Brief Outline about Biofilm Resistance to Antimicrobials

Wesam Abdel Moniem Hafez Boghdady, Ahmed Mohamed Morad Asaad, Enas AbdelZaher Tantawy, Doaa Al Hussein Abo-Alella

Medical Microbiology and Immunology Department,Faculty of Medicine, Zagazig University, Egypt

Corresponding author: Wesam Abdel Moniem Hafez Boghdady

E-mail: wessammoniem@gmail.com, wABaghdadi@medicine.zu.edu.eg

Conflict of interest: None declared

Funding: No funding sources

Abstract

Background: Antibiotic molecules ought to penetrate throughout the biofilm matrix to impact the covered cells. The extracellular polymeric matrix influences the amount of the molecule, which is transferred to the inner layer of biofilm and interacts with an antibiotic agent, so it provides an anti-spread barrier for an antimicrobial agent. Biofilm EPS confers a physical barrier containing numerous anionic and cationic molecules such as proteins, glycoproteins, and glycolipid that can bind charged antimicrobial agents and provide shelter for microorganisms. For example in Pseudomonas aeruginosa biofilms, Pel exopolysaccharides, an EPS component is able to spread cationic antibiotics such as aminoglycosides and, thus, provides tolerance to these molecules. During growth in biofilm structures, physiological heterogeneity happens due to the occurrence of oxygen and other nutrients gradient in biofilms. This gradient is created because cells that are close to the surface of the biofilm consume obtainable nutrient sources and oxygen before the nutrients disperse into depth of the biofilm. In the biofilm, cells coexist with different genotypes and phenotypes. This leads to expression of distinct metabolic pathways depending on the local environmental conditions in the biofilm. The metabolic activity of bacteria is high in the outer part of the biofilm, while it is low in the inner part. Many antibiotics are directed against processes occurring in growing bacteria e.g. replication, transcription, translation & cell wall synthesis. So, increased antimicrobial tolerance will be found in biofilm bacteria with low metabolic activity located in the inner part of biofilms.

Keywords: Biofilm Resistance, Antimicrobials

Tob Regul Sci. ™ 2022;8(1): 3990-4010
DOI: doi.org/10.18001/TRS.8.1.300

Introduction:

Antibiotic resistance is the increase in the minimum inhibitory concentration (MIC) value of an antibiotic due to a permanent change in the bacteria such as mutation or resistance acquired by

horizontal gene transfer. While, antibiotic tolerance is the ability of cells to survive the effect of an antibiotic due to a reversible phenotypic state **(Hathroubi et al.,2017).**

Mechanism of biofilm resistance to antibiotics

A-Failure of antibiotics to penetrate biofilm

Antibiotic molecules ought to penetrate throughout the biofilm matrix to impact the covered cells. The extracellular polymeric matrix influences the amount of the molecule, which is transferred to the inner layer of biofilm and interacts with an antibiotic agent, so it provides an anti-spread barrier for an antimicrobial agent. Biofilm EPS confers a physical barrier containing numerous anionic and cationic molecules such as proteins, glycoproteins, and glycolipid that can bind charged antimicrobial agents and provide shelter for microorganisms (**Nadell et al.,2014**)

For example in Pseudomonas aeruginosa biofilms, Pel exopolysaccharides, an EPS component is able to spread cationic antibiotics such as aminoglycosides and, thus, provides tolerance to these molecules (**Colvin et al.,2011).**

B-Slow rate of growth

During growth in biofilm structures, physiological heterogeneity happens due to the occurrence of oxygen and other nutrients gradient in biofilms. This gradient is created because cells that are close to the surface of the biofilm consume obtainable nutrient sources and oxygen before the nutrients disperse into depth of the biofilm (**Stewart et al.,2008).**

Nutrient and oxygen concentration gradients develop and cause bacterial populations that display different growth rates . The effect of many antibiotics depends on growth. Because most antibiotics aim at some kind of produced macromolecule, it is unexpected that these agents will have much impact on the microorganisms in biofilm that limit macromolecular production, so conventional antibiotics are usually less affected against metabolically inactive or slow-growing cells (**Blanco et al.,2016**).

C-Altered metabolism

In the biofilm, cells coexist with different genotypes and phenotypes. This leads to expression of distinct metabolic pathways depending on the local environmental conditions in the biofilm. The metabolic activity of bacteria is high in the outer part of the biofilm, while it is low in the inner part. Many antibiotics are directed against processes occurring in growing bacteria e.g. replication, transcription, translation & cell wall synthesis. So, increased antimicrobial tolerance will be found in biofilm bacteria with low metabolic activity located in the inner part of biofilms **(Ciofu et al., 2015).**

D-Oxygen gradients

The oxygen tension is low in the inner part of biofilm. In P. aeruginosa, hypoxia increased antibiotic resistance by changing the composition of multidrug efflux pumps. The anaerobic environment within biofilms will mostly affect aminoglycosides action by inducing downregulation of genes of energy metabolism and by enhancing changes in gene expression **(Taylor et al., 2014).**

E-Persistent cells

This is a small subpopulation of bacteria that has entered a slow-growing or starvation state and that is highly resistant to killing by antibiotics. The reduced metabolic rates of these cells make them less susceptible to antibiotics compared to active exponential growth-phase bacteria **(Conlon et al., 2013).**

This occurs especially in sites where immune components are limited, such as in biofilm. Persisters exhibit enhanced toxin/antitoxin (TA) systems induced by starvation or DNA damage. In biofilms, several TA systems have been associated with high numbers of multidrug tolerant persister cells, but this tolerance is limited to specific antibiotics and toxin/antitoxins **(Lewis, 2012).**

F-Molecular factors

Mutation

It has been shown that biofilm-induced antibiotic susceptibility is a physiological state where mutation is not involved. The biofilm mode of growth leads to oxidative stress, that may increase mutability in biofilms. In biofilm cultures of P. aeruginosa, a 105-fold increase in mutability was detected in comparison to that of planktonic cells **(Mah, 2012).**

Quorum-sensing

Quorum sensing (QS) is cell-to-cell communication at the molecular level controlled by chemical signaling molecules called autoinducers (AIs) (**Bhardwaj et al., 2013)**

Due to QS, bacteria can recognize the population density by measuring the accumulation of signaling molecules that are secreted from members of the community. The accumulation of the signal in the extracellular environment is adequate to activate the response only when the population density is high (**Solano et al 2014**)

