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REVIEW

Mechanisms of Bacterial Drug Resistance with Special Emphasis on Phenotypic and Molecular Characterization of Extended Spectrum Beta-lactamase

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SUMMARY

Antibiotics are designed to effectively treat bacterial infections while minimizing harm to the human body. They work by targeting specific components of bacteria or by disrupting essential processes such as cell wall synthesis, membrane function, protein production, and metabolic pathways. However, the misuse and overuse of antibiotics have led to the emergence of drug resistance in humans, animals, and agriculture, contributing to the global spread of this problem. Drug resistance can be either innate or acquired, with acquired resistance involving changes in the bacterial chromosomes or transferable elements. Bacterial species employ various mechanisms of drug resistance, including modifying the antibiotic targets, inactivating the drug, reducing uptake or increasing efflux, overexpressing the target, utilizing alternative pathways, and forming biofilms. One significant concern in the realm of drug resistance revolves around the emergence and proliferation of extended-spectrum beta-lactamases (ESBLs), a gene that is found in most gram-negative bacteria, primarily carried by *Escherichia coli* and *Klebsiella pneumoniae* in healthcare settings. ESBL-mediated resistance poses challenges for diagnosis, treatment, infection control, and antibiotic stewardship. Accurate detection of ESBL genes is crucial, and phenotypic methods are commonly used for initial screening. However, these methods have limitations, and confirmatory molecular techniques such as PCR and DNA sequencing are employed to accurately identify ESBL genes. Despite the significant global concerns surrounding ESBLs, they have spread worldwide, mainly facilitated by healthcare settings, inappropriate antimicrobial use, and host susceptibility. Addressing this issue requires implementing comprehensive measures, including enhanced surveillance, strict infection control practices, antibiotic stewardship programs, rapid diagnostic methods, alternative therapies, public education initiatives, and research focused on developing new drugs. Furthermore, collaboration among the healthcare, public health, and research sectors is pivotal in effectively combating the escalating threat posed by ESBL-mediated resistance. Antibiotics have revolutionized medical care by effectively treating bacterial infections. However, the emergence of ESBL gene resistance poses a global challenge that requires an integrated approach to prevent a threatening future.

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INTRODUCTION

Overview of Drugs and Their Mechanisms of Action

The term drugs comprises a wide range of chemical substances, including antimicrobials and antibiotics that have been specifically developed to combat diseases and promote overall well-being (Breckenridge, 1976). Antimicrobials are a specific category of drugs that either kill microorganisms or inhibit their growth (Anandabaskar, 2021; Uddin *et al*., 2021a). Antibiotics, on the other hand, are a subset of antimicrobial drugs that are carefully designed to target bacteria and eliminate them while minimizing harm to the human body (Fowler, 1959). They are often referred to as "magic bullets" because of their remarkable ability to attack bacteria without causing significant damage to human cells (Salyers & Whitt, 2014). The magic bullet achieves selective toxicity by targeting specific components that are either unique to bacteria, similar but not identical to host cells, or shared but differ in importance between bacteria and host cells (Kasmar *et al*., 2008). Antibiotics have a favorable therapeutic index and serve various purposes. They are commonly used to treat bacterial infections, improve feed utilization in livestock, increase production yields, manage chronic diseases, and provide economic benefits that have revolutionized the field of medicine (Salyers & Whitt, 2014; Uddin *et al*., 2021b).

Key words:
Antibiotics, bacterial infections, bacterial characterization, drug resistance.

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Antibiotics can be classified based on their chemical structure, mechanism of action, spectrum of activity, source, or mode of action (Eliason, 1940). Some well-known groups of antibiotics include β-lactams (the most commonly used (Bush & Bradford, 2016)), macrolides, Sulfonamides, Tetracyclines, fluoroquinolones, tetracyclines, phenols, and aminoglycosides (Uddin *et al.*, 2021b). Each group exhibits similar properties and effectiveness in combating bacterial infections (Cheng *et al.*, 2016).

The mechanism of action of antibiotics refers to how they exert their effects within the body. Antibiotics can interfere with the synthesis of bacterial cell walls, disrupt the function of the cell membrane, impede protein synthesis, hinder the production of nucleic acids, or disrupt metabolic pathways. By interfering with these vital processes in bacteria, antibiotics ultimately lead to the destruction of bacteria or the inhibition of their growth (*Figure 1*) (Eliason, 1940). Gram-positive and gram-negative bacteria rely on their cell walls for strength, division, attachment, and osmotic pressure (Thiemann *et al.*, 2016). Gram-positive bacteria have thicker cell walls, but both types contain beta-lactamases and penicillin-binding proteins. Gram-negative bacteria also have an outer membrane with lipopolysaccharides and porins, as well as a peptidoglycan layer (Garde *et al.*, 2021). Cell wall biosynthesis involves multiple stages and proteins, including monomer synthesis, glycan polymerization, and polymer cross-linking (Kasmar *et al. 2008*). Beta-lactam antibiotics target penicillin-binding proteins and disrupt cell wall synthesis, leading to osmotic instability and cell death (Ojkic & Serbanescu, 2022). This selective toxicity is achieved by interfering with transglycosylases and transpeptidases (Golan *et al.*, 2016; Mhatre V. Ho, Ji-Ann Lee & Dien *et al.*, 2008). Mechanisms related to cell membrane inhibition involve the cytoplasmic membrane of bacteria acting as a selective barrier, regulating the internal composition of the cell (Wipt & George, 2008). When this membrane is disrupted, macromolecules and ions can leak out, causing cell damage or death. The cell surface-to-volume ratio plays a role in regulating intracellular antibiotic concentrations (Ojkic & Serbanescu, 2022). Lipoglycopeptides such as oritavancin, dalbavancin, and telavancin are clinically applicable membrane-active agents, particularly effective against stubborn bacteria (Wipt & George, 2008). Daptomycin, on the other hand, forms a calcium complex by binding to calcium ions, which then aggregate within the plasma membrane. This aggregation leads to the leakage of ions from the cell, ultimately resulting in cell death (Müller *et al.*, 2016). Polymyxins, which are cyclic peptides with a long hydrophobic tail, serve as antibacterial agents. They have an affinity for polysaccharide molecules present in the outer membranes of many Gram-negative bacteria, making them toxic (Uddin *et al.*, 2021b).

Different classes of antibiotics interfere with various steps of protein synthesis (Kester *et al.*, 2012). For example, tetracycline prevents the binding of transfer RNA to the mRNA-ribosome complex, thereby inhibiting protein synthesis. Other antibiotics, like lincosamides, chloramphenicol, macrolides, and aminoglycosides, target protein synthesis at different stages. Antibiotics can also inhibit nucleic acid synthesis, disrupting the genetic processes necessary for bacterial survival. Rifampin, for instance, inhibits RNA elongation and gene transcription by binding to DNA-dependent RNA polymerase (Vannuffel & Cocito, 1996) (Uddin *et al.*, 2021b). Quinolones target DNA gyrase, causing DNA damage and inhibiting replication. Antibiotics like nitrofurantoin, metronidazole, and fluoroquinolones disrupt nucleic acid synthesis by causing DNA damage (Thiemann *et al.*, 2016) (Kester *et al.*, 2012; Uddin *et al.*, 2021b). Nucleic acid synthesis inhibitors are antibiotics that target DNA and RNA synthesis in infectious diseases (Lambert, 2005). They exploit differences in enzymes between eukaryotic and prokaryotic cells for selective toxicity. RNA inhibitors like rifampin block DNA-dependent RNA polymerase, preventing RNA elongation and gene transcription, leading to cell death. DNA inhibitors like quinolones bind to DNA gyrase, inhibiting DNA replication and causing cell damage (Evans-Roberts *et al.*, 2016). Antibacterial drugs such as nitrofurantoin and metronidazole create metabolites that bind to bacterial DNA, increasing the risk of DNA rupture

