ANTIMICROBIAL RESISTANCE PATTERN OF BACTERIAL ISOLATES

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Abstract:
Emergence of antimicrobial resistance is a major public health problem worldwide, particularly in developing countries. The effectiveness of currently available antibiotics is decreasing as a result of increasing resistant strains among clinical isolates.

Keywords: Bacterial Isolates, Antimicrobial, Resistance.

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Introduction:

Antimicrobial Resistance (AMR) occurs when microorganisms including bacteria, viruses, fungi, and parasites become able to adapt and grow in the presence of medications that once impacted them. AMR is considered a significant threat to the public health systems not just in developing countries but throughout the world **(1)**. Infection with AMR leads to serious illnesses and prolonged hospital admissions, increases in healthcare costs, higher costs in second-line drugs, and treatment failures **(2)**. Antibiotic resistance compromises a human immune system's capacity to fight infectious diseases and also contributes to different complications in vulnerable patients undergoing chemotherapy, dialysis, surgery, and joint replacement. Furthermore, people with chronic conditions like diabetes, asthma, and rheumatoid arthritis will be heavily impacted by antibiotic resistance. Since the effectiveness of antibiotics will be reduced due to persistence in trends of AMR, physicians should use last-resort classes of medicine such as carbapenems and polymyxins, which are not necessarily readily available in developing countries, have a high cost, and have many different side-effects **(3)**.

Antibiotic resistance was to occur when a drug loses its ability to inhibit bacterial growth effectively. Bacteria become 'resistant' and continue to multiply in the presence of therapeutic levels of the antibiotics **(4)**. Microorganisms generally acquire antibiotic resistance by genetic changes, but sometimes they do so by non-geneticmechanisms **(5)**.

There are four principal forms of antibiotic resistance evolve as 1-Natural Resistance (Intrinsic, Structural)

In this type of resistance, the usage of antibiotics is not associated with the resistance but it caused by the bacteria's structural properties **(6)**. This occurs as a result of intrinsic resistance, or microorganism which doesn't follow the target antibiotic structure, or antibiotics which due to its characteristics do not encounter its target **(7)**. Gram-negative bacteria and vancomycin, for example, vancomycin antibiotics does not move through the outer membrane. So that these Gram-negative bacteria are naturally insusceptible to vancomycin **(8)**.

Likewise, L-form bacteria that are cell wall-less types of the bacteria, such a Ureaplasma and Mycoplasma Mycoplasma that are naturally owningbeta-lactam antibiotics resistance **(9)**.

2- Acquired resistance

Regardless of resistance development due to alteration in the genetic features of bacteria, an acquired because it is not affected by the antibiotics it was previously susceptible to it **(10)**. This form of resistance comes from the main chromosome or extra chromosome structures (plasmids, transposons, etc.) **(11)**.

Chromosomal resistance results from mutations that change randomly bacterial chromosome, these mutations can occur by certain physical and chemical factors **(12)**.

This may be due to changes in the composition of bacterial cells, so that may be decreased bacterial drug permeability, or maybe changes to thedrug's target in the cell **(13)**.

Antibiotic resistance of bacteria spreads through getting resistance genes, which exist in plasmids, transposons (Tns), and integrons, which are a extrachromosomal genetic materials . Plasmids are segments of DNA that can replicate independently of chromosomal DNA. A plasmid is typically responsible for the development of antibiotic inactive enzymes (**14**). Persistence of these selfish genetic elements is improved when they carry genes that are useful to the host cell, such as antibiotic resistance genes (ARGs) in the presence of antibiotics. Consequently, many different ARGs circulate on plasmids (**15**).

Plasmids disseminate through bacterial populations primarily through the process of conjugation. Conjugation requires physical contact between two cells in the same environment, followed by the formation of a bridge that enables the transfer of a plasmid from a donor to a recipient cell (16).

Plasmid-mediated resistance to β-lactam antibiotics provides prime examples of how horizontal gene transfer HGT exacerbates AR challenges in hospitals. Extended-spectrum β-lactamases (ESBLs) and carbapenemase confer resistance by hydrolyzing β-lactam antibiotics, including penicillin, carbapenems, and cephalosporins (**17).**

So there are main forms of holding genetic material (resistance genes and plasmids) from bacterial cells, this form are transduction, transformation, conjugation, and mechanism of transposition **(18).** The antibiotic resistance genes (ARG) are in many cases associated with conjugative to plasmids or transposons. β-lactam resistance genes are commonly located on plasmids and thus disseminateby inter- and intraspecies conjugation in the Entero bacteriaceae, Pseudomonas and Acinetobacter **(19)**.