Recent studies indicate that in many bacterial species, activation of QS happens in the formed biofilm activating the maturation and disassembly of the biofilm. The initial adhesion step seems not suitable for the accumulation of signal molecules. Then, with the next steps, the attached bacteria are divided and form microcolonies, population density rises, and so signal molecules can reach adequate levels to activate the maturation and disassembly of the biofilm in a coordinate

manner. The time nutrients and other resources become limited and waste products accumulate, biofilm dispersion is imperative to provide bacteria to escape and colonize new niches (**Solano et al 2014).**

Carbamenases

Carbapenems are potent members of the lactam family of antimicrobials that are structurally related to the penicillins. They are beta-lactam antimicrobial agents with an exceptionally broad spectrum of activity. Carbapenems are a class of highly effective antibiotic agents commonly used for the treatment of severe or high-risk bacterial infections. In terms of structure, the carbapenems are very similar to the penicillins (penams), but the sulfur atom in position 1 of the structure has been replaced with a carbon atom, and an unsaturation has been introduced—hence the name of the group, the carbapenems **(Aurilio et al., 2022).**

The carbapenem antibiotics imipenem, meropenem, ertapenem, doripenem,panipenem-betamipron, and biapenem have a broad antimicrobial spectrum, with activity against almost all aerobic and anaerobic pathogens(**Papp-Wallace et al., 2011).**

Meropenem is slightly more active than imipenem against gram-negative bacilli and slightly less active against gram-positive cocci. Doripenem combines the broad-spectrum coverage of imipenem and meropenem, with more potent activity against P. aeruginosa. The activity of ertapenem is similar to that of the other carbapenems, but ertapenem is not active against enterococci and P. aeruginosa(**Gales et al.,2011).**

Unfortunately, the increased use of carbapenems has led to the emergence of carbapenem resistance (CR) in Gram-negative bacteria (GNB), such as Enterobacterales, Pseudomonas spp., and Acinetobacter spp., as well as the emergence of pathogens carrying up to three different carbapenemase genes. These CR pathogens are capable of spreading in the hospital setting and, in the community (**Bush and Bradford,2020**).

Action of carbapenem

The function of carbapenems is to inhibit cell wall synthesis by binding to penicillin-binding protein (PBPs), thus, causing bacterial cell wall defect, bacterial swelling and killing bacteria. Mammal's cells without cell wall are not affected by these antibiotics, therefore, these antibiotics with a selective bactericidal effect on bacteria and have little toxicity to the host. It has been proved that the special protein PBPs is the target of this antibiotic on the bacterial cell. The binding of imipenem to PBP, especially with the strong affinity, can hinder the synthesis of the cell wall, cause rapid swelling and dissolve bacteria, and the effect is rarely affected by the amount of inoculating bacteria . Meropenem can quickly penetrate into Enterobacteriaceae and Pseudomonas aeruginosa, and mainly targets PBP2 and PBP3. and on Escherichia coli and Pseudomonas aeruginosa were PBP2(**Sarkar et al., 2017).**

Resistance of carbapenem:

Bacterial resistance against carbapenems is increasing at a significant rate and has become a common problem in the primary care medicine. There are three major mechanisms of carbapenem resistance: overexpression of efflux pumps, porin-mediated resistance, and enzyme-mediated resistance The latter is due to the production of carbapenemases, which are β-lactamase enzymes capable of hydrolyzing β-lactam antimicrobials and carbapenems(**Elshamy et al., 2020**).

Carbapenemase production (blaKPCs, blaOXA, and MβLs), nonexpression or mutation of porin genes (OmpK35 and OmpK36), and upregulation of efflux systems (AcrB) are all molecular strategies used by bacterial strains to develop carbapenem resistance. The latter two mechanisms are frequently combined with high levels of other types of lactamases (e.g. AmpC) (**Pitout et al., 2015**). In some bacterial species, such as K. pneumoniae, a combination of these mechanisms can result in high levels of carbapenem resistance (**Mena et al., 2006).**

Carbapenemase genes are transported predominantly by large conjugative plasmids, allowing for horizontal transfer of carbapenem resistance between different bacterial strains and species. The recent and rapid growth in global antimicrobial resistance has been attributed to the transfer of plasmid-mediated antibiotic resistance genes (**Miao et al., 2018).**

Carbapenemases are categorized in Ambler classification system as follows, the class A serine carbapenemases (GES, KPCs) which are inhibited by clavulanic acid; the class B or metallo-β-lactamases (VIM, IMP, NDMs) which are inhibited by ethylene diamine tetra-acetic acid (EDTA); and the class D oxacillinases(OX48) which are not affected by clavulanic acid or EDTA.(**Baran and Aksu, 2016).**

Molecular classification:

According to molecular classification; Carbapenemases, β-lactamases with catalytic efficiencies for carbapenem hydrolysis, resulting in elevated carbapenem MICs, include enzymes from classes A, B, and D (**Patel et al.,2013)**

a. MOLECULAR CLASS A CARBAPENEMASES Class A carbapenemases may be chromosomally-encoded (SME,SFC-1, BIC-1, PenA, FPH-1, SHV-38), plasmid-encoded (KPC, GES, FRI-1) or both (IMI). Chromosomally-encoded class A carbapenemases have been identified in rare gram-negative species that appeared sporadically in clinical or environmental samples since their first discovery, more than 20 years ago (**Naas et al., 2016**).

Three major families of class A serine carbapenemases include the imipenem-hydrolyzing β-lactamase (IMI)/ not metalloenzyme carbapenemase (NMC), Serratia marcescens enzyme (SME), and Klebsiella producing carbapenemase (KPC) enzymes. Their hydrolytic mechanism requires an

active-site serine at position 70 in the Ambler numbering system for class A β-lactamases (**Naas et al., 2016**).

All have the ability to hydrolyze a broad variety of β- lactams, including carbapenems, cephalosporins, penicillins, and aztreonam, and all are inhibited by clavulanate and tazobactam. A fourth member of this class, Guiana extended spectrum (GES) β-lactamases, was originally identified as an ESBL family, but over time variants were discovered that had low, but measurable, imipenem hydrolysis **(Bontron et al ., 2015**).

KPC:

The class A KPC-type β-lactamases have been extensively and almost exclusively reported in K. pneumoniae. KPC-1 was reported in the late 1990s in a K. pneumoniae isolate in North Carolina. To date, more than 20 different KPC variants have been described, even though KPC-2 and -3 remain the most commonly identified variants (**Munoz et al., 2013**).

