to the catechol moiety of cefiderocol, it can easily sequester iron from the environment, which is essential for bacterial growth (Sato and Yamawaki, 2019). Cefiderocol mimics natural siderophores produced by bacteria, thus is able to bind and exploit the outer membrane iron transporter of the pathogen, evading the bacterial resistance mechanisms (Sato and Yamawaki, 2019). In the case of *P. aeruginosa*, cefiderocol uses a specific membrane transporter, PiuA, to access the periplasmic space (Sato and Yamawaki, 2019). In order to exhibit its antibacterial activity, the complex formed by the siderophore moiety and the antibiotic dissociates in the periplasmic space (Sato and Yamawaki, 2019). Cefiderocol, similarly to other β-lactams, binds and inactivates PBPs, resulting in cell death (Nguyen *et al*., 2018).

In vivo studies conducted on mouse lung models have demonstrated the efficacy of cefiderocol, showing bactericidal properties against *P. aeruginosa* (Sato and Yamawaki, 2019). It has been observed that mutations occur in the iron transport system as a mechanism of resistance to cefiderocol, but further studies are needed to elucidate the potential of cefiderocol in the emergence of resistance (Sato and Yamawaki, 2019).

Fosfomycin

Discovered in 1969, fosfomycin is an old antibiotic, a derivative of a phosphoric acid found in *Streptomyces spp.* (Falagas *et al*., 2016; Falagas *et al*., 2019). In the past, it has been primarily used in the treatment of uncomplicated urinary tract infections (Falagas *et al*., 2016). Owing to the urgent need of finding novel strategies to combat multidrug-resistant bacteria, fosfomycin re-emerged recently as a possible treatment option, primarily in combination with other antibiotics (Falagas *et al*., 2016). Its structure is composed of a phosphonic acid group and an epoxide group, the latter important in its biological activity (Falagas *et al*., 2019). Fosfomycin has two commercially available oral forms (fosfomycin tromethamine and fosfomycin calcium) and an intravenous form (fosfomycin disodium) (Falagas *et al*., 2016).

Fosfomycin is a phosphoenolpyruvate analog, having bactericidal properties (Pál, 2013). Due to its low molecular weight, it can easily penetrate Gram-negative bacterial cells through the pores of the outer membrane, or in the case of *P. aeruginosa* it can also actively enter through transporters (Borisova *et al*., 2014). In the early stages of bacterial cell wall synthesis, fosfomycin inhibits the formation of *N*-acetylmuramic acid, more precisely the activity of UDP-*N*-acetylglucosamine enolpyruvyl transferase (MurA) (Falagas *et al*., 2016). MurA catalyzes the transfer of the enolpyruvyl group of phosphoenolpyruvate to the UDP-*N*-acetylglucosamine, essential in the synthesis of the peptidoglycan layer (Falagas *et al*., 2016). By binding to the active site of MurA, fosfomycin inactivates this enzyme, which results in the damage of the peptidoglycan layer of the cell wall, leading to cell lysis and death (Falagas *et al*., 2019).

P. aeruginosa is intrinsically resistant to fosfomycin, due to its ability to use a salvage pathway in peptidoglycan synthesis, other than the one targeted by fosfomycin (Borisova *et al*., 2014). This pathway allows the recycling of the peptidoglycan rather than being *de novo* synthesized (Falagas *et al*., 2016). Multidrug-resistant *P. aeruginosa* has intrinsic resistance to fosfomycin when used in monotherapies, nonetheless it has been demonstrated that administering it in combination with other antibiotics may provide an effective treatment option (Dijkmans *et al*., 2017; Horcajada *et al*., 2019). Fosfomycin has been found to exhibit synergistic effect with aztreonam, cefepime, meropenem, imipenem, amikacin, ceftazidime, gentamycin, ciprofloxacin (Dijkmans *et al*., 2017).

Novel antibiotics in clinical development

The latest report of the Pew Charitable Trust organization from April 2020, contains 41 antibiotics that are currently in clinical development. Of the antibiotics currently in the pipeline, 18 have the potential of treating pathogens for

which there is an urgent need of novel antimicrobials. Several antibiotics in phase I (Tab. 1) and phase III clinical trials (Tab. 2) are being investigated for their potential antipseudomonal activity (The Pew Charitable Trusts, 2014).