The transfer of plasmids in pathogens has led to the

worldwide spread of numerous ARGs encoding resistance to β-lactams, quinolones, aminoglycosides, tetracyclines, sulfonamides, and many other drug classes **(20).**

The free fragments of DNA, to the environment can be taken up and incorporated into the chromosome of a living bacterium to provide the recipient with new characteristics. This process is called bacterial transformation, and if the incorporated DNA contains genes that encode for resistance to an antibiotic, a previously susceptible bacterium can be "transformed" to now be resistant. Several clinically relevant antibiotic resistant pathogens are capable of DNA uptake and natural transformation, including Haemophilus, Pseudomonas, Staphylococcus, and Streptococcus **(19)**.

Transposons (Tns) or transposable elements (TEs), and retrotransposons (RTns) are DNA elements that can move from the DNA molecule to other places on the same DNA or other DNA molecules **(21).**

The existence of the enzyme in Tns, named transposase (Tase) causes their transposition **(21).**

Tns usually stay in the genome for a long time and cause mutations by transmission to other places of the genome. These TEs based on the transmission mechanisms which are divided into two groups: class I (RTns) and class II (DNA Tns) **(22)**

Class I RTns (retroelements) transmission can be done by the reverse transcriptase enzyme, a process called transposition **(23).** Bacterial retrotranscriptase has been found in retrons, theses Retrons produce multicopy single-stranded DNA (msDNA) (**24).**

Retrons have also been found in the genome of many bacteria. Retrons are sequences of DNA that encode a reverse transcriptase. It is not yet clear whether the retrons are mobile elements. It has been shown that retrons are effective on integron gene cassettes (an antibiotic resistancecarrier) in Salmonella (**25**).

Class II (DNATns) : Most DNA Tns are transmitted by the cut-and-paste mechanism and are mainly shorter thanRTns .DNA Tns in their two ends often have the sequences of inverted repeats (IRs) and a gene for encoding Tase enzyme (**26).**

TEs (DNA Tns) are divided into four categories in bacteria: insertion sequence (IS), composite Tns, non-composite Tns (Tn3 family), and transposable phage Mu **(27) (28).**Mobile elements (like DNA Tns) can cause the spread of antibiotic resistance in bacteria species **(29).**

Today, it has become clear that ISs can cause the bacterial antibiotic resistance to different ways **(30).** ISs can cause the genes inactivation in the insertion site by direct integration and with composite Tns cause the transmission of antibiotic resistance genes to other bacteria.

For example, IS256 that exists in the composite Tns of Tn4001 is responsible for resistance to aminoglycosides. Composite and non-composite Tns can increase the antibiotic resistance in bacteria by carrying additional genes (such as resistance genes) **(29).**

Drug resistance genes are carried by composite Tns and their transmission among bacteria is the most serious challenge in the treatment of infectious diseases **(31,32).**

They are carriers of antibiotic resistance genes. Tn5, Tn6, Tn9, Tn10, Tn903, Tn1525, Tn2010, Tn2680, Tn4001, Tn4003, Tn2700, and Tn3411 are all composite types. Tn5 (Kanamycin Resistance), Tn9 (chloramphenicol resistance), Tn10 (tetracycline resistance), and Tn903 being most important in mediating antibiotic resistance especially in E. coli **(33).**

The Tn916 family includes the tetM and mef E resistance genes, which are mainly the creator of antibiotic resistance in Streptococcus pneumoniae **(34).**

Tn3 is the carrier of the resistance gene to ampicillin and exists in both the Gram-negative and Gram-positive bacteria **(33).**

While Tn21 is the carrier of mercury-resistance genes(merC, merA, merR, merT, merP, merE, me rD), and the carrier of some resistance genes to cephalosporins and sulfonamides It has been demonstrated that both shapes of organic and inorganic mercury resistance in P. aeruginosa K62 strain are caused by the merT gene inplasmidp MR26 **(35).**

Another class of genetic structures, termed integrons, Integrons are a segment of dsDNA that play a major role in bacterial adaptation and evolution. These genetic determinants are known by the presence of three necessary apparatuses: an integrase (intI gene), Pc (a promoter) and attI (a recombination site). These elements are able to acquire gene cassettes, which can carry antibiotic resistance factors, by site-specific recombination mechanism. The most common types of resistance integrons are class I (Tn402 derivatives), followed by class II and III. In recent years, the role of integrons as an important factor in the transmission and spread of resistance factors has been considered.Up to date, 4 general classes of integrons have been identified and distinguished, termed classes 1–4 integrons. Known as multi-resistant integron (RIs), classes 1–3 integrons are capable of acquiring same gene cassettes via similar recombination platform **(36).**