These enzymes provide resistance to the penicillins, carbapenems, cephalosporins, cephamycins, and monobactams and are inhibited by β-lactamase inhibitors such as clavulanic acid (weakly), tazobactam (weakly), boronic acid, and avibactam. KPC β-lactamases (especially KPC-2 and -3) have been described in several enterobacterial species, especially Klebsiella spp. and to a lesser extent in Enterobacter spp(**Naas et al., 2016**)..

b. CLASS B METALLO-B-LACTAMASES

The Verona integron–encoded metallo-β-lactamase [VIM], Active on imipenem [IMP], and New Delhi metallo-β-lactamase-1 [NDM-1] are the most common MβLs. In Japan, the first acquired MβL, IMP-1, was discovered in Serratia marcescens in 1991. MβLs have been described all over the world since then (**Nordmann et al., 2011**).

Except for aztreonam, MβLs can hydrolyze all β-lactams and are not inhibited by clavulanic acid, tazobactam, or boronic acid. Because they have zinc in their active core, metal chelators like ethylenediaminetetraacetic acid (EDTA) are used to inhibit them in-vitro (**Meletis, 2016**).

Carbapenems can be hydrolyzed via the interaction of βlactams with zinc ions in the active site of the enzyme (**Pitout et al., 2015**).

NDM

NDM-1 positive Enterobacterales are a family of bacteria that has gotten a lot of attention recently (**Yong Dongeun et al., 2009**). NDM-1 producers have a direct connection to the Indian subcontinent (**Nordmann et al., 2011**).

Recently, production of New Delhi metallo β lactamases (NDM) have been reported in Enterobacteriaceae, Acinetobacter spp. and Pseudomonas aeruginosa strains. NDM-producing

bacteria can spread from human to human and from water sources, foods of animal origin, and also from the polluted environment. (**Karabay et al., 2016).**

Many NDM-1 producers remain susceptible only to tigecycline, colistin and to a lesser extent fosfomycin (**Kumarasamy et al., 2010**).

Plasmids carrying the blaNDM-1 gene are diverse and can harbor a large number of resistance genes associated with other carbapenemase genes (OXA-48 types, VIM types), plasmid-mediated cephalosporinase genes, ESβLs genes, aminoglycoside resistance genes (16S RNA methylases), macrolide resistance genes (esterase), rifampin (rifampin modifying enzymes) and sulfamethoxazole resistance genes as a source of multidrug resistance and pandrug resistance (**Nordmann et al., 2011**).

IMP

Currently, up to 18 varieties of IMP-type carbapenemases have been identified. This type was first recognized in the 1990s in Japan. The majority of these enzymes were investigated in Acinetobacter and Pseudomonas species as well as those in the Enterobacteriaceae family. (**Codjoe and Donkor, 2017).** The current epidemiology of metallo-β-lactamase production generally follows patterns of increasing occurrences that are country specific. Presumably this is due to multiple factors, including antibiotic usage, dosing regimens, and local hospital practices concerning isolation of patients with multiresistant pathogens (**Quale et al., 2006).**

C. CLASS D SERINE-CARBAPENEMASES: THE OXA BLACTAMASES

OXA (for "oxacillin-hydrolyzing") β-lactamases represented one of the most prevalent plasmid-encoded β-lactamase families in the late 1970 and early 1980 When the molecular class D OXA β-lactamases were placed in a separate molecular class from the other serine β- lactamases, they had been identified mainly in the Enterobacteriaceae and P. aeruginosa and were functionally described as penicillinases capable of hydrolyzing oxacillin and cloxacillin (**Queenan and Bush, 2007**).

The mechanism of class D carbapenemases is the formation of an acyl intermediate when the β-Lactam ring is broken (**Bush and Jacoby, 2010**).

These β-lactamases are known as OXA enzymes because they can hydrolyze cloxacillin or oxacillin at a higher rate than penicillin. Carbapenems and broad-spectrum cephalosporins, such as ceftazidime and aztreonam are weakly hydrolyzed by these enzymes. Their activity is not inhibited by EDTA or clavulanic acid (**Poirel Laurent et al., 2004**).

The Class D β-lactamase family is very diverse, with over 400 variants currently recognized. This diversity is a source of confusion within the literature for the description of class D β-lactamases, especially at the level of amino acid identity (**Antunes and Fisher, 2014).** The most prevalent

variation discovered in carbapenem resistant isolates is OXA-48. However, OXA-181, OXA-232, OXA-244, OXA-245 and OXA-484 are also reported (**Findlay et al., 2017**).

Although OXA-48 producers have been found in a variety of enterobacterial species, they are most commonly found in K. pneumoniae and E. coli. When ESβL and permeability defects are present together with OXA-48 production, the amount of carbapenem resistance is usually higher (**Kalpoe et al., 2011**).

Detection of carbpenemases

The presence of a carbapenemase can be detected by a number of methods in clinical laboratories. These include automated systems or disc diffusion, MICs, selective agar, modified Hodge test, synergy tests (e.g., Etests or double disc tests), spectrometrics, whole genome sequencing and molecular methods. Currently, detection of the enzymes is difficult because of the different mechanisms involved and unreliable techniques practised in some clinical laboratories. On the other hand, extended-spectrum -lactamase inhibitors, such as clavulanic acid, tazobactam and sulbactam when used in combination with a carbapenem agent are also unreliable for phenotypically detecting carbapenemase production in bacterial isolates (**Codjoe and Donkor, 2017**).

Rapid detection of the mechanisms of resistance is crucial for appropriate antimicrobial therapy and infection control measures (**Birgy et al., 2012**).

Non molecular methods for carbapenemases detection.

1-Antimicrobial susceptibility tests

Antimicrobial susceptibility is tested according to clinical and laboratory standards institute (CLSI) recommendations. The diffusion method on Mueller-Hinton agar (MHA) was used to test susceptibility to amoxicillin (AMX), ticarcillin (TIC), cefepime (FEP), ticarcillin-clavulanic acid (TCC), cephalotin (CF), ceftazidime (CAZ), amoxicillin-clavulanic acid (AMC), cefotaxime (CTX), ertapenem (ETP), doripenem (DORI), aztreonam (ATM), cefoxitin (FOX), meropenem (MEM), moxalactam (MOX), and imipenem (IPM). Carbapenem MICs (IPM, MEM, ETP, and DORI) were determined with the epislometer (E) test and interpreted according to CLSI guidelines (**Clinical and Laboratory Standards Institute, 2021**).