Table 1. Antibiotics in Phase I clinical trials for the treatment of *P. aeruginosa* infections

Antibiotic name	Drug class	Target	Indication(s)
spr 206	Polymyxin	Outer membrane	cUTI, HAP, VAP
Cefepime/Zidebactam	β-lactam + β-lactamase inhibitor	PBP β-lactamase	cUTI, HAP, VAP
Meropenem/Nacubactam	β-lactam + β-lactamase inhibitor	PBP β-lactamase/ PBP2	cUTI

Abbreviations: cUTI – complicated urinary tract infections, HAP – hospital-acquired pneumonia, PBP – penicillin-binding proteins, VAP – ventilator-associated pneumonia

Murepavadin (POL7080)

Macrocyclic peptides targeting the components of the outer membrane protein are under investigation in efforts to fight resistance of Gram-negative bacteria to last-resort antibiotics (Mclaughlin and Donk, 2020). By modifying protegrin-1, a cationic peptide, researchers developed the peptidomimetic murepavadin (Romano *et al*., 2019; Mclaughlin and Donk, 2020). It has been found, that murepavadin specifically targets LptD, a component of the outer membrane of *P. aeruginosa* (Romano *et al*., 2019). LptD is a transport protein which plays a role in the biogenesis of the LPS by inserting it in the outer membrane (Storek *et al*., 2019). Thus, inhibitors of LptD such as murepavadin interfere with the formation of the outer membrane, leading to growth inhibition through a nonlytic mechanism (Romano *et al*., 2019; Storek *et al*., 2019). *P. aeruginosa* shows high resistance to murepavadin by harboring mutations in *pmrB*, the sensor of the PmrA-PmrB TCS (Romano *et al*., 2019).

In a large-scale study, murepavadin showed high efficacy against *P. aeruginosa*, inhibiting 98.7% of the 1,219 isolates tested (Sader *et al*., 2018). Moreover, murepavadin exhibited higher activity than polymyxins, being 4- to 8- fold more active than colistin and polymyxin B (Sader *et al*., 2018). However, clinical trials evaluating the safety and efficacy of intravenous murepavadin were stopped as of July 2019, due to the high incidence of renal failure observed in patients undergoing murepavadin therapy for lower respiratory

tract infections caused by *P. aeruginosa* (Tümmler, 2019). After the unsuccessful phase III clinical trials, intravenous formulation of murepavadin has been taken back to preclinical stages (Mclaughlin and Donk, 2020). The aerosolized form of murepavadin, however, is still in development.

Table 2. Antibiotics in Phase III clinical trials for the treatment of *P. aeruginosa* infections

Antibiotic name	Drug class	Target	Indication(s)
Cefepime/Taniborbactam	β-lactam + β-lactamase inhibitor	PBP β-lactamase	cUTI
Ceftobiprole	β-lactam	PBP	acute skin infections, CAP, HAP
Tebipenem/tebipenem pivoxil hydrobromide	β-lactam	PBP	cUTI

Abbreviations: CAP – community-acquired pneumonia, cUTI – complicated urinary tract infections, HAP – hospital-acquired pneumonia, PBP – penicillin-binding proteins.

Alternative antipseudomonal strategies

Bacteriophage therapy

Bacteriophages are viruses capable of infecting only bacteria *(Pang et al*., 2019). In search for alternative strategies to combat MDR and XDR *P. aeruginosa* infections, bacteriophages first described in 1915 have resurged recently as a potential treatment option owing to the several advantages they possess (Chanishvili and Aminov, 2019). Bacteriophage therapy demonstrates high specificity by targeting only a specific bacterium, whereas antibiotics exhibit a broad-spectrum activity, consequently affecting the normal microflora of the host (Chanishvili and Aminov, 2019). Moreover, by replicating at the site of infection, bacteriophages show an increased local antibacterial activity (Debarbieux *et al*., 2010; Chanishvili and Aminov, 2019). Other advantages of bacteriophages include: little to no side effects; phages can show efficacy in the treatment of biofilms; they adapt to the resistant strains owing to their coevolution with the bacterial host; being the most ubiquitous entities, their discovery and development as antibacterial therapies is inexpensive; their administration unlikely results in inflammatory responses of the immune system (Debarbieux *et al*., 2010).