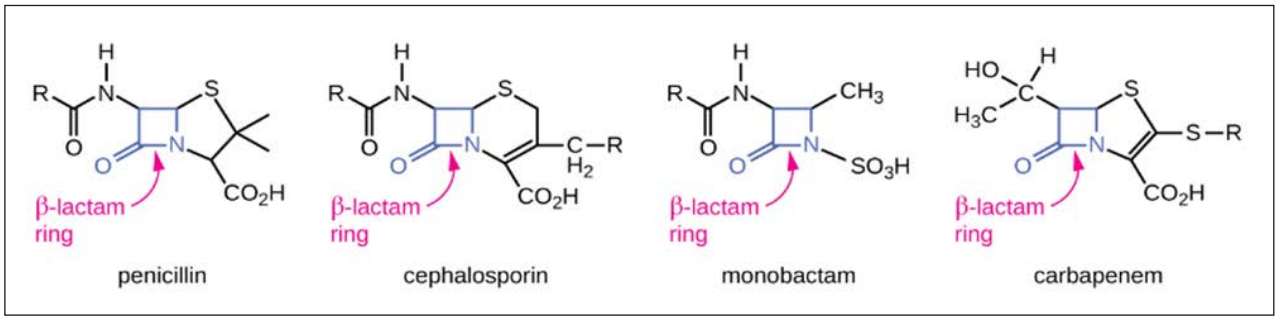

Figure 1 - *Chemical structures and cleavage sites of beta-lactam antibiotics.*

(Fàbrega *et al.*, 2009). Metronidazole and fluoroquinolones induce double-stranded DNA breaks, resulting in harmful DNA lesions and cell death. Rifampicin inhibits DNA-dependent RNA polymerase, suppressing RNA synthesis and causing cell death (Campbell *et al.*, 2001; Evans-Roberts *et al.*, 2016). In bacteria and certain parasites, folate is synthesized from pteridine and PABA, while humans rely on dietary folate (Uddin *et al.*, 2021b). The synthesis process involves the formation of dihydropteroic acid, which is then converted to dihydrofolate (DHF) and further reduced to tetrahydrofolate (THF) by dihydrofolate reductase (DHFR). THF is essential for DNA, RNA, and protein biosynthesis. Inhibition of folate metabolism targets three key steps (Sköld, 2000): dihydropteroate synthase is inhibited by sulonamides, DHFR is competitively inhibited by trimethoprim, methotrexate, and pyrimethamine (Gleckman *et al.*, 1981), and thymidylate synthase is inhibited by 5-fluorouracil (5-FU) and fucytosine, which affects thymidine biosynthesis (*Kasmar et al. 2008*).

EMERGENCE, DISSEMINATION, AND MECHANISMS OF BACTERIAL DRUG RESISTANCE

Emergence and Dissemination of drug resistance

The introduction of antibiotics in 1910 marked a significant turning point in modern medicine, representing one of the greatest medical breakthroughs of the 20th century (Hutchings et al., 2019). However, the discovery of penicillin in 1928, which initiated a golden age of antibiotic exploration, brought about a formidable challenge known as microbial revenge, leading to the emergence of drug resistance (Thiemann *et al.*, 2016). Microbial revenge refers to the ability of micro-

Box 1 - *Definition of terms (Akpaka et al., 2021; Anandabaskar, 2021; Cheng et al., 2016; Stone, 1975; Thiemann et al., 2016; Uluseker et al., 2021; D. Y. Wang et al., 2016).*

1. **Active resistance:** the result of a specific evolutionary pressure to adapt a counterattack mechanism against an antibiotic or class of antibiotics)
2. **Antimicrobial:** any substance that can affect microbial life and used to treat infectious diseases
3. **Beta-lactamases:** Bacterial enzymes that hydrolyze the beta-lactam ring of certain penicillins and cephalosporins
4. **β-lactam ring:** structure where the nitrogen atom is attached to the β-carbon atom relative to the carbonyl
5. **Beta-lactamase inhibitors:** Beta-lactamase inhibitors are drugs that block the activity of certain beta-lactamases
6. **Cross-resistance:** resistance to two related or unrelated drugs due to a single biological mechanism
7. **Co-resistance:** resistance to unrelated drugs due to different resistance mechanisms located in the same genetic element
8. **Conjugation:** transfer of DNA through a multi-step process requiring cell to cell contact via cell surface pili or adhesins
9. **Development window:** how long after its discovery the antibiotic was first used in the clinic
10. **Drug resistance:**
    1. *Real world - clinical:* the ability of a bacterial strain to survive or grow during antimicrobial treatment
    2. *Research - genetic:* the presence of a genetic change (mutation or gene) or resistance determinant
    3. *Laboratory - microbiological:* the ability to survive or grow in higher antibiotic concentrations than most other bacterial strains of the same species
11. **Extended spectrum beta-lactamases:** enzymes produced by certain bacteria that are able to hydrolyze extended spectrum cephalosporin
12. **Integrons:** site-specific recombination systems capable of recruiting open reading frames in the form of mobile gene cassettes, providing an efficient and rather simple mechanism for the addition of new genes into bacterial chromosomes
13. **Intermediate resistant isolates:** organisms with MICs that approach typically attainable blood and tissue concentrations of antimicrobial drugs and for which response rates may be lower than for susceptible isolates
14. **MIC:** the minimum concentration of antibiotic that will inhibit growth of the organism in vitro
15. **Nonsusceptible:** the combined categories of full and intermediate resistance
16. **Passive resistance:** a consequence of general adaptive processes that are not necessarily linked to a given class of antibiotic; e.g., the non-specific barrier afforded by the outer membrane of gram-negative bacteria.
17. **R factor or resistance transfer factor:** tiny, circular DNA elements that contain antibiotic resistance genes and are self-replicating
18. **Resistant isolates** organisms that are not inhibited by the usually achievable concentrations of antimicrobials
19. **Selective pressure:** the environmental conditions that allow the survival and proliferation of organisms with novel mutations or newly developed characteristics
20. **transduction:** transfer of genetic material by bacteriophage
21. **transformation:** capable of uptake, integration, and functional expression of naked fragments of extracellular DNA
22. **transposons or jumping genes:** genes/DNA segments that are transferred within themselves or between chromosomes and extra chromosomal plasmids but non-self-replicating

organisms to develop survival mechanisms against the drugs intended to combat them (Breckenridge, 1976; Salyers & Whitt, 2014). This phenomenon poses a major obstacle to modern medicine, as it undermines the effectiveness of antibiotics and restricts treatment options for patients (Thiemann et al., 2016). Bacteria continually adapt and evolve, employing various mechanisms encoded in their DNA to acquire resistance to drugs. Drug resistance (*defined in box 1*) has now become a prevalent global issue, with pathogens actively developing resistance to antibiotics. Despite its significance, there is currently no universally agreed-upon definition for drug resistance, and comprehending its emergence, mechanisms, and detection remains a complex and challenging area of scientific research. The emergence of drug resistance has rendered infections more challenging and expensive to treat, as well as more difficult to control (Davison *et al*., 2000). The misuse and overuse of antibiotics in medical practice have significantly contributed to the development of drug resistance. Presently, there is a high prevalence of resistant cells present in humans, animals, plants, and the environment, raising concerns regarding the imbalance of power between humans and microbes (De Rosa *et al*., 2021; Uddin *et al.*, 2021b).The landscape of the antimicrobial battle has undergone a transformation, with a decline in the discovery of new antibiotics and a surge in drug resistance (WHO, 2012). Consequently, an antimicrobial resistance crisis has unfolded, and it is projected to result in 10 million deaths annually by 2050, with a significant contribution from ESBL-producing bacteria (Bush & Bradford, 2020; Kirchhelle *et al*., 2020).