The first sign of carbapenemase production in a clinical isolate is an increase in carbapenem minimum inhibitory concentration (MIC) or a decrease in inhibition zone diameter. This result qualifies the bacterial isolate for additional analysis of carbapenemase production using more specific methods (**Miriagou et al., 2010**).

According to the 2021 U.S guidelines of the Clinical Laboratory and Standards Institute (**CLSI**), these breakpoints for Enterobacterales are more than or equal to 4 mg/L for imipenem, doripenem and meropenem and more than or equal to 2 mg/L for ertapenem (**CLSI, 2021**).

If the isolate harbors either class A or class B carbapenemase, it is expected to be resistant to broader-spectrum cephalosporins like ceftazidime, ceftriaxone and cefotaxime. However these antibiotics are not affected by the production of class D carbapenemases (**MerieQueenan and Bush, 2007**).

Hence, the broth microdilution and disk diffusion approaches are thought to be more reliable for detection of all types of carbapenemmediated resistance (**Miriagou et al., 2010**).

2-UV spectrophotometer

Detection of carbapenemase activity can be done using a UV spectrophotometer, which is available in many microbiology laboratories. It is based on several steps, including:

(i) An 18 h culture (which can be shortened in some cases to 8 h).

(ii) A protein extraction step.

(iii) Measurement of imipenem hydrolysis using a UV spectrophotometer.

It has been shown that this spectrophotometry-based technique has 100% sensitivity and 98.5% specificity for detecting any kind of carbapenemase activity (**Bernabeu et al., 2012**).

This cheap technique can accurately differentiate carbapenemase producers from non-carbapenemase producers among carbapenem nonsusceptible isolates [outer membrane permeability defect, overproduction of cephalosporinases or/and extended-spectrum β-lactamases (ESBLs)]. It can be implemented in any reference laboratory, but this technique still requires time (**Nordmann and Poirel, 2012**).

3. Phenotypic methods:

1. Modified Hodge test (MHT) :

The modified Hodge test is used to reveal carbapenemase production, it is performed as recommended by CLSI. MHT is an original phenotypic approach used to detect carbapenemase production among carbapenem resistant organisms (CRO). It entails plating of a carbapenem susceptible E. coli strain with a carbapenem disc, commonly meropenem, in the center and plating linear streaks of the carbapenem-resistant test isolate away from the carbapenem disc (**Birgy et al., 2012**).

The test is considered positive if there is enhanced growth of the carbapenem-susceptible indicator strain toward the carbapenem disc along the linear streak of the test isolate forming a cloverleaf-like indentation (**Gniadek et al., 2016**).

The MHT is easy to perform, but the CLSI 2021 no longer validates it in favor of other methods with higher performance characteristics, such as the mCIM and the Carba NP (**CLSI, 2021**).

2. Inhibitor-based tests:

In the presence of specific carbapenemase inhibitors, the activity of certain carbapenemases decreases and carbapenemase producers become more sensitive to ß-lactams (**Hammoudi et al., 2014**).

2.1. Combined disc test:

The inhibitor is added to a commercially available disc of carbapenem; the combined disc and a disc of the same carbapenem are placed on Muller Hinton agar streaked with the test strain (**Pasteran et al., 2009**).

2.2. Double Disc Synergy Test (DDST) Discs of carbapenems are placed at variable distances from sterile discs impregnated with the inhibitor; the observation of synergy between the carbapenem and the inhibitor is considered a positive result (**Pasteran et al., 2009**).

2.3. Etest strips Strips using imipenem and imipenem combined with EDTA are available to test for metallo-β-lactamase activity (**Queenan and Bush, 2007**).

3. Imipenem-EDTA synergy test:

EDTA (ethylene-diamine-tetraacetic acid) is a polyamino carboxylic acid that binds metal ions like zinc and can inactivate the metallo-βetalactamases. Therefore, it is used for the phenotypic detection of MβL production in clinical isolates (**Miriagou et al., 2010).**

4. Boronic acid test:

Phenylboronic acid acts as an inhibitor for KPC carbapenemases and class A and C beta-lactamases. The boronic acid test is used for phenotypic detection of KPC producers because it is easier to perform than the DDST and presents less false positive results (**Tsakris et al., 2009**) and (**Pournaras et al., 2010**).

5. Chromogenic agar :

It has emerged for identification of MDR organisms from surveillance cultures such as CHROMagar KPC and CHROMagar™ Acinetobacter (CHROMagar, Paris, France). Culture media are made selective by adding chromogenic substrates and agents that inhibit growth of other Gram positive, Gram-negative and yeast isolates. A new formulation, with the addition of

„Klebsiella pneumoniae carbapenemase supplement" was able to pick out carbapenem resistant Acinetobacter baumannii. Recent modifications to CHROMagar Acinetobacter have improved selective growth for organisms that are resistant to carbapenems(**Arnold et al., 2011**).

CHROMagar KPC has a sensitivity of 100% and specificity of 98.4% compared with polymerase chain reaction (PCR). This technique is used for confirmatory identification of KPC production though is expensive in terms of cost and is mostly performed in research laboratories (**Arnold et al., 2011**).

The recently developed SUPERCARBA medium consists of Drigalski agar supplemented with ertapenem, zinc sulfate to enhance expression of MβL producers and cloxacillin to inhibit AmpC natural producers like Enterobacter cloacae, Enterobacter aerogenes, Morganella morganii and Serratia marcescens, which are clinically important sources of carbapenem resistance associated with an outer membrane permeability defect and their inhibition increases specificity of the medium in detecting carbapenemase producers. This medium is also sensitive for detecting carbapenemase producers with low-level resistance, such as KPC, VIM, IMP and OXA-48 (**Nordmann et al., 2012**)

Commercially available carbapenem-containing media are imperfect to differentiate between carbapenem resistance caused by enzyme inactivation and resistance caused by other mechanisms. SUPERCARBA medium has an advantage over other media as theoretically it inhibits carbapenem-resistant but non-carbapenemase producing organisms (**Nordmann et al., 2012).**

6. Carbapenem inactivation method (CIM)

A newer phenotypic test. A suspension of the bacterial isolate of interest and water is made, and a meropenem disc is incubated with this suspension. The meropenem disc is then removed and placed on a Mueller-Hinton agar plate that is streaked with a susceptible laboratory strain of E. coli. The absence of an inhibition zone indicates hydrolysis of meropenem in the first step and the presence of a carbapenemase. The presence of a clearing zone indicates lack of meropenem hydrolysis (no carbapenemase present). The initial data from this test show a sensitivity of 98 to100% (**Lutgring and Limbago 2016).**