Class 1 integron have been found in about of 9% of bacterial genoms, and has been well established and documented in Gram-negative microorganisms, with its role in the distribution and spread of antimicrobial resistance also verified and identified. Class 1 integrons are associated with a variety of resistance gene cassettes, but most integrons contain an *aad A* resistance determinant, encoding streptomycin-spectinomyc in resistance. Trimethoprim resistance determinants are also detected frequently **(37,38)** ,

Class 1 integron has been studied in various microorganisms, with its occurrence and prevalence commonly reported to be ranging from 22 to 59 % and identified in clinical Gram-negative bacteria, nincluding. *Acinetobacter*, *Campylobacter*, *Citrobacter*, *Enterobacter*, *Escherichia*, *Klebsiella*, *Mycobacterium*, *Pseudomonas* **(39-41)** Class 1 integrons isolated from bacteria involved in infections of man frequently also harbor gene cassettes encoding β-lactam resistance (**42).**

While class 2 integrons have been commonly reported in some species of Gram-negative organisms such as *Acinetobacter*, *Enterobacteria ceae*, *Salmonella* and *Psuedomonas*, with a low occurrence and prevalence comparing with class 1 integron **(43,44)**

Its identification has been limited within a few microorganisms including *Acinetobacter* spp., *Alcaligenes*, *Citrobacter freundii*, *Escherichia coli*, *Klebsiella pneumoniae*, *Pseudomonas aeruginosa*, *Pseudomonas putida*, *Salmonella* spp **(45)**

Identification of class 4 integron has been limited within microorganisms such as the *Vibrionaceae*, *Shewanella*, *Xanthomonas*, *Pseudomonad*, and other proteobacteria(**46).** To date, class 4 integrons have been found to carry gene cassettes imparting resistance to the antibiotics chloramphenicol and fosfomycin **(37).**

3- Cross-resistance

It is mean the resistance to a specific antibiotic by specific microorganisms, that work with the identical or related mechanisms and that are also resistant to other antibiotics. This is generally seen when antibiotics have common structures: such as resistance to erythromycin, neomycin, kanamycin, or resistance to cephalosporins and penicillins **(47).**

However, cross-resistance can some times be seen in a completely distinct group of drugs as well, like a cross- resistance that exists amongst erythromycin-lincomycin, this resistance might be the chromosomal origin or not**(48)**.

4- Multi-drug and other types of resistance

Multidrug-resistant species are typically pathogens that have been resistant to their antibiotics, this ensures that the bacteria will no longer be eliminated or regulated by a single drug. Inappropriate utilization of antibiotics for treatment culminated in the introduction of multidrug- resistant pathogenic bacteria **(49)**. Either of the two mechanisms can induce multidrug resistance in bacteria **(50).**

Firstly, these bacteria will acquire several genes, each coding for specific drug resistance, this form of resistance usually exists on R- plasmids **(51)**.

Secondly, the form of multidrug resistance may also occur by enhanced gene expression encoding for efflux pumps, enzymatic inactivation for antibiotics, changes in target structure, and others **(52).**

If the bacterial strains are not susceptible to three or more antimicrobial types, they are called multidrug-resistant (MDR) bacteria. If the species, resistant to all but one or two classes of antibiotics, are deemed highly resistant to medicines, whether the species resistant to all usable antibiotics are known as pan-drug resistant **(53)**.

Antibiotics Resistance Mechanisms

There are many mechanisms that bacteria exhibit to protect themselves from antibiotics and understanding the mechanisms by which bacteria resist antibiotics will become critical to solving the crisis. Misuse of antibiotics may contribute to the development of resistant bacteria; an incomplete course of antibiotics risks not entirely eradicating the colony thus allowing the development of resistant bacteria. Mechanisms of drug resistance fall into several broad categories, including active efflux pumps, drug inactivation /alteration, modification of drug binding sites/ targets, changes in cell permeability resulting in reduced intracellular drug accumulation, biofilm formation and others **(54)**.

Drug Inactivation

Beta-lactamases are enzymes that hydrolyse the beta lactam ring. Penicillin contains beta lactam ring and is therefore inactivated by these enzymes. The first beta lactamase was discovered in S. aureus. However, these enzymes more commonly produce resistance in Gram-negative pathogens **(55)**. Beta lactamase enzymes destroy the amide bond of the β-lactam ring, rendering the antimicrobial ineffective. To overcome this problem, new β-lactam compounds with a wider spectrum of activity and less susceptibility to penicillinases (such as ampicillin) were manufactured **(5).**

Bacterial enzymes have been shown to add chemical groups to vulnerable sites on the antibiotic molecule preventing the antibiotic from binding to its original target. Within the structure of an antibiotic, hydroxyland amides groups can easily be changed by hydrolysis. Moreover, acetyl, phosphate and nucleotide groups can be added to the antibioticsinactivating them **(56)**.