The excessive use of antibiotics in humans, animals, and plants has greatly contributed to the rapid emergence and spread of drug-resistant infections (Courvalin, 2016; WHO, 2012) and pathogenic and commensal bacteria in humans (Davison *et al.*, 2000). This has led to the emergence of new forms of resistance genes that can quickly travel globally through various means, including human travelers, animal and insect vectors, agricultural products, and surface water (W. Wang *et al.*, 2018). In bacteria, drug resistance is typically described based on phenotypic and genotypic characteristics and can be categorized according to its origin (intrinsic versus acquired) and type (single, multiple, co-resistance, or cross-resistance) (Courvalin, 2016; Davison et al., 2000). Intrinsic drug resistance can be either innate (passive) or mediated (active) (Citation, 2003). Passive resistance (*defined in box 1*) is naturally present in organisms, while active resistance is triggered after exposure to antibiotics (Uluseker *et al*., 2021). On the other hand, acquired resistance occurs when bacteria acquire genetic material through horizontal gene transfer, mutation, or new sources via processes like transformation, transduction, or conjugation (Gyles & Boerlin, 2014; Munita & Arias, 2016). Acquired drug resistance can be classified as chromosomal-mediated or transferable drug resistance. Chromosomal-mediated resistance involves gene changes that occur through stepwise or one-step mutations. It works by modifying the molecular structure of the drug's target or the transport system responsible for drug uptake (Von Wintersdorff et al., 2016). Plasmid-mediated resistance, a type of transferable drug resistance, involves the transfer of extra-chromosomal resistance plasmids (R factor) (Stone, 1975), which are commonly found against beta-lactam antibiotics. This transfer can facilitate the spread of resistance genes between different bacterial species through direct or indirect contact, resulting in the inactivation of drugs through enzyme production (Meng *et al.*, 2022). Similarly, transposon-mediated resistance occurs through the transfer of transferable

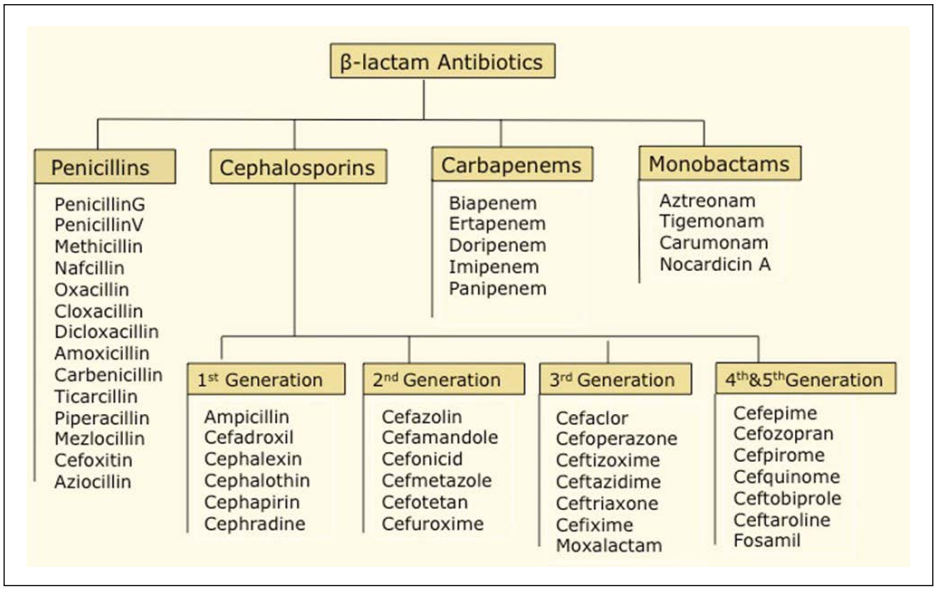

Figure 2 - *Structural classification of beta-lactam antibiotics and member antibiotics.*

drug resistances via transposons or "jumping genes," which are non-self-replicating (Babakhani & Oloomi, 2018). Horizontal gene transfer can occur through processes such as transformation, transduction, conjugation, and plasmid uptake (*Figure 2*). On the other hand, spontaneous mutation can lead to the development of new and unique resistance mechanisms (selective replication) at a relatively constant rate of 1 in 10^6 (CDC, 2019; Uluseker *et al.*, 2021).

Basic mechanisms of drug resistance

The genetic, biochemical, and target modification mechanisms that contribute to bacterial resistance in clinical practice can be broadly classified as bacterial resistance mechanisms (Munita & Arias, 2015). These mechanisms do not have strict boundaries, as different bacterial species have developed preferences for certain resistance mechanisms. For example, gram-negative bacteria often produce lactamases as a means of resistance, whereas gram-positive organisms primarily achieve resistance to beta-lactams through modifications to their target sites or penicillin-binding proteins (Jovanović *et al.*, 2008). The genetic drug resistance mechanism includes mutations in drug target genes, gene amplification, and horizontal gene transfer (Stone, 1975). Mutations in drug target genes can occur spontaneously or due to selective pressure from drug exposure. These mutations can alter the structure or function of the target protein, making it less susceptible to the drug's effects and leading to reduced drug efficacy (Munita & Arias, 2015). Gene amplification involves the duplication or amplification of specific genes responsible for drug targets, resulting in increased production of the target protein. This higher protein level can compensate for the drug's inhibitory effects, reducing its effectiveness. Horizontal gene transfer involves the transfer of genetic material, including drug resistance genes, between different organisms. This process allows for the rapid spread of drug resistance within a population, even across different species. It can occur through mechanisms such as plasmid exchange, transformation, or transduction, enabling the acquisition of new genetic traits, including resistance to drugs like antibiotics or anticancer medications (*Figure 3*) (Tao *et al.*, 2022). The development of drug resistance also involves a complex biochemical process that can vary depending on the specific drug and organism involved. While there are multiple mechanisms of drug resistance, some common ones include target modification, drug inactivation or modification, reduced drug uptake or increased drug efflux, overexpression of target molecules, alternative metabolic pathways, and biofilm formation (Chakraborty *et al.*, 2022)**.** These mechanisms contribute to making the drug less effective against microorganisms. Target modification involves altering the target molecule (protein sequestration), reducing the drug's efficacy (Munita & Arias, 2015; Saha & Sarkar, 2021). An example of target modification in drug resistance is the structural alteration of penicillin-binding proteins (PBPs) in *methicillin-resistant Staphylococcus aureus* (MRSA) (Zhang & Cheng, 2022). Drug inactivation occurs when microorganisms possess enzymes that