The mCIM is a modified version of the original Carbapenem Inactivation Method (CIM) which was first reported in 2015 by van der Zwaluw and colleagues (**van der Zwaluw et al., 2015**). When compared to the CIM, the mCIM uses 1-µl loopful of the CRO instead of a 10-µl loopful for setup and tryptic soy broth (TSB) instead of water for better detection of MβL producers that require divalent cations for activity and the incubation time was increased to 4 hours from 2 hours for enzymes with reduced hydrolytic activity during the meropenem disc inactivation step (**Pierce et al., 2017).** After 4 hours of TSB inoculation with a carbapenem-resistant test isolate in the presence of a meropenem disc, the meropenem disc is transferred onto Mueller-Hinton agar (MHA) streaked with a carbapenem-susceptible E. coli indicator strain and incubated overnight

(**van der Zwaluw et al., 2015**). The test is positive on the basis that carbapenemase producers will rapidly hydrolyze the meropenem in the disc during the 4-hour incubation period so that when the disc is removed and plated with the carbapenem susceptible indicator strain, the disc has no activity and a zone diameter of 6 to 15 mm will be yielded. The tested organism is considered carbapenemase intermediate when the zone diameter is 16-18 mm or of ≥19 mm with pinpoint colonies within the zone. The non-carbapenemaseproducing CRO will not easily hydrolyze the meropenem disc and will stay active against the indicator strain, providing zone diameters of ≥ 19 mm after overnight incubation, indicating that the test is negative (**CLSI, 2021**).

The mCIM has excellent sensitivity and specificity of ≥99% and is a very cost-effective method that just requires standard microbiology laboratory supplies. The TAT of 18 to 24 hours is the most significant drawback of the mCIM test (**Workneh et al., 2019**).

The mCIM has been improved by adding EDTA, a cation chelator and MβL inhibitor, to further differentiate MβL from serine carbapenemases. The EDTA mCIM (eCIM) is performed concurrently with the mCIM, and a ≥5 mm zone diameter difference between the eCIM and the mCIM for the E. coli indicator strain is indicative of MβL production, while a ≤4-mm difference indicates production of a serine carbapenemase that is not inhibited by the addition of EDTA (**CLSI, 2021**).

Additional CIM modifications have been recently published, including a CIMplus test that detects carbapenemase in 8 hours (**Caméléna et al., 2018**), and the rapid carbapenem inactivation method (rCIM) which reduces the time it takes to detect carbapenemase to less than three hours (**Muntean et al., 2018).**

7. Combination meropenem disc test This test is a combination of EDTA and boronic acid test in a single plate and has been introduced in Greece after the emergence of Gram negative isolates co-producing KPC and MβL carbapenemases, so it discriminates between carbapenem-susceptible, KPC-producing, MβL producing and double-carbapenemase producing bacteria (**Zioga et al., 2010**).

8. D-test:

D-test is used for the detection of inducible AmpC and beta lactamases, antibiotic is used as an inducer for AmpC production (imipenem or cefoxitin) whereas others are used as substrates (ceftazidime, cefotaxime, piperacillin/ tazobactam) (**Dunne and Hardin, 2005**).

9. Carba NP test:

Carba Nordmann-Poirel test is a recent biochemical detection method of carbapenemases in Enterobacteriaceae, and Pseudomonas. The principle is based upon hydrolysis of the β-lactamring of imipenemby the tested strain, followed by color change of a pH indicator, usually phenol red from red to yellow/orange (**Nordmann et al., 2012).**

10-Lateral Flow Immuno-Assays (LFIA):

LFIA use nanoparticles bound to a nitrocellulose membrane with antibodies to detect epitopes specific to carbapenemase enzymes within the lateral-flow device. It had sensitivity of 100% for class A, B and D carbapenemases and specificities of 95 to 100% (**Glupczynski et al., 2016**).

The most interesting development in this sector is a multiplex lateral-flow immunoassay, the Carba5, for the rapid identification of NDM, KPC, IMP,VIM and OXA-48-like carbapenemases in Enterobacterales (**Gauthier et al., 2017**).

Because it is reliable, sensitive, and simple to use, it is suggested that this newly developed test can replace molecular detection of carbapenemases. However, it is specific for carbapenemase type rather than subtype. For example, it can differentiate IMP from VIM or NDM but cannot differentiate IMP-1 from IMP-2 or IMP-7, nor can identify novel mutations of target genes (**Kitao et al., 2011**).

Molecular tests for carbapenemase genes:

Molecular techniques remain the gold standard for the precise identification of carbapenemase genes (**Nordmann and Poirel, 2012**). Most of these techniques are based on PCR and may be followed by a sequencing step if a precise identification of the carbapenemase gene is needed (e.g. VIM type, KPC type, NDM type or OXA-48 type) (**Poirel et al., 2011**).

A PCR technique performed directly on colonies can give results within 4–6 h (or less when using real-time PCR technology) with excellent sensitivity and specificity (**Cuzon et al., 2012).** The main disadvantages of the molecular based technologies are their cost, the requirement for trained microbiologists and the inability to detect novel unidentified genes. Sequencing of the genes is interesting mostly for research and epidemiological purposes. Precise identification of the type of carbapenemase is not actually needed for treating patients or for preventing outbreaks. We believe these molecular techniques may be mostly used in reference laboratories (**Nordmann and Poirel, 2012).** Molecular methods such as simplex and multiplex PCRs, real-time PCR, DNA hybridization and sequencing have been commonly used for the identification of carbapenemase genes in research laboratories and reference centres. Nowadays, some of these methods, mostly PCR, are routinely performed in some clinical laboratories in order to circumvent the problems of the phenotypic detection of CP organisms (**Miriagou et al., 2010**).

1. Multiplex PCR

This system can identify metallo-β-lactamase genes such as blaVIM, blaIMP and blaNDM-1, oxacillinase genes such as blaOXA-48-like encoding genes and all variants of blaKPC genes. DNA was extracted from bacteria grew in 24-h culture. After PCR reactions, amplification products were heat-denaturated to obtain single stranded DNA and loaded to microtiter plates coated with

specific probes. Hybridization reaction principle was used to investigate carbapenemase gene loci in loaded DNA (**Baran and Aksu 2016**).

A loop-mediated isothermal amplification (LAMP) method can be used for the rapid and sensitive detection of carbapenemase genes (**Qi et al., 2012**).