Target Modification and Mutation

A common pathway for bacteria to develop antimicrobial resistance is toavoid the action of the antibiotic by interfering with their target site. To achieve this, bacteriahave evolved different tactics, including modificationsof the target site that result in decreased affinity for the antibiotic molecule and protection of the target (preventing the antibiotic from reaching its binding site). One of the classic and best- studied examples of the target protection mechanism is the tetracycline resistance. The target changes may consist of (i) point mutations in the genes encoding the target site, (ii) enzymatic alterations of the binding site (e.g., addition of methyl groups), and/or (iii) replacement or bypass of the original target. Mutations of the target site: One of the classical examples of mutational resistance is the development of rifampin resistance **(5).**

Modification of the antibiotic target site makes the antibiotic unable to bind properly. Microorganisms cannot evade antimicrobial action by dispensing with them entirely because of the vital cellular functions of the target sites. In thismechanism, bacteria found ways to alter the targets of antimicrobial agents. The classical example of drug target modification is the staphylococcal mechanism of variously altering the Penicillin Binding Protein (PBP) which is the target of β-lactam antibiotics **(57)**.

Reduced Permeability

Most of the antibiotics used in clinical practice have intracellular bacterial targets or, in the case of Gram negative bacteria, targets located in the cytoplasmic membrane. Hence, the compound must penetrate the outer and/or cytoplasmic membrane to exert its antimicrobial effect. Bacteria have developed mechanisms to prevent the antibiotic from reaching its intracellular or periplasmic target by decreasing the uptake of the antimicrobial molecule **(5).** The relative impermeability of the outer membrane is one of the major causes of increased intrinsic drug resistance seen in opportunistic Gram-negative pathogens like P. aeruginosa. Hydrophilic molecules such as tetracyclines, β-lactams, and some fluoroquinolones are particularly affected by changes in permeability of the outer membrane since they often use water-filled diffusion channels known as porins to cross this barrier **(58)**. The example of the efficiency of this natural barrier is the fact that vancomycin, a glycopeptide antibiotic, is not active against Gram-negative organisms due to the lack of penetration through the outer membrane.

Drug Efflux Pumps

Efflux pumps are transporter proteins involved in the removal of toxic substances from the interior of the cell to the external environment. Efflux pumps in bacteria are major contributors to drug resistance; they extrude a broad spectrum of antibiotics to the exterior of the organism as shown in Figure 3. Hence, infections caused by these pathogens can be difficult to treat. Some efflux pumps are specific for a single drug while others are capable of transporting multiple substrates. **(57)**.

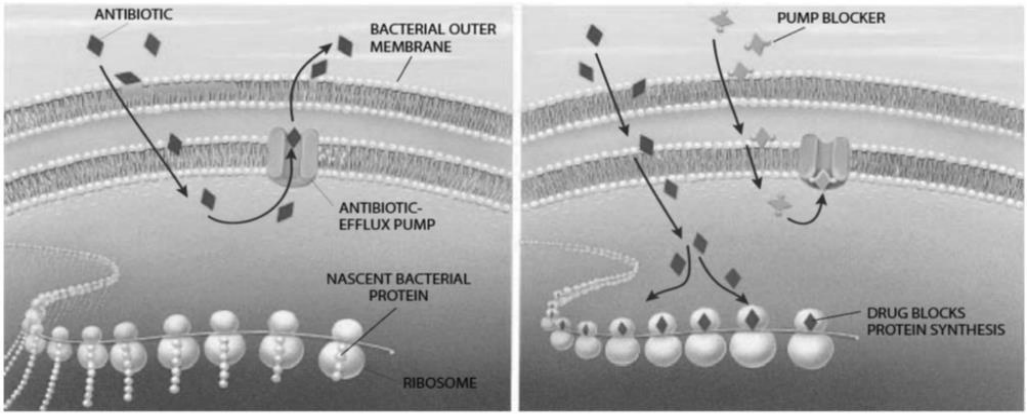

Figure 1: Bacterial efflux system A, system for antibiotic pumping out of the cell; B, antibiotic interfering with ribosomes in protein biosynthesis (57).

Drug efflux is the energy-dependent process of production of complex bacterial machineries capable of extruding a toxic compound out of the cell can also result in antimicrobial resistance. The efflux systemable to pump tetrac͘ycline out of the cytoplasm of E. coli dates from the early 1980s and was among the first to be described **(59)**. From then, many classes of efflux pumps have been characterized in both Gram-negative and Gram-positive pathogens.