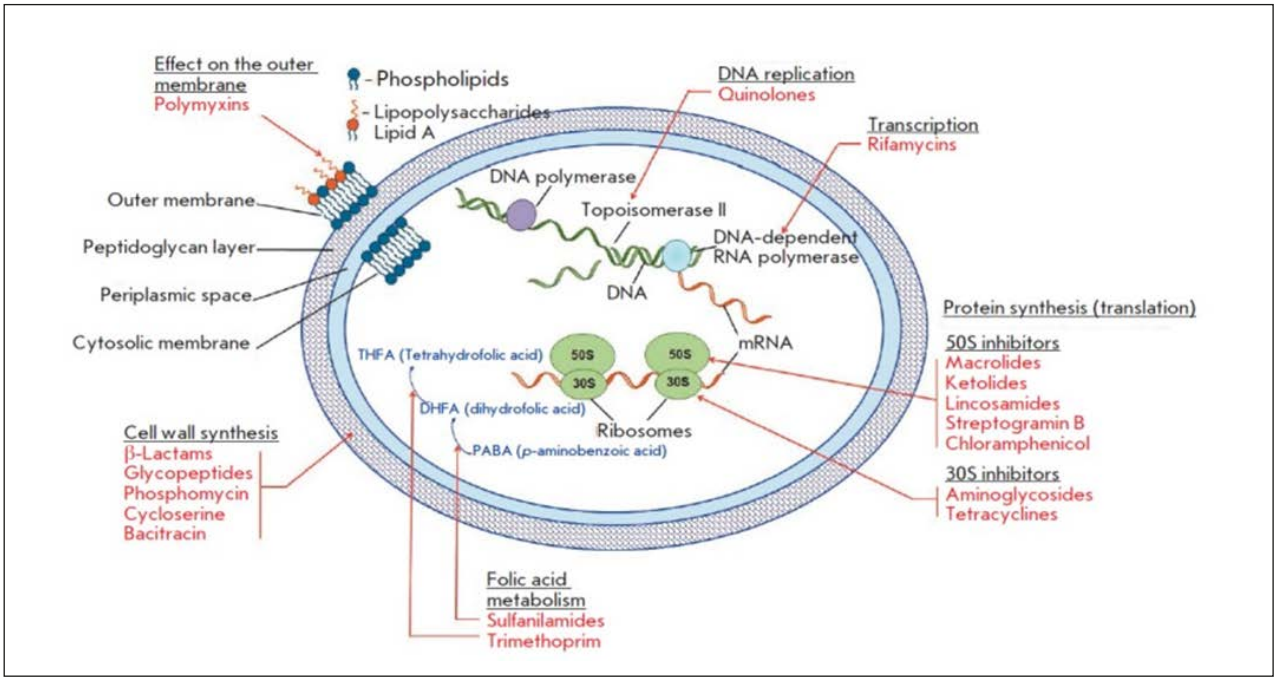

Figure 3 - *The main classes of antimicrobial drugs, their targets, and their effect on the main processes of vital activity in a bacterial cell.*

render the drug inactive. Reduced drug uptake or increased drug efflux involves mechanisms that limit drug entry into cells or enhance drug removal from cells, leading to lower drug concentrations (Munita & Arias, 2015). Typically, gram-negative bacteria delete or damage their outer membrane proteins (Omps) and significantly reduce the effectiveness of antibiotics (except polymyxin) (Kapoor *et al.*, 2017).

Microorganisms can also overexpress target molecules to compensate for the drug's inhibitory effect and maintain normal functions. Overexpression occurs when there is an increased production of efflux pump proteins embedded in the bacterial cell membrane, enabling them to actively pump out antibiotics and other toxic substances (Peterson & Kaur, 2018) (Blair *et al.*, 2014). The most clinically significant efflux pumps found in Gram-negative bacteria belong to the Resistance-nodulation-division (RND) family. This family includes three essential components: an inner membrane transporter called AcrB, an outer membrane protein channel known as TolC, and a periplasmic adaptor protein called AcrA. These components work together to form a complex efflux pump system that plays a crucial role in pumping out antibiotics and other harmful substances from the bacterial cell, contributing to antibiotic resistance (Blair *et al.*, 2014). Lastly, Biofilm formation is a common mechanism where microorganisms in a structured community become more resistant to drugs compared to their single-cell counterparts. The biofilm matrix composed of polysaccharides, proteins, and DNA, presents a challenge for antimicrobial agents to reach bacteria. This condition can lead to the loss of function and toxicity. However, it also enables survival during antibiotic treatment by inducing dormancy in persisters and viable but non-culturable cells (Bollen *et al.*, 2021; Reygaert, 2018).

Classes of Beta-lactamase enzymes involved in drug resistance

Beta-lactamases are enzymes produced by bacteria that can deactivate beta-lactam antibiotics by breaking the beta-lactam ring. These enzymes are commonly found in gram-negative bacteria, particularly in Escherichia coli, Klebsiella pneumoniae, Klebsiella oxytoca, and Proteus mirabilis (Tamma *et al.*, 2021). There are different classification methods for beta-lactamases, with the Ambler classification scheme and the functional classification scheme being the most widely used (Paterson & Bonomo, 2005). The Ambler classification scheme categorizes beta-lactamases into groups based on molecular similarities, whereas the functional classification scheme considers substrate and inhibitor profiles (*Figure 4*). According to the Bush-Jacobi-Medeiros method, beta-lactamases are grouped as follows: group 1 includes cephalosporinases (Ambler class C), group 2 includes serine beta-lactamases (Ambler class A and D), and group 3 includes metallo-beta-lactamases (Ambler class B) (*Figure 4*) (Bush & Jacoby, 2010).

The Ambler classification system categorizes beta-lactamases into four main classes: A, B, C, and D, based on structural features and amino acid sequences. Each class has unique characteristics and mechanisms that contribute to resistance to beta-lactam antibiotics. Class A, C, and D beta-lactamases use serine in the enzyme active center, but class B beta-lactamases use metal zinc ions, resulting in metallo-be-