LAMP consists of a strand-displacement Bst DNA polymerase with 4 to 6 primers that recognize 6 to 8 distinct regions of the target gene and generate the loop-mediated amplification under isothermal conditions ranging from 60°C to 65°C for about 60 minutes, resulting in large amounts of amplification products with many types of structures. LAMP is more specific and quicker to perform than PCR. Furthermore, because the LAMP method provides large amounts of DNA, gel electrophoresis is not necessary because the results may be easily recognized by turbidity or fluorescence (**Notomi et al., 2000**).

2. Microarray technology

It utilizes a number of DNA probes that hybridize to DNA targets, including resistance genes. Microarrays can be paired with PCR amplification of target sequences or can be used to directly query DNA in bacterial isolates or patient specimens. The benefit of an array over PCR assays is in the number of targets available for interrogation; while PCR can typically accommodate a maximum of four to five targets per assay, microarrays can include dozens to hundreds of targets, depending on the platform. Several microarray platforms for carbapenemases have been developed, including Verigene, BioFire, and Check-Points. Sensitivities have been reported at 100% (**Tuite et al., 2014**).

3. Whole genome sequencing (WGS)

This technology can provide resistance information for numerous antimicrobial classes, rather than targeting only carbapenemases, and can identify other contributors to resistance, such as porin mutations. WGS also provides information on the type of plasmid carrying resistance genes, the evolutionary lineage of the bacterium, and the relatedness of isolates, all of which can help to elucidate the source of the isolate or inform outbreak investigations. Furthermore, data generated with WGS can be stored for future inquiry as new resistance determinants or virulence factors of interest are identified. Currently, WGS is a rather expensive technology and its use is a fairly specialized process, but as the price drops and analysis pipelines become more automated, WGS is likely to become more widely available (**Lutgring and Limbago 2016**).

4. Real-time PCR:

Cunningham et al. reported the use of a real time PCR assay that applies fluorescence resonance energy transfer (FRET) hybridization probe based detection of KPC and NDM-encoding genes simultaneously, the assay was 100% sensitive and specific and it had a turnaround time of 90 min from colony to results (**Cunningham et al., 2013**).

5. Enterobacterial Repetitive Intergenic Consensus (ERIC) PCR

(ERIC)PCR can be used to determine clonal relatedness of strains in low resource settings. The ERIC sequences are present in many copies in the genomes of E. coli, Salmonella typhimurium and other Enterobacterales **(Zulkifli et al., 2009**).

These ERIC sequences are 126 bp long, contain a central core inverted repeat and are highly conserved at the nucleotide level. The position of ERIC elements in enterobacterial genomes varies between different species and has been used as a genetic marker to characterize isolates within a bacterial species **(Radu et al., 2002)**.

For subtyping Gram-negative enteric bacteria, ERIC-PCR produces a band pattern by amplification of genomic DNA situated between ERIC elements or between ERIC elements and other repetitive DNA sequences (**Ferreira et al., 2011).**

6. Pulsed field gel electrophoresis (PFGE)

It is a more accurate tool to establish clonality. The principles of this technique depend upon cutting bacterial genomes by specific restriction enzymes and separating the resulting large DNA fragments using an electric field switched periodically between different directions. The resulting patterns can then be interpreted using well-accepted criteria to establish the degree of relatedness (**Hammoudi et al., 2014).**

7. Multilocus sequence typing (MLST) method

Despite using PFGE, it appears nowadays to be more appropriate. The MLST procedure characterizes isolates of a given microbial species using DNA sequences of multiple housekeeping genes. Approximately 450–500 bp internal fragments of each of these genes are used, as these can be accurately sequenced using an automated DNA sequencer obtained by importing sequences into MLST database. In areas with long-term persistence of carbapenemase-producing isolates, MLST offers the opportunity of tracking clones and the exchange of allelic information among different geographic areas. In areas with low prevalence, MLST helps to identify emergence of highly epidemic clones associated with specific carbapenemases (**Hammoudi et al., 2014**).

An example of such clones is carbapenem-resistant K. pneumoniae strain that is classified by MLST as ST258. Carbapenem resistance in ST258 is presented by blaKPC within a transposon 4401 (Tn4401) that is present on a plasmid (**Kobayashi et al., 2018**).

References:

1. Bader MS, Loeb M, Leto D, Brooks AA. Treatment of urinary tract infections in the era of antimicrobial resistance and new antimicrobial agents. Postgrad Med. 2020 Apr;132(3):234-250. doi: 10.1080/00325481.2019.1680052.

2. Aurilio, C.; Sansone, P.; Barbarisi, M.; Pota, V.; Giaccari, L.G.; Coppolino, F.; Barbarisi, A.; Passavanti, M.B.; Pace, M.C. Mechanisms of Action of Carbapenem Resistance. Antibiotics 2022, 11, 421. https://doi.org/10.3390/antibiotics11030421
3. Bartal, C., Rolston, K.V.I. & Nesher, L. Carbapenem-resistant Acinetobacter baumannii: Colonization, Infection and Current Treatment Options. Infect Dis Ther 11, 683–694 (2022). https://doi.org/10.1007/s40121-022-00597
4. Bernardi, S., Continenza, M.A., Al-Ahmad, A., Karygianni, L.,Follo, M., Filippi, A. and Macchiarelli, G. (2019)Streptococcusspp. andFusobacterium nucleatumin tonguedorsum biofilm from halitosis patients: a fluorescencein situ hybridization (FISH) and confocal laser scanningmicroscopy (CLSM) study.New Microbiol42, 108–113
5. Bhardwaj AK, Vinothkumar K, Rajpara N. Bacterial quorum sensing inhibitors: Attractive alternatives for control of infectious pathogens showing multiple drug resistance. Recent Patents on Anti-Infective Drug Discovery. 2013;8(1):68-83.
6. Blanco P, Hernando-Amado S, Reales-Calderon JA, Corona F, Lira F, Alcalde-Rico M, et al. Bacterial multidrug efflux pumps: Much more than antibiotic resistance determinants. Microorganisms. 2016;(1):1-19. DOI: 10.3390/microorganisms4010014
7. Bontron S., Poirel L., Nordmann P. In vitro prediction of the evolution of GES-1 β-lactamase hydrolytic activity. Antimicrob. Agents Chemother. 2015;59:1664–1670
8. -Bose S, Khodke M, Basak S & Mallick SK (2009): Detection of biofilm producing staphylococci: need of the hour. Journal of clinical and diagnostic research; 3(6): 1915-1920.
9. Bush, K.; Bradford, P.A. Epidemiology of β-Lactamase-Producing Pathogens. Clin. Microbiol. Rev. 2020, 33
10. Caputo, P., Di Martino, M.C., Perfetto, B., Iovino, F. andDonnarumma, G. (2018) Use of MALDI-TOF MS todiscriminate between biofilm-producer and non-producerstrains ofStaphylococcus epidermidis.Int J Environ ResPublic Health15, 1695. https://doi.org/10.3390/ijerph15081695.
11. Ciofu O, Tolker-Nielsen T, Jensen PØ, Wang H & Høiby N (2015): Antimicrobial resistance, respiratory tract infections and role of biofilms in lung infections in cystic fibrosis patients. Advanced drug delivery reviews; 85: 7-23.
12. Codjoe F. S. and Donkor E. S. (2017): Carbapenem resistance: areview. Medical Sciences, 6(1), 1.
13. Colvin KM, Gordon VD, Murakami K, Borlee BR, Wozniak DJ, Wong GC, et al. The pel polysaccharide can serve a structural and protective role in the biofilm matrix of Pseudomonas aeruginosa. PLoS Pathogens. 2011;7(1):e1001264. DOI: 10.1371/journal.ppat.1001264
14. Cruz-Lo´ pez F, Mart´ınez-Mele´ ndez A, Morfin-Otero R, Rodriguez-Noriega E, Maldonado-Garza HJ and Garza-Gonza´lez E (2022) Efficacy and In Vitro Activity of Novel Antibiotics for Infections With Carbapenem-Resistant Gram-Negative Pathogens. Front. Cell. Infect. Microbiol. 12:884365. doi: 10.3389/fcimb.2022.884365