1. Mechanisms of Antibiotic Resistance In Streptococcus pneumoniae

Streptococcus pneumoniae is a Gram- positive pathogen and one of the most common causes of community acquired diseases, such as pneumonia .The morbidity and mortality of infections caused by S. pneumoniae remain high despite appropriate antibiotic therapy **(58).**

The mechanism of action of beta-lactam antibiotics is based on the binding of the antibiotic to cell wall synthesizing enzymes that is, the penicillin-binding proteins (PBPs), thereby interfering with the biosynthesis and remodeling of the bacterial peptidoglycan. Binding of beta-lactams to PBPs leads to a covalently deacylated complex removing the PBPs from the metabolically active pool **(60)**. The mechanism of penicillin resistance inclinical isolates of *Streptococcus pneumoniae* involves the alteration of PBPs so as to reduce their affinityfor the antibiotic molecule. Mutations leading to resistance to penicillin are usually seen in the transpeptidase-penicillin-binding domain **(61).**

Fluoroquinolones

In the clinical isolates of pneumococci, fluoroquinolone resistance is mediated by target modifications that involve mutations in the gyrase genes, *gyrA* and *gyrB*, and in the topoisomerase IV genes, *parC* and *parE*. Moreover, the in vitro studies indicated that some strains may use an efflux mechanism resulting in reduced intracellular accumulation of the antibiotic **(62)**.

Macrolide-lincosamide-streptogramins (MLS)

Even though MLS antibiotics are chemically distinct, they competitively interact while binding to the ribosomal 50S subunit, where only one molecule is able to bind. Two mechanisms of resistance to MLS in clinical isolates of pneumococci have already been reported which includes modification of the target that results in co- resistance to MLS and efflux of the antibiotic that mediates resistance to 14- membered and 15-membered macrolides only resulting in a so-called M phenotype**(63).**

Tetracycline

Tetracyclines exhibit bacteriostatic activity by binding to either the acceptor site (A- site) or the peptidyl-donor site (P-site) of the30S subunit of the bacterial ribosome, thus preventing binding of the aminoacyl-tRNA to the A-site. Tetracyclines acquire resistance by Ribosomal protection mediated by the genes *tet*(M) and *tet*(O) **(64)**.

Trimethoprim-sulfamethoxazole

The combination of trimethoprim with sulfamethoxazole (cotrimoxazole) has been used extensively for the treatment of lower respiratory tract infections. They interferewith the biosynthesis of folic acid. Trimethoprim selectively acts by inhibiting the bacterial dihydrofolate reductase (DHFR) thus preventing the reduction of dihydrofolate to tetrahydrofolate. Trimethoprim resistance in clinical isolates of *S. pneumoniae* results from substitutionof single amino acids in the chromosomal- encoded DHFR **(65)**.

2. Mechanisms of Antibiotic Resistance in Staphylococcus Aureus

Staphylococcus aureus is a gram positive organism responsible for a wide spectrum of infections. S.aureus have shown remarkable ability to acquire resistance to a variety of antibiotics through, mutation and horizontal gene transfer **(66)**.

Resistance to beta-lactam antibiotics

S. aureus resistance to penicillin appeared very soon after the introduction of this antibiotics. Nowadays, more than 90% of S. aureus isolates are penicillin resistant, which is due to the production of penicillinase, an extracellular enzyme that hydrolyzes penicillin. The prototype of the anti staphylococcal penicillins called methicillin, was designed to resist the action of penicillinase. Moreover, S. aureus developed resistance to it. The Methicillin resistant staphylococcus aureus (MRSA) produces an altered penicillin binding protein, termed PBP2a which has reduced affinity for methicillin and can continue peptidoglycan synthesis in the presence of antibiotic. PBP2a is encoded by the mecA gene that is incorporated in a chromosomal genetic element designated staphylococcal chromosomal cassette (SCC) mec. MRSA are resistant to all the beta lactams, including carbapenems and cephalosporins. They are typical nosocomial pathogens and are often multiresistant which is also resistant to other classes of antibiotics. Recently, MRSA strains causing serious infections termed as community acquired (CA-MRSA) have emerged**(67**).

Resistance to Glycopeptides

Vancomycin is considered as one of the cornerstone of therapy in MRSA. But, at the end of the decade, strains are intermediately resistant (VISA) or fully resistant (VRSA)to vancomycin. Mechanism of resistance in VISA includes trapping of the antibiotics in a thickened cell wall, rich in residues that binds vancomycin. The antibiotic is hence prevented from reaching the true targets in the glycopeptide precursors at the inner layer of the cell wall. **(66)**.