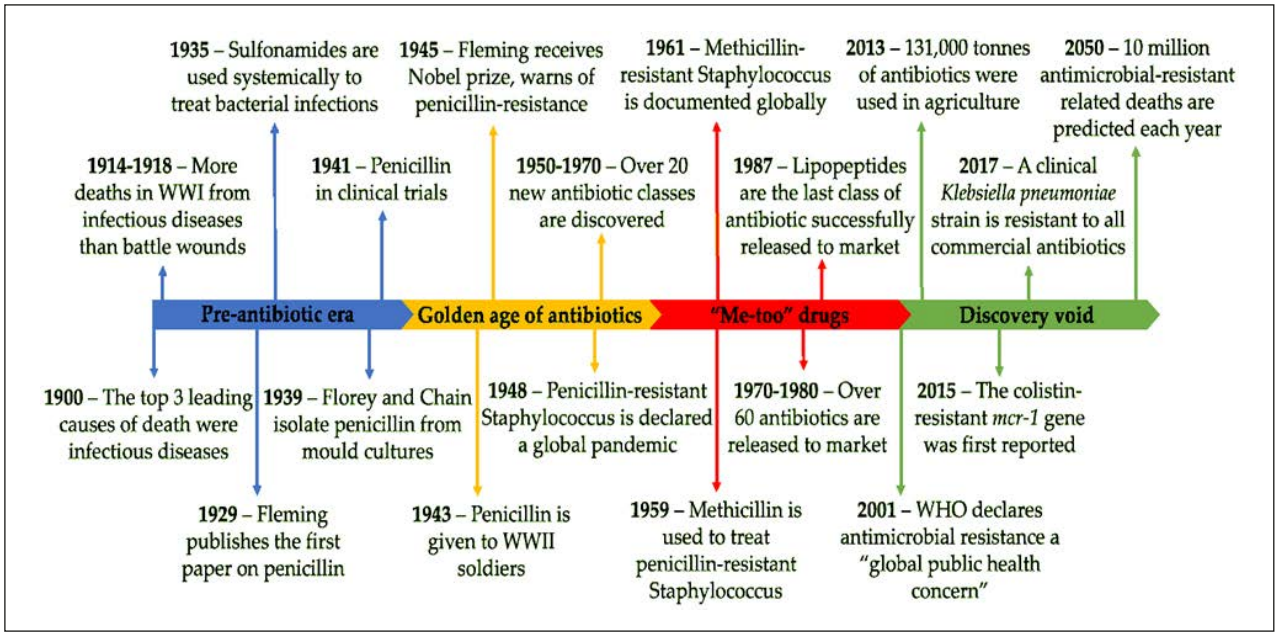

Figure 4 - *Timeline of main events of antibiotics including resistance.*

ta-lactamases (MBLs). Class A contains active-site serine beta-lactamases such as Pseudomonas Specific Enzyme and carbenicillinase (Thabaut *et al.*, 1984), as well as extended-spectrum betalactamases and class A carbapenemases (Walther-Rasmussen & Høiby, 2007). Class B contains the Metallo-beta-lactamases (NDM, IMP, and VIM) (Pitout & Nordmann, 2015) and their subclasses (B1, B2, and B3), whereas class C contains AmpC and extended spectrum AmpC (ESAC). Class D also comprises oxacillinases (OXAs), ESBLs, and carbapenem-hydrolyzing class D beta-lactamases (CHDLs) (*Figure 4*) (Brandt *et al.*, 2017).

PREVALENCE AND CLINICAL IMPACT OF ESBLS

Global epidemiology and nomenclature of extended-spectrum beta-lactamases

The global epidemiology of ESBL-producing bacteria is a complex issue with varying prevalence across regions and healthcare settings. These bacteria, which produce enzymes that confer resistance to certain antibiotics, are becoming increasingly prevalent and pose challenges for infection treatment. ESBL-producing bacteria are commonly found in healthcare settings like hospitals and intensive care units, but community-acquired infections are also on the rise. Globally, these bacteria are found in countries with high population densities, inadequate infection control measures, and excessive antibiotic use. The main culprits are Escherichia coli and Klebsiella pneumoniae, although other species can also produce ESBL enzymes (Tamma *et al.*, 2021).

Over the years, numerous ESBL enzymes have been identified and named based on various criteria. These include the bacterial species, strain, plasmid, place of discovery, or the patient from which they were isolated. For example, the first plasmid-mediated beta-lactamase was discovered in an E. coli strain isolated from the blood culture of a Greek patient named Temoniera in the early 1960s. Other enzymes were named based on the most hydrolyzed substrate (e.g., OXA for Oxacillinase), a specific strain feature (e.g., SHV for Sulfhydryl variant), the place of discovery (e.g., CTX-M for Cefotaximase Munich), the patient from whom the strain was isolated (e.g., TEM for Temoneira, BIL for Bilal), or the producing organism (e.g., PSE for Pseudomonas specific enzyme) (Castanheira *et al.*, 2021). ESBL enzymes were initially named by researchers without full understanding of their protein nature or corresponding genes, leading to a confusing array of names. To address this, an international group of beta-lactamase experts, led by the National Center for Biotechnology Information, met in November 2021 to establish a consensus on the naming of naturally-occurring beta-lactamase genes (Bradford *et al.*, 2022).

CLINICAL IMPLICATIONS OF ESBL-MEDIATED RESISTANCE

ESBL-mediated resistance has significant clinical implications, including treatment challenges due to limited antibiotic options and the need for alternative, more expensive or toxic drugs (Paterson & Bonomo, 2005). It is associated with increased morbidity and mortality rates, treatment failure, prolonged hospital stays, and higher healthcare costs. ESBLs narrow the range of effective antibiotics, impacting empirical therapy and contributing to the spread of resistance. Infection control measures are crucial to prevent transmission, and surveillance is necessary to identify high-risk areas and guide antibiotic stewardship efforts. ESBLs are commonly associated with healthcare-associated and community-acquired infections, posing a risk of outbreaks. ESBL infections present a notable set of diagnostic challenges, frequently resulting in underestimation and delays in their identification. Consequently, there is a recognized urgency to establish antimicrobial stewardship programs in order to effectively address this issue. However, it is important to acknowledge that implementing such programs may come with the potential for additional financial burdens (Breckenridge, 1976).

GENETIC BASIS OF ESBL PRODUCTION

Beta-lactam antibiotics and their targets

Beta-lactam antibiotics (*defined in box 1*) are a group of antibiotics characterized by the presence of a distinctive structural component called the beta-lactam ring (cleavage site) (Eliason, 1940). This ring structure mimics the shape of the D-Ala-D-Ala peptide sequence, which is a target for bacterial cell wall transpeptidases. It consists of a four-membered lactam ring and varies depending on the type of antibiotic. Penicillin contains a five-membered thiazolidine ring, cephalosporin has a six-membered dihydrothiazine ring, carbapenems feature a pyrroline ring, and monobactams have a standalone ring (Figure 5) (De Rosa *et al.*, 2021). These antibiotic groups differ in their side chains, which include penams, cephalosporins, monobactams, carbapenems, and carbacephems (De Rosa *et al.*, 2021). Further, these antibiotics exhibit variations in their side chain structures and can be categorized into several distinct groups, which include penams, cephalosporins, monobactams, carbapenems, and carbacephems (Cheng *et al.*, 2016).

These antibiotics are commonly employed in the treatment of bacterial infections due to their broad-spectrum activity against a wide variety of bacteria. The mechanism of action of beta-lactam antibiotics involves targeting specific components involved in the synthesis of the bacterial cell wall, ultimately resulting in damage to the cell wall and the subsequent death of the bacteria (Ojkic & Serbanescu,

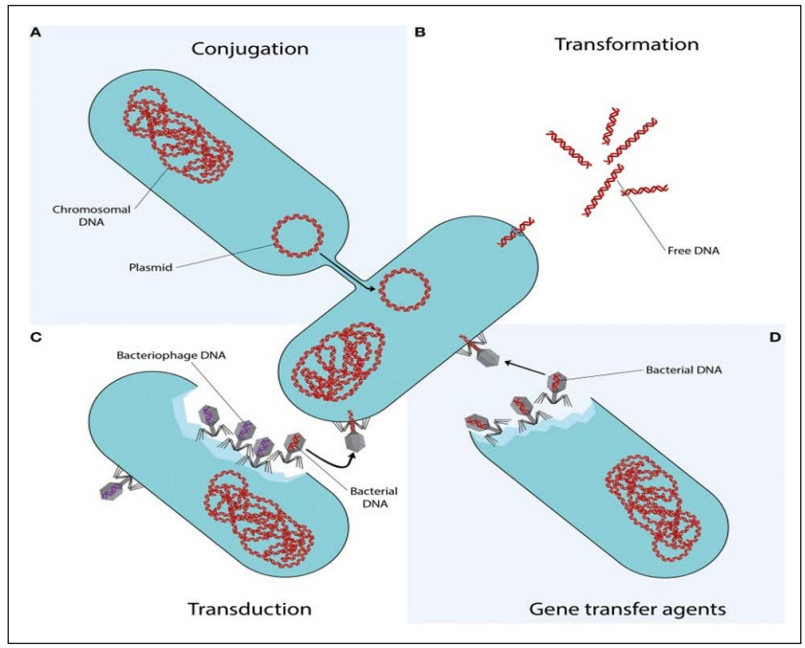

Figure 5 - *Basic mechanisms of horizontal gene transfer with each letter A, B, C & D quadrants representing one different method gene transfer.*