15. Donald E Greydanus MD, DrHC (ATHENS), Maria Demma Cabral MD and Dilip R. Patel MD, MBA, MPHDisease-a-Month, 2022-03-01, Volume 68, Issue 3, Article 101287, Copyright © 2021
16. Elshamy, A.A.; Aboshanab, K.M. A review on bacterial resistance to carbapenems: Epidemiology, detection and treatment options. Futur. Sci. OA 2020, 6, FSO438.
17. Flores-Mireles AL, Walker JN, Caparon M, Hultgren SJ. Urinary tract infections: epidemiology, mechanisms of infection and treatment options. Nat Rev Microbiol. 2015;13(5):269-284. doi:10.1038/nrmicro3432
18. Foxman B. Urinary tract infection syndromes: occurrence, recurrence, bacteriology, risk factors, and disease burden. Infect Dis Clin North Am. 2014;28:1–13. This paper presents the most recent information about UTIs and their socioeconomic impact
19. Gales, Ana Cristina et al. Antimicrobial activity of doripenem against Gram-negative pathogens: results from INVITA-A-DORI Brazilian study. Brazilian Journal of Infectious Diseases [online]. 2011, v. 15, n. 6 [Accessed 19 September 2022] , pp. 513-520. Available from: <https://doi.org/10.1590/S1413-86702011000600003>. Epub 04 Jan 2012. ISSN 1678-4391. https://doi.org/10.1590/S1413-86702011000600003.
20. Galli, J., Calo, L., Meucci, D., Giuliani, M., Lucidi, D., Paludetti,G., Torelli, M.S., Sanguinetti, M.et al. (2018) Biofilm invoice prosthesis: a prospective cohort study and laboratorytests using sonication and SEM analysis.Clin Otolaryngol43, 1260–1265. https://doi.org/10.1111/coa.13141.
21. Gilbert J. Wise & Peter N. Schlegel (March 12, 2015). "Sterile Pyuria". N Engl J Med. 372 (11): 1048–1054. doi:10.1056/NEJMra1410052. PMID 25760357.
22. Goering, Richard, Hazel Dockrell, Mark Zuckerman, and Peter L. Chiodini. Mims' Medical Microbiology E-Book. Elsevier Health Sciences, 2018.
23. Grela, E., Zazbek, A. and Grabowiecka, A. (2015) Interferences inthe optimization of the MTT assay for viability estimationofProteus mirabilis.Avicenna J Med Biotechnol7, 159.Halim, R.M.A., Kassem, N.N. and Mahmoud, B.S. (2018)Detection of biofilm producing staphylococci amongdifferent clinical isolates and its relation to methicillinsusceptibility.Open Access Maced J Med Sci6, 1335.https://doi.org/10.3889/oamjms.2018.246.
24. Hassan, P.A. and Khider, A.K. (2019) Correlation of biofilmformation and antibiotic resistance among clinical and soilisolates ofAcinetobacter baumanniiin Iraq.Acta Microbiolet Immunol Hungarica1–10. https://doi.org/10.1556/030.66.2019.026
25. Hathroubi S, Mekni MA, Domenico P, Nguyen D, Jacques M. Biofilms: Microbial shelters against antibiotics. Microbial Drug Resistance. 2017;23(2):147-156. DOI: 10.1089/mdr.2016.0087
26. Hickling DR, Sun TT, Wu XR. Anatomy and Physiology of the Urinary Tract: Relation to Host Defense and Microbial Infection. Microbiol Spectr.

2015;3(4):10.1128/microbiolspec.UTI-0016-2012. doi:10.1128/microbiolspec.UTI-0016-2012