Resistance to Fluoroquinolones

Fluoroquinolone resistance is widespread among MRSA and is due to mutations in the quinolone-resistance determining region(QRDR) of DNA gyrase and topoisomerase IV. Overexpression of the efflux pump NorA can also contribute to resistance **(58)**.

Mechanisms of Antibiotic Resistance in Klebsiella pneumonia

K. pneumoniae shows resistance against the main antibiotic classes: carbapenems, cephalosporins, aminoglycosides, and fosfomycin, leading to the therapeutic failure of these agents **(68)**. The Resistance may occur due to increased efflux, drug inactivation, or altered binding to the target site. Many strains of K. pneumoniae produce ESBL or form biofilms, further exacerbating resistance. The antibiotic resistance of

K. pneumoniae is mainly produced in the following five ways: (1) enzymatic antibiotic inactivation and modification, (2) antibiotic target alteration, (3) porin loss and mutation, (4) increased efflux pump expression of the antibiotic, and (5) biofilm formation **(69)**. The five mechanisms conferring antibiotic resistance to K. pneumoniae are shown in Figure 2.

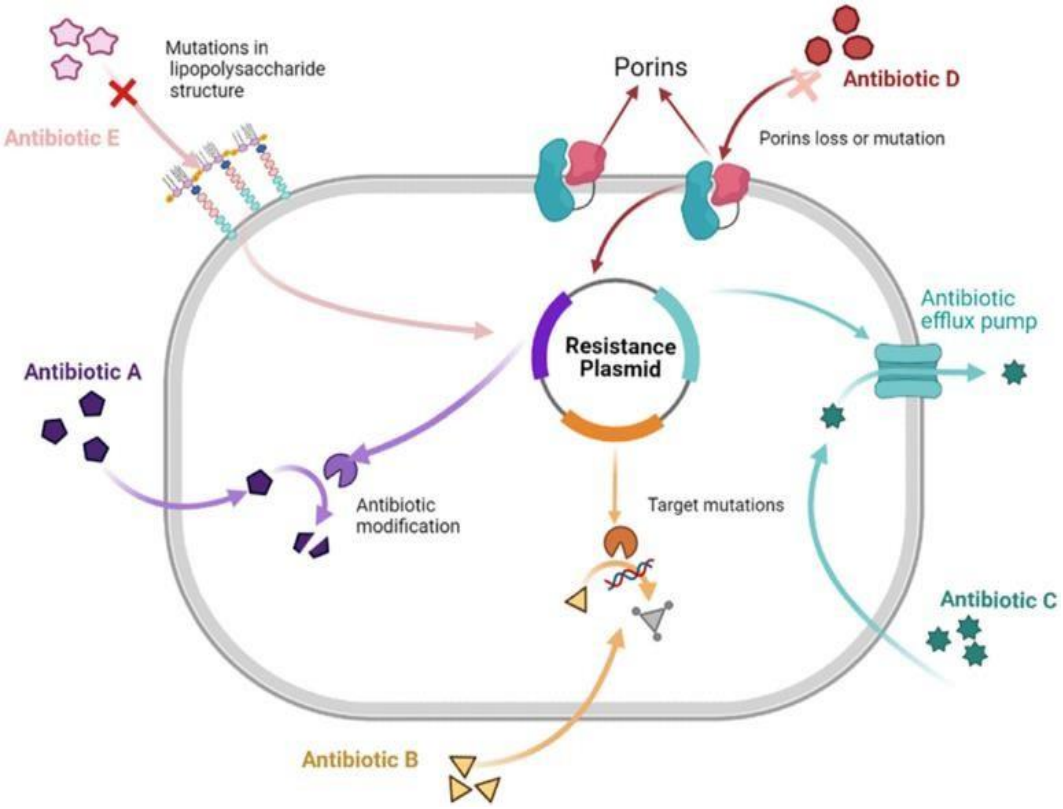

Figure 2: Various mechanisms conferring antibiotic resistance to K pneumoniae(70).

Mechanisms of Antibiotic Resistance Pseudomonas *aeruginosa*

Intrinsic resistance mechanisms of *P. aeruginosa* include its low outer membrane permeability (12- to 100- fold lower than that of *Escherichia coli*), the presence of antibiotic efflux pumps and β-lactamases,such as OXA-50 and AmpC **(71)**.

Acquired resistance mechanisms from horizontal gene transfer include acquisition of transferable aminoglycoside modifying enzymes and β-lactamases, while acquired resistance as a result of *de novo* mutational events often takes the form of overexpression of efflux pumps and β- lactamases, along with decreased expression or modification of target sites and porins**(72).**

Adaptive resistance mechanisms are those that are induced through external stimuli, such as stress factors and the presence of certain antibiotics. This is different from acquired mutational resistance as adaptive resistance is transient and unstable.