2022). Beta-lactam antibiotics primarily target penicillin-binding proteins (PBPs), which are enzymes responsible for catalyzing the cross-linking of peptidoglycan chains during bacterial cell wall synthesis. Peptidoglycan is a crucial component of the bacterial cell wall, providing strength and rigidity (Garde *et al*., 2021). When a beta-lactam antibiotic enters a bacterial cell, it binds tightly to PBPs, effectively inhibiting their transpeptidase activity. This activity is essential for the proper cross-linking of peptidoglycan. Consequently, the bacterial cell wall becomes weakened and loses its structural integrity. The disruption of cell wall synthesis sets off a cascade of events, including the activation of autolytic enzymes and osmotic instability. These processes ultimately lead to cell lysis and bacterial death (Lee *et al*., 2003). The efficacy of beta-lactam antibiotics relies on the presence of an intact cell wall, which makes them particularly effective against actively growing and dividing bacteria (Golan *et al*., 2016). Different types of beta-lactam antibiotics exhibit varying spectra of activity and are effective against different bacteria. Examples of beta-lactam antibiotics include penicillins (such as amoxicillin and ampicillin), cephalosporins (such as ceftriaxone and cephalexin), carbapenems (such as imipenem and meropenem), and monobactams (such as aztreonam). Each class of antibiotics has its own range of bacterial targets and may be more effective against specific types of infections (Cheng *et al., 2016)*.

GENES ENCODING ESBLS AND THEIR CLASSIFICATION

Several genes have been identified to encode Extended-Spectrum Beta-Lactamases (ESBLs) (*defined in box 1*), and can be classified into different families based on the similarities in their amino acid sequences (Akpaka *et al*., 2021; Bush & Jacoby, 2010). The major ESBL gene families include Temoneira (TEM), Sulfhydryl variable (SHV), Cefotaxime-Munich (CTX-M), and Oxacillinase (OXA) (Bubpamala *et al*., 2018). The TEM family is one of the most prevalent ESBL families and includes genes like TEM-1, TEM-2, and TEM-3. These genes were initially discovered in Escherichia coli but have since spread to other bacterial species. The SHV family, also known as Sulfhydryl variable, consists of genes such as SHV-1, SHV-2, and SHV-5. They were initially identified in Klebsiella pneumoniae but can now be found in various species within the Enterobacteriaceae family (Shaikh *et al.*, 2015). The CTX-M family is a rapidly expanding group of ESBL genes, and includes genes like CTX-M-1, CTX-M-2, and CTX-M-9. These genes are commonly detected in bacteria such as Escherichia coli and Klebsiella pneumoniae, among others. Although primarily associated with resistance to oxacillin and similar antibiotics, certain OXA enzymes also possess extended-spectrum beta-lactamase activity. Examples of such enzymes include OXA-10 and OXA-48 (Paulitsch-Fuchs *et al*., 2023).

Mechanisms of ESBL gene transfer and dissemination

The transfer and dissemination of ESBL genes are major drivers of antibiotic resistance in bacteria. ESBL genes, which encode enzymes that break down β-lactam antibiotics, can be transferred through various mechanisms, including conjugation, transposition, transformation, transduction, and horizontal gene transfer (Stadler *et al*., 2018; Von Wintersdorff *et al*., 2016). Conjugation involves the transfer of ESBL

genes carried on plasmids, small circular DNA molecules that can replicate independently. Plasmids are transferred between bacteria through direct cell-to-cell contact, facilitated by genes encoding conjugative pili or other transfer proteins. Transposons, mobile genetic elements, enable the movement of ESBL genes within and between DNA molecules, such as plasmids and chromosomes. These elements can "jump" between DNA molecules, allowing ESBL genes to be transferred to different bacteria or integrated into different regions of the bacterial genome. ESBL genes can also be found within integrons, genetic elements that capture and express gene cassettes. Integrons can integrate into plasmids or chromosomes, promoting the expression of captured genes, including ESBL genes. Transformation enables bacteria to take up free DNA from the environment, potentially incorporating ESBL genes into their genome. Co-selection occurs when the use of one antibiotic selects for bacteria carrying resistance genes to other antibiotics. ESBL genes often co-occur with genes conferring resistance to other antibiotic classes, providing a selective advantage to strains carrying ESBL genes. ESBL genes can be located within mobile genetic elements, such as insertion sequences or integrons, which facilitate their transfer between different genetic contexts, including plasmids and chromosomes, contributing to the dissemination of ESBL genes among bacteria (Stadler et al., 2018; Von Wintersdorff *et al*., 2016).

Mechanisms of ESBL-mediated Resistance

ESBL-mediated resistance is a significant challenge in the treatment of bacterial infections. It involves the production of extended-spectrum beta-lactamases (ESBLs), enzymes that confer resistance to a wide range of beta-lactam antibiotics. The resistance mechanisms of ESBLs include enzymatic activity, mutation and gene acquisition, genetic variability, co-resistance, and efflux pumps (Ferreira *et al*., 2011). ESBLs exhibit enzymatic activity by hydrolyzing beta-lactam antibiotics, rendering them ineffective against bacteria producing ESBLs. These enzymes have an extended spectrum of activity, including the hydrolysis of oxyimino-cephalosporins and monobactams. ESBL genes are often located on plasmids, which can be easily transferred between bacteria, allowing for the rapid dissemination of ESBL-mediated resistance. Mutations in chromosomal genes encoding beta-lactamases can also contribute to the development of ESBLs. Genetic variability plays a significant role in resistance development. Mutations or genetic rearrangements in ESBL genes can lead to the generation of novel ESBL variants with enhanced activity against beta-lactam antibiotics. This genetic plasticity enables bacteria to adapt and evolve in response to antibiotic selection pressures (Teklu *et al*., 2019). ESBL-producing bacteria often exhibit co-resistance, which means they are resistant to multiple classes of antibiotics. This co-resistance can arise from the acquisition of additional resistance genes carried on the same plasmids or through mutations in other bacterial genes that confer resistance to different antibiotics. Some ESBL-producing bacteria possess efflux pumps, which are membrane proteins that actively pump antibiotics out of the bacterial cell. These efflux pumps reduce the intracellular concentration of antibiotics, protecting the bacteria from their antimicrobial effects and contributing to resistance (Estaleva *et al*., 2021; Ferreira *et al*., 2011).

ESBL Detection mechanisms

Detection of resistance must be defined with respect to a reference population, detection methodology, sensitivity, specificity, repeatability, and reproducibility (Davison *et al*., 2000). Laboratory techniques and sampling design are important for measuring resistance within and between bacterial and host populations. The estimation and control of the problem use quantitative (resistant, intermediate, and sensitive classification) and qualitative (MIC mg/l) laboratory test findings from phenotypic detection methods, genotypic detection methods, and epidemiologic surveillance (Diallo *et al*., 2020).