Phage-resistance in bacteria has already been observed a century ago when researchers described bacterial regrowth following bacteriophage therapy (Oechslin, 2018). One of the resistance mechanisms of bacteria is the occurrence of spontaneous mutation, affecting the phage receptors located in the bacterial outer membrane (Oechslin, 2018). These phage receptors are represented by components of the cell wall (polysaccharides, outer membrane proteins, type IV pili) that are important virulence factors of *P. aeruginosa* (Oechslin, 2018). Thus, with the emergence of phage-resistance, a trade-off cost has been observed: phage-resistant bacteria become less virulent due to alterations of the cell wall-associated virulence factors (Oechslin, 2018; Mangalea and Duerkop, 2020).

An effective way of getting a successful outcome out of bacteriophage therapy would be the combined administration of phages with antibiotics (Oechslin, 2018). Considering the fact that phage-resistance comes with costs to bacteria, targeting specific receptors of *P. aeruginosa* such as the MexAB and MexXY-OprM efflux pumps with bacteriophages could open up other possibilities for antimicrobials to enter bacterial cells, thus restoring susceptibility of the pathogen to antibiotics (Oechslin, 2018; Mangalea and Duerkop, 2020). In the case of *P. aeruginosa,* the administration of phages with streptomycin or ceftazidime showed promising results in combating infections (Oechslin, 2018; Mangalea and Duerkop, 2020).

Another important aspect is the synergy of bacteriophages with the host immune system. *In vivo* studies of *P. aeruginosa* pneumonia using a mouse model showed that phage therapy failed in mice carrying immune defects due to the emergence of phage-resistant strains (Oechslin, 2018). This observation elucidated the fact that nor bacteriophages, nor the innate immune system is effective enough on its own to fight resistant bacteria (Mangalea and Duerkop, 2020).

Different approaches of addressing phage-resistance have been proposed for a successful bacteriophage therapy: phage cocktails and personalized therapy (Oechslin, 2018). By extending the host spectrum of bacteriophages, phage cocktails have demonstrated a promising empirical treatment (Oechslin, 2018). Despite the higher costs associated with the development of personalized phage therapy, this strategy would represent a better way of tackling the emergence of resistance by specifically targeting the pathogen in question (Oechslin, 2018). During bacteriophage therapy, monitoring phage-resistance by adapting phage composition is a key of achieving the best outcomes (Oechslin, 2018).

Pyocins

Pyocins are antimicrobial compounds produced by bacteria for competitive purposes (Redero *et al*., 2018; Oluyombo *et al*., 2019). Of the three types of pyocins produced by *P. aeruginosa*, R-pyocins have been found to have the

highest activity against competitors of the same species (Redero *et al*., 2018). R-pyocins have a phage origin, structurally and genetically being related to the tail of P2 and lambda bacteriophages (Redero *et al*., 2018; Oluyombo *et al*., 2019). After successfully binding to the receptors located on the LPS, pyocins cause the depolarization of the membrane, subsequently leading to cell lysis and death (Redero *et al*., 2018; Oluyombo *et al*., 2019).

Pyocins used as a therapeutic strategy against *P. aeruginosa* have been found to show advantages in contrast with traditional antibiotic therapies. R-pyocins have a narrow-spectrum activity, having little effect on the normal microflora (Redero *et al*., 2018). Moreover, owing to the fact that R-pyocins have no genetic material, they cannot drive the emergence of antibiotic-resistance through the horizontal-transfer of resistance genes (Redero *et al*., 2018). Resistance to pyocins is mediated by the modification of their binding site, the LPS of bacterial cells (Redero *et al*., 2018).

Studies have found a 80% susceptibility rate of *P. aeruginosa* isolates collected from cystic fibrosis lungs, whereas only 50% susceptibility was found in the case of isolates analyzed from blood stream infection (Redero *et al*., 2018).

Alternative strategies for eradicating biofilms

Biofilms produced by *P. aeruginosa* are highly refractory to treatment, making their eradication a great challenge (Maurice *et al*., 2018). Due to the high antibiotic resistance of the pathogen, several promising alternative therapies are under investigation.

Biofilms often develop on medical equipment, leading to severe *P. aeruginosa* infections. A strategy to prohibit biofilm development would be engineering materials that prevent the adhesion of the pathogen to these surfaces (Maurice *et al*., 2018). This would require the implementation of molecules having high antimicrobial resistance onto the material. In a large study, endotracheal tube coated with silver hydrogel showed promising results in preventing *P. aeruginosa* infections and had better patient outcomes (Maurice *et al*., 2018).