Adaptive resistance mechanisms are not permanent, unlike mutational events, and become inactive upon removal of the stress factor **(73)**.

Mechanisms of Antibiotic Resistance in Haemophilus pneumonia

Haemophilus influenzae is an opportunistic pathogen found naturally in human upper respiratory tract. *Haemophilus influenzae* is the main causeof bronchopulmonary infections. these infections are caused by non- encapsulated (non typable) and encapsulated (typable) strains **(74)**.
Two mechanisms are responsible for resistance to aminopenicillins, the main and the common mechanism is due to enzymatic hydrolysis of the antibiotic by β-lactamase (TEM-1 and ROB-1 type). The second mechanism of resistance is due to mutations in PBPs affecting their affinity to penicillin **(75)**.

In *H. influenzae*, resistance to ampicillin without the production of β-lactamase was shown to be chromosomally mediated and was correlated with alterations in PBP 3 (3A and 3B) **(76)**.

Mechanisms of Antibiotic Resistance in mycoplasma pneumonia

Mycoplasma pneumoniae (*M. pneumoniae*) is a small, cell wall-less, and pleomorphic bacterium which belongs to the order Mycoplasma tales, a.It is one of the major mucosal pathogens of the respiratory tract that causes variety diseases in humans **(77)**.

Because of their lack of a cell wall, *M. pneumoniae* is innately resistant to many classes of antimicrobial agents that act on the cell wall **(78)**. Effective antimicrobials against *M. pneumoniae* include fluoroquinolones (levofloxac in, ciprofloxacin, and moxifloxacin) and macrolides (erythromycin, azithromycin, and josamycin) and tetracyclines (minocycline and doxycycline) **(79)**.

Macrolide-Resistant Mycoplasma pneumoniae MRMP have been demonstrated by in vitro selection that involves point mutations in the peptidyl transferase loop of 23S rRNA and point mutations, insertions, or deletions in ribosomal proteins L4 and L22 (**77)**

Mechanisms of Antibiotic Resistance in legionella pneumonia

L. pneumophila has been reported to be resistant to erythromycin, ciprofloxacin, and azithromycin (AZM) in vitro. Macrolides (especially azithromycin) and fluoroquinolones are recommended as first-line treatments for Legionnaires' disease because of their efficiency against intracellular *L. pneumophila* strains *in vitro* **(80)**

Azithromycin and other macrolides exert a bacteriostatic effect by interacting directly with the central loop of domain V, the site of peptide bond formation, thereby inhibiting protein synthesis. Mutations in genes encoding 23S rRNA or L4 and L22 ribosomal proteins are known to be responsible for macrolide resistance determinants in L. pneumophila strains .(**81)** .

Detecting Methods of Antimicrobial Resistance

Antimicrobial susceptibility testing methods are in vitro procedures used to detect antimicrobial resistance in individual bacterial isolates. Those laboratory-based detection methods can determine resistance or susceptibility of an isolate against any therapeutic candidates. Those methods can also be used for monitoring the emergence and spread of resistant microorganisms in the population **(82)**.

Disk-diffusion method

The disk diffusion is also known as Kirby-Bauer antibiotic testing. The drug diffuses radially through the agar, the concentration of the drug decreasing logarithmically as the distance from the disk increases and results in a circular zone of growth inhibition around the disk, the diameter of which is inversely proportional to the MIC. The zone diameters are interpreted on the basis of guidelines published by CLSI, and the organisms are reported as susceptible, intermediate, or resistant. Disk diffusion can only be used to test rapidly growing organisms, for which criteria for interpretation of zone sizes are available **(83)**. The diameter of zone of inhibition around the antimicrobial disk is related to minimum inhibitory concentration (MIC) for that particular bacterial isolate; the zone of inhibition correlates inversely with the MIC of the test bacterium. Generally, having larger the zone of inhibition, the lower the concentration of antimicrobial required to inhibit the growth of the organisms as shown in figure 3. **(84).**

Figure 3: Kirby Bauer Disc Diffusion Method For Antibiotic Susceptibility Testing

Dilution Method

Agar dilution and broth dilution are the most commonly used methods to determine the minimal concentration of antimicrobial agents that kill (bactericidal activity, MBC) or inhibit the growth (bacteriostatic activity, MIC) of microorganisms. Dilution methods are performed when quantitative methods are required for microorganisms with a variable growth rate **(85)**.