27. Høiby, N., Bjarnsholt, T., Moser, C., Bassi, G.L., Coenye, T.,Donelli, G., Hall-Stoodley, L., Imbert, C.et al. (2015)ESCMID guideline for the diagnosis and treatment ofbiofilm infections 2014.Clin Microbiol Infect21,S1–S25.https://doi.org/10.1016/j.cmi.2014.10.024.
28. John A. S., Mboto C. I. and Agbo B. (2016): A review on the prevalence and predisposing factors responsible for urinary tract infection among adults. Eur J Experiment Biol, 6(4), 7-11.
29. -Kamaruzzaman, N.F., Tan, L.P., Yazid, K.A.M., Saeed, S.I.,Hamdan, R.H., Choong, S.S., Wong, W.K., Chivu, A.et al. (2018) Targeting the bacterial protective armour;challenges and novel strategies in the treatment ofmicrobial biofilm.Materials11, 1705. https://doi.org/10.3390/ma11091705
30. Kim WY, Moon JY, Huh JW, Choi SH, Lim CM, Koh Y, Chong YP, Hong SB. Comparable Efficacy of Tigecycline versus Colistin Therapy for Multidrug-Resistant and Extensively Drug-Resistant Acinetobacter baumannii Pneumonia in Critically Ill Patients. PLoS One. 2016 Mar 2;11(3):e0150642. doi: 10.1371/journal.pone.0150642. PMID: 26934182; PMCID: PMC4775052.
31. Kwon JH, Fausone MK, Du H, Robicsek A, Peterson LR. Impact of laboratory-reported urine culture colony counts on the diagnosis and treatment of urinary tract infection for hospitalized patients. Am J Clin Pathol. 2012 May;137(5):778-84. doi: 10.1309/AJCP4KVGQZEG1YDM. PMID: 22523217.
32. Lala V, Minter DA. StatPearls [Internet]. StatPearls Publishing; Treasure Island (FL): Jun 25, 2021. Acute Cystitis91-Moore A., Doull M., Grad R., Groulx S., Pottie K., Tonelli M. and Thombs B. D. (2018): Recommendations on screening for asymptomatic bacteriuria in pregnancy. CMAJ, 190(27), E823-E830.
33. Li R, Leslie SW. Cystitis. [Updated 2022 Feb 14]. In: StatPearls [Internet]. Treasure Island (FL): StatPearls Publishing; 2022 Jan-. Available from: https://www.ncbi.nlm.nih.gov/books/NBK482435/
34. Long B., Koyfman A.: The emergency department diagnosis and treatment of UTI. Emerg Med Clin North Am 2018; 36: pp. 685-710
35. Mah TF (2012): Biofilm-specific antibiotic resistance. Future microbiology; 7(9): 1061-1072
36. Mishra SK, Basukala P, Basukala O, Parajuli K, Pokhrel BM & Rijal BP (20Grela, E., Zazbek, A. and Grabowiecka, A. (2015) Interferences inthe optimization of the MTT assay for viability estimationofProteus mirabilis.Avicenna J Med Biotechnol7, 159.Halim, R.M.A., Kassem, N.N. and Mahmoud, B.S. (2018)Detection of biofilm producing staphylococci amongdifferent clinical isolates and its relation to methicillinsusceptibility.Open Access Maced J Med Sci6, 1335.https://doi.org/10.3889/oamjms.2018.246.

37. mlynarikova, K.,Sedo, O., Ruzicka, F., Zdrahal, Z., Hola, V.and Mahelova, M. (2016) Evaluation of capacity to detectability to form biofilm inCandida parapsilosissensustricto strains by MALDI-TOF MS.Folia Microbiol61,465–471. https://doi.org/10.1007/s12223-016-0458-7
38. Munoz-Price LS, Poirel L, Bonomo RA, Schwaber MJ, Daikos GL, Cormican M, Cornaglia G, Garau J, Gniadkowski M, Hayden MK, Kumarasamy K, Livermore DM, Maya JJ, Nordmann P, Patel JB, Paterson DL, Pitout J, Villegas MV, Wang H, Woodford N, Quinn JP. 2013. Clinical epidemiology of the global expansion of Klebsiella pneumoniae carbapenemases. Lancet Infect Dis 13:785–796.
39. Nadell CD, Drescher K, Wingreen NS, Bassler BL. Extracellular matrix structure governs invasion resistance in bacterial biofilms. The ISME Journal. 2015;9:1700-1709. DOI: 10.1038/ismej.2014.246
40. Nickel JC. Prostatitis and related conditions, orchitis, and epididymitis. In: Wein AJ, Kavoussi LR, Novick AC, Partin AW, Peters CA, eds. Campbell-Walsh Urology. 10th ed. Philadelphia: Saunders; 2012: 327–356
41. Niveditha S, Pramodhini S, Umadevi S, Kumar S & Stephen S (2012): The isolation and the biofilm formation of uropathogens in the patients with catheter associated urinary tract infections (UTIs). Journal of Clinical and Diagnostic Research; 6(9): 1478-1482.
42. Oliveira A & Maria de Lourdes RS (2010): Comparison of methods for the detection of biofilm production in coagulase-negative staphylococci. BMC research notes; 3(1): 260.
43. Ordonez, A.A., Sellmyer, M.A., Gowrishankar, G., Ruiz-Bedoya, C.A., Tucker, E.W., Palestro, C.J., Hammoud,D.A. and Jain, S.K. (2019) Molecular imaging of bacterialinfections: overcoming the barriers to clinical translation.Sci Transl Med11, 508. https://doi.org/10.1126/scitranslmed.aax8251.
44. Oviaño M, Bou G. Matrix-Assisted Laser Desorption Ionization-Time of Flight Mass Spectrometry for the Rapid Detection of Antimicrobial Resistance Mechanisms and Beyond. Clin Microbiol Rev. 2018;32(1):e00037-18. Published 2018 Nov 28. doi:10.1128/CMR.00037-18
45. Oyaert M, Van Meensel B, Cartuyvels R, Frans J, Laffut W, Vandecandelaere P, De Beenhouwer H; BILULU Study Group. Laboratory diagnosis of urinary tract infections: Towards a BILULU consensus guideline. J Microbiol Methods. 2018 Mar;146:92-99. doi:
46. Patel, Gopi; Bonomo (March 2013). ""Stormy waters ahead": global emergence of carbapenemases". Frontiers in Microbiology. 4:
47. Percival, S.L. (2017) Importance of biofilm formation insurgical infection.Br J Surg104, e85–e94. https://doi.org/10.1002/bjs.10433.
48. Rajapaksha, P., Elbourne, A., Gangadoo, S., Brown, R.,Cozzolino, D. and Chapman, J. (2019) A review ofmethods for the detection of pathogenic microorganisms.Analyst144, 396–411

49. Review Evolution of β-lactams resistance in Gram-negative bacteria in Tunisia. Chouchani C, Marrakchi R, El Salabi ACrit Rev Microbiol. 2011 Aug; 37(3):167-77
50. Rossi F. 2011. The challenges of antimicrobial resistance in Brazil. Clin. Infect. Dis. 52:1138–1143 [PubMed] [Google Scholar]
51. Sarkar P, Yarlagadda V, Ghosh C, Haldar J. A review on cell wall synthesis inhibitors with an emphasis on glycopeptide antibiotics. Medchemcomm. 2017 Jan 26;8(3):516-533. doi: 10.1039/c6md00585c. PMID: 30108769; PMCID: PMC6072328.
52. Schlafer, S. and Meyer, R.L. (2017) Confocal microscopyimaging of the biofilm matrix.J Microbiol Methods138,50–59. https://doi.org/10.1016/j.mimet.2016.03.002.
53. Shahmoradi, M., Faridifar, P., Shapouri, R., Mousavi, S.F.,Ezzedin, M. and Mirzaei