Another strategy to approach biofilm treatment would be the inhibition of quorum sensing systems, that have a major role in biofilm formation (Maurice *et al*., 2018). Studies have found several quorum sensing inhibitors that significantly reduced biofilm mass, decreasing the pathogenicity of *P. aeruginosa* (Maurice *et al*., 2018). Halogenated furanones isolated from the macroalga *Delisea pulchra,* and the subsequently developed synthetic furanones exhibited promising QS inhibitor properties and therapeutic effects against *P. aeruginosa* (Maurice *et al*., 2018; Chang *et al*., 2019). Natural compounds such as patulin from *Penicillium*, iberin from horseradish, ellagic acid from *Terminalia chebula*

and eugenol from clove extract have also been investigated for their QS inhibitor properties, but future clinical investigation is still needed to confirm their antipseudomonal efficacy (Maurice *et al*., 2018).

Quorum quenching enzymes (acylases, lactonases) which target AHLs have also been the focus of studies due to their promising effect on biofilms (Maurice *et al*., 2018). Furthermore, host response modulation and inhibition of c-di-GMP signaling could also represent potential strategies in eradicating *P. aeruginosa* biofilms (Maurice *et al*., 2018).

Experimental studies have concluded that fosfomycin administered alone or in combination with other antibiotics is able to penetrate and destroy biofilms (Falagas *et al*., 2016). It has been found that the combination of fosfomycin with prulifloxacin resulted in the eradication of *P. aeruginosa* biofilm layers in rat models (Falagas *et al*., 2016).

Conclusions

Evaluated as a top priority pathogen, *Pseudomonas aeruginosa* has been a subject of concern due to its rapidly emerging resistance to almost all classes of antibiotics. *P. aeruginosa* is the sixth most common nosocomial pathogen, being accountable for 10-15% of hospital-acquired infections worldwide. *P. aeruginosa* can establish infections in any part of the human body, causing severe conditions such as hospital-acquired pneumonia, community-acquired pneumonia, complicated urinary tract infections and complicated intra-abdominal infections, primarily in immunocompromised patients. The successful invasion into host cells is achieved by the arsenal of virulence factors the pathogen possesses, which also provide protection in evading the immune system.

The various resistance mechanisms acquired by *P. aeruginosa* have led to the emergence of multidrug-resistant and extensively drug-resistant strains. The removal of antimicrobials by efflux pumps, the inactivation of the antibiotics by beta-lactamases or the alteration of the target of the antibiotics all contribute to the high resistance demonstrated by *P. aeruginosa*. Moreover, by forming biofilms the pathogen has additional protective layers due to the hardly penetrable matrix.

The majority of traditional antibiotics have lost their efficacy over time due to the pathogen's increased resistance. In recent years, however, several novel antibiotics have been approved for the treatment of *P. aeruginosa* infections. β-lactam/β-lactamase inhibitor combinations such as ceftazidime/avibactam, ceftolozane/tazobactam, imipenem-cilastatin-relebactam have all shown promising results in treating *P. aeruginosa* infections. Cefiderocol, the siderophore cephalosporin, has also shown strong bactericidal properties by

exploiting the iron transport system of *P. aeruginosa*. In addition, an old antibiotic, fosfomycin, has been recently approved as a possible treatment option, primarily in combination with other antibiotics. Furthermore, several new antibiotics are currently in the pipeline in first or late-stage clinical trials, including the *Pseudomonas*-specific murepavadin. Owing to their several advantages, various alternative strategies are also under investigation.

However, due to the rapid development of multidrug-resistant and extensively drug-resistant strains of *P. aeruginosa*, the above-mentioned novel antibiotics could soon be proven ineffective.

Over the last decade, untreatable infections have become alarmingly frequent with the rise of antibiotic resistant strains of bacteria, to the extent to which clinicians might soon be left with no effective treatment options available. In order to halt the post-antibiotic era we are headed to, there are several courses of actions that can be taken to prevent and stop the spread of resistance: educating the population about the appropriate use of antibiotics, improving antibiotic prescription by healthcare providers, preventing environmental contamination by properly disposing antibiotics and ultimately promoting the research and development of novel antibiotics to combat emerging pathogens.

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