Broth Dilution Technique:

The broth dilution technique of antibiotic susceptibility testing is alsoknown as the minimal inhibitory concentration (MIC) technique as shown in FIGURE 4. Test tubesor wells containing increasing concentrations of each antibiotic to be tested,from 0.0312 to 512 µg/ml, are inoculated with a fixed volume of nutrient broth containing a standard concentration of bacteria. The concentration of the antibiotic in each tube is double that in the previous tube. In the broth dilution assay, an antimicrobial is added to a culture tube of non-selective broth medium at different concentrations. Tubes are incubated under optimum conditions for the test microorganism from 16 to 24 hours. Antimicrobial effect could be determined by spectro-photometry or byplating counting **(57)**.

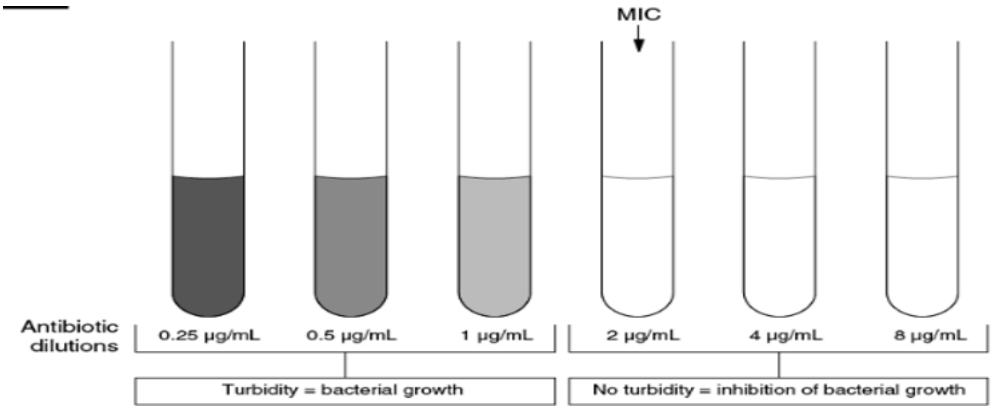

Figure 4: Diagram of broth dilution method (57).

The Agar Dilution Method:

Agar dilutions are most often prepared in petri dishes and haveadvantage that it is possible to test several organisms on each plate. In the agar dilution method, the antimicrobial agent is incorporated into the agar medium with each plate containing a different concentration of the agent. The inoculum can be applied rapidly and simultaneously to the agar surfaces using an inoculum replicating apparatus. Mueller-Hinton agar is prepared from a dehydrated base. The advantages of agar dilution testing include the reproducible results and satisfactory growth of most nonfastidious organisms **(57)**.

Epsilometer Test (E-Test)

Epsilometer test (E- test) is an 'exponential gradient' method ofdetermination of antimicrobial resistance. The E-test is developed to provide a direct quantification of antimicrobial susceptibility

of microorganisms. This is a quantitative method that applies both the dilution of antibiotic and diffusion of antibiotic into the medium. The device consists of a predefined, continuous, and exponential gradient of antibiotic concentrations immobilized along a rectangular plastic test strip. The principle of E test method is based on antimicrobial concentration gradient in an agar plate (Figure 5). These strips are impregnated on the underside with a dried antibiotic concentration gradient and are labeled on upper surface with a concentration scale. When this E test strip was placed onto an inoculated agar plate, there was an immediate release of the drug. E test have been used to determine MIC for fastidious organisms like *S. pneumoniae, ß-hemolytic streptococci, Haemophilus* sppcies and anaerobes. **(57)**.

Automated Instrument Methods

There are a variety of commercially available automated systems available to help reduce the technical time required to perform and record routine sensitivity tests. For example, the results of disk sensitivity tests and breakpoint sensitivity tests can be read using a camera interfaced to a computer system. Other Systems utilize liquid cultures and detect the effectof antibiotics on the rate of bacterial growth through measurement of turbidity (nephelometry) or the production of CO_2.

These automated systems can significantly shorten the necessary incubation time.

The advantage of using this test is increased reproducibility, decreased labor costs and issued rapid results but the disadvantage is they are not available widely in developing country**(57)**.

Molecular Methods for Detection of Antimicrobial Resistance

Molecular characterization of the genetic mechanism(s) underlying a given phenotypic result, obtained by traditional antimicrobial sensitivity testing, is now an integral part of many clinical investigations in relation to bacterial infections, In some cases, when phenotypic results are too time- consuming, non-conclusive, or unavailable, molecular analysis can be used to investigate the presence of a given gene. Molecular methods are being used extensively by both research and reference laboratories. Some of the methods employed, such as PCR and hybridization techniques, have been used for decades, while new methods such as Whole-Genome Sequencing (WGS) and Matrix-Assisted Laser Desorption Ionization–Time of Flight Mass Spectrometry (MALDITOF MS) are just emerging **(86).**

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