Phenotypic Methods for ESBL Detection

Phenotypic methods are essential for detecting extended-spectrum beta-lactamase (ESBL) production in bacteria. These methods are utilized to assess antibiotic resistance and provide an initial indication of ESBL production with high sensitivity and specificity, reaching up to 95%. They are known for their simplicity and cost-effectiveness. Some commonly employed phenotypic methods include the Double-Disk Synergy Test, Combination Disk Test, Modified Hodge Test, and Etest (Cockerill, 1999; Drieux *et al*., 2008; Fang & Fu, 1994). The Double-Disk Synergy Test involves the placement of disks containing beta-lactam antibiotics and an ESBL inhibitor near each other on an agar plate. The presence of a synergistic effect, characterized by an enhanced inhibition zone around the disk containing the inhibitor, suggests ESBL production (Drieux *et al*., 2008; Fang & Fu, 1994). Similarly, the Combination Disk Test involves the simultaneous placement of disks containing a third-generation cephalosporin and a beta-lactamase inhibitor. The presence of an enhanced zone of inhibition around the disk containing the inhibitor indicates the presence of ESBL (Drieux *et al*., 2008; Fang & Fu, 1994). The Modified Hodge Test is performed to detect carbapenemase production, which is often associated with ESBL-producing bacteria. This test involves the inoculation of a carbapenem-susceptible indicator strain in close proximity to a test organism on an agar plate. If the test organism produces carbapenemase, it will cause distortion or cloverleaf-like indentation in the growth of the indicator strain (Cockerill, 1999; Drieux *et al*., 2008).

The Etest is a quantitative method that utilizes antibiotic gradient strips to determine the minimum inhibitory concentration (MIC) of an antibiotic against a test organism. By comparing the MIC values of beta-lactam antibiotics with and without a beta-lactamase inhibitor, the presence of ESBL production can be inferred (Cockerill, 1999; Drieux *et al*., 2008).

Challenges and limitations of phenotypic methods

Phenotypic methods are commonly used to detect extended-spectrum beta-lactamases (ESBLs), enzymes produced by bacteria that confer resistance to various antibiotics. However, these methods have several limitations, including inconclusiveness (potential lack of sensitivity, false-positive results, inability to detect all ESBL types, time-consuming nature, lack of standardization, inability to detect other resistance mechanisms) and higher biohazard risk (Anjum *et al*., 2017; Cockerill, 1999). Some phenotypic methods may not detect low levels of ESBL production, leading to false-negative results, especially when dealing with strains producing ESBLs at a low level or in the presence of other beta-lactamases. Additionally, they may produce false-positive results, indicating the presence of ESBLs when they are absent, due to the presence of other beta-lactamases or non-enzymatic resistance mechanisms. Additionally, phenotypic methods may not be able to detect all ESBL types, as some variants may not be phenotypically expressed or have different substrate specificities, leading to false-negative results. Additionally, there is a lack of standardization among different phenotypic methods for ESBL detection, making it difficult to compare data and establish consistent guidelines. Therefore, while these phenotypic methods offer valuable insights into the potential presence of ESBL, confirmation of ESBL production requires molecular techniques (Correa-Martínez *et al*., 2019).

Molecular Characterization of ESBLs

The molecular characterization of extended-spectrum beta-lactamases (ESBLs) involves examining the genetic and structural properties of these enzymes. This typically includes employing molecular techniques for ESBL gene detection, genotyping, and sequence analysis.

MOLECULAR TECHNIQUES FOR ESBL GENE DETECTION

Genotypic assays are essential for detecting resistance genes that confer phenotypic resistance, such as ESBL genes (Anjum *et al*., 2017). In particular, various molecular techniques are commonly employed for ESBL gene detection. These techniques include Polymerase Chain Reaction (PCR), Real-time PCR (qPCR), Multiplex PCR, DNA sequencing, DNA microarrays, and Loop-mediated isothermal amplification (LAMP). These advanced molecular methods enable accurate and efficient identification of ESBL genes, facilitating the determination of effective antibiotic treatments (Hasman *et al*., 2014). PCR amplifies specific DNA sequences, while real-time PCR allows for the detection and quantification of target genes in real time. Multiplex PCR enables simultaneous amplification of multiple target genes in a single reaction, making it useful for screening for different types of ESBL genes simultaneously. DNA sequencing provides direct information about the nucleotide sequence of a DNA molecule, confirming the presence of ESBL genes and identifying specific mutations or variants associated with resistance (Imkamp *et al*., 2022). DNA microarrays are solid supports with thousands of specific DNA probes immobilized on them, which can hybridize with target DNA sequences obtained from a sample. Loop-mediated isothermal amplification (LAMP) is a rapid and sensitive method for ESBL gene detection under isothermal conditions, particularly useful in resource-limited settings or point-of-care diagnostics. These techniques can be combined or modified based on the specific requirements of the study or diagnostic setting. However, given their current widespread adoption and ease of use, PCR and single-isolate whole-genome sequencing appear to be irreplaceable (Hasman *et al*., 2014). Commercial kits are available for ESBL gene detection, which may utilize one or more of the aforementioned techniques in a standardized format.(Correa-Martínez *et al*., 2019; Imkamp *et al*., 2022; Mohammed & Anwar, 2022).

Genotyping and sequence analysis of ESBL genes

Genotyping and sequence analysis of Extended-Spectrum Beta-Lactamase (ESBL) genes is a process that identifies and characterizes these genes found in bacteria, particularly of the Enterobacteriaceae family (Ferreira *et al*., 2011). The process involves sample collection and bacterial isolation, phenotypic confirmation, DNA extraction, PCR amplification, product analysis/gel electrophoresis, DNA sequencing, sequence analysis, phylogenetic analysis, and data interpretation. Bacterial isolation involves identifying the bacteria of interest, phenotypic confirmation confirms the presence of ESBL production, DNA extraction is performed using standard methods, PCR is performed using specific primers, and agarose gel electrophoresis confirms the presence of the target gene. DNA sequencing is performed using Sanger sequencing or next-generation sequencing (NGS) technologies. Sequence analysis uses bioinformatics tools and databases to identify specific ESBL gene variants. Phylogenetic analysis studies the evolutionary relationships between different ESBL gene variants and traces their dissemination patterns among bacterial populations. Data interpretation helps understand the prevalence, diversity, and distribution of ESBL genes, providing insights into antibiotic resistance mechanisms and guiding infection control

measures and antibiotic stewardship programs (Mahazu *et al*., 2022).

Recently, the Center for Genomic Epidemiology has made several bioinformatics plug-and-play tools available online for rapid sequencing assembly and annotation (Vasala *et al*., 2020). These tools serve various purposes, including species identification, virulence analysis, phylogeny determination, and resistance detection. Some of the notable tools accessible at the Center include ARG-ANNOT, CARD, SRST2, MEGARes, Genefinder, ARIBA, KmerResistance, AMRFinder, and ResFinder. Researchers can utilize these tools based on their specific sequencing objectives, enabling efficient genomic analysis and epidemiological studies (Hendriksen *et al*., 2019).

Factors and settings that promote drug resistance

Several factors and settings contribute to the promotion of drug resistance. These factors can be categorized into drug-related, environmental, prescriber-related, and patient-related (Assefa, 2022). Drug-related factors encompass the source of the drug and the limited discovery of new antimicrobials. Environmental factors include places like daily care centers, jails, long-term care facilities, homeless shelters, and intensive care units, where poor infection control can contribute to the development of resistance. Prescriber-related factors involve instances of under - or over -prescription, inadequate follow-up and inappropriate use of antibiotics. Patient-related factors include behaviors such as shortened treatment courses or incomplete compliance with prescribed medications (Chen *et al*., 2021). Despite the multitude of factors contributing to antimicrobial resistance, it is important to recognize the interplay between individual health, global health, and social norms. These interactions are depicted in a conceptual diagram, illustrating the emergence (left panel) and dissemination (right panel) of antibiotic resistance (*Figure 6*). This emphasizes the intricate relationship between these factors and highlights the need for comprehensive strategies to address antimicrobial resistance effectively (Hernando-Amado *et al*., 2020).

Population groups at risk of drug resistance

The section of population groups at risk of drug resistance varies depending on the specific drug and the context in which it is used. However, there are certain populations that are generally considered to be at higher risk for drug resistance. These include individuals with a history of non-compliance (extreme ages), such as those who fail to follow prescribed treatment regimens due to forgetfulness, lack of understanding, or limited access to healthcare services. People with recurrent infections are also at higher risk, as frequent use of antibiotics can provide more opportunities for bacteria to develop resistance mechanisms (Chen *et al*., 2021). Healthcare-associated populations, such as hospitalized patients or those receiving invasive procedures are more susceptible to drug-resistant infections due to the higher prevalence of antibiotic use in these settings. Individuals in close contact with drug-resistant pathogens, such as people living in long-term care facilities or military barracks, are at higher risk due to the potential for transmission. Agricultural workers and livestock handlers in regions where antibiotics are

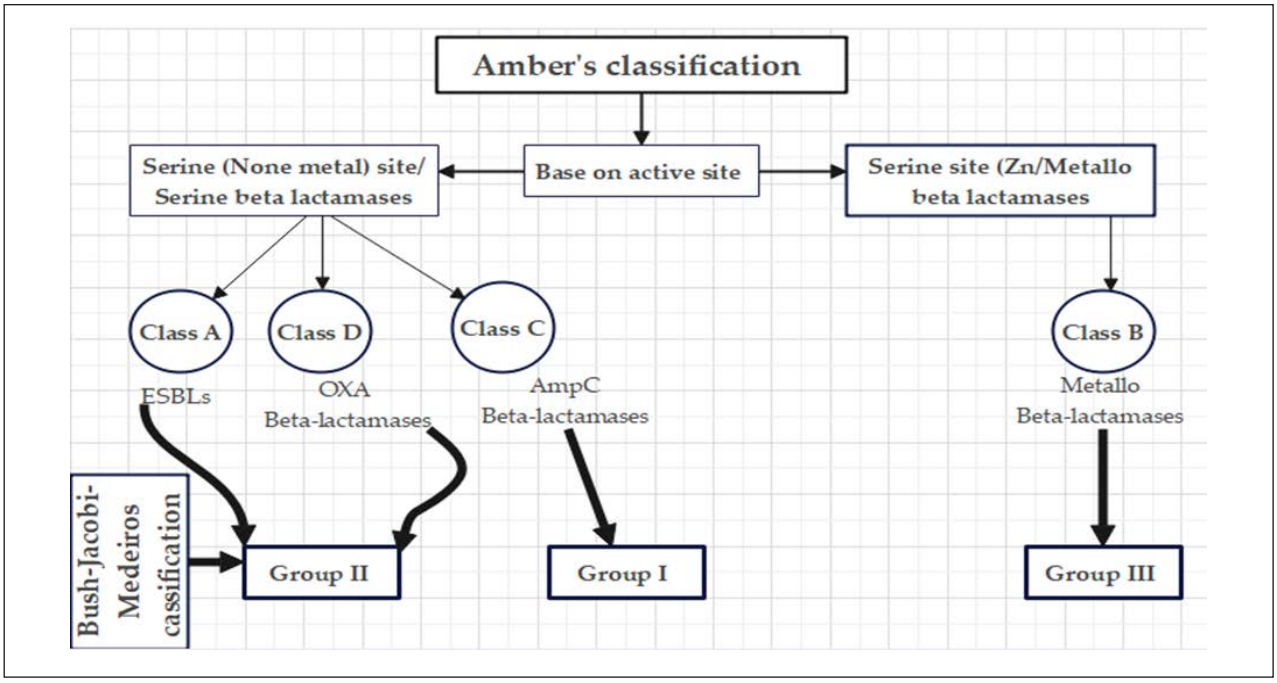

Figure 6 - *Schematic representation of common bacterial resistance mechanisms.*

extensively used in farming may encounter drug-resistant bacteria through direct contact with animals or exposure to the environment. Finally, international travelers visiting areas with high rates of drug-resistant infections, especially in developing countries with limited healthcare infrastructure, may be at increased risk of acquiring drug-resistant pathogens through various means of exposure (CDC, 2019).

Strategies to Control ESBL-mediated Resistance

To effectively control ESBL-mediated resistance, a comprehensive and multifaceted approach is necessary. First, robust surveillance systems should be implemented in healthcare facilities to promptly identify and monitor the prevalence of ESBL-producing bacteria. This enables timely intervention and targeted control measures (Johnson, 2015). Second, strict adherence to infection control measures, including consistent hand hygiene and thorough environmental cleaning, is essential to prevent the spread of ESBL-producing bacteria within healthcare settings (CDC, 2019). Third, promoting antibiotic stewardship programs is crucial to optimize antibiotic prescribing practices, reducing the selective pressure that drives resistance development. Fourth, the use of advanced diagnostic techniques like PCR can rapidly identify ESBL-producing bacteria, enabling early detection and appropriate treatment (Anandabaskar, 2021). Fifth, combination therapy, guided by local susceptibility patterns, can enhance treatment effectiveness while minimizing the emergence of resistance (De Rosa *et al*., 2021). Additionally, it is important to encourage research and development of new antibiotics and explore alternative therapies such as non-beta-lactam antibiotics, phage therapy and monoclonal antibodies to expand treatment options (Zhang & Cheng, 2022). Public education on infection prevention measures and responsible antibiotic use in the community plays a vital role in reducing transmission to healthcare settings (Eliason, 1940). Collaboration among healthcare facilities, public health agencies, and research institutions is essential to establish surveillance networks for monitoring ESBL-mediated resistance. Finally, implementing environmental control measures and providing comprehensive education and training to healthcare professionals further contribute to combating ESBL-mediated resistance. By implementing this comprehensive approach, we can effectively address the challenges posed by ESBL-mediated resistance and preserve the efficacy of antibiotics in healthcare settings and the community (Tamma PD, Aitken SL, Bonomo RA, Mathers AJ, van Duin D, 2022).

CONCLUSION

In conclusion, bacterial drug resistance, including the emergence and spread of extended-spectrum beta-lactamases (ESBLs), is a significant global health concern that hinders the effective treatment of bacterial infections. ESBLs, produced by bacteria, possess the ability to render beta-lactam antibiotics ineffective by hydrolyzing and inactivating them. These enzymes are encoded by genes located on transferable genetic elements, facilitating their horizontal transfer between different bacterial strains and species. This transfer contributes to the rapid dissemination of resistance, making ESBL-producing strains a major public health issue. Phenotypic characterization methods, such as the double-disk synergy test, and molecular techniques like PCR and sequencing, are crucial for detecting and understanding ESBLs. The molecular characterization of ESBLs has revealed a diverse array of genes, further complicating treatment options. To combat bacterial drug resistance, it is essential to understand ESBL mechanisms and characteristics, promote rational antibiotic use, implement infection control measures, and develop new antimicrobial agents. Continued research is necessary to stay ahead of evolving resistance mechanisms and ensure effective treatment for bacterial infections in the future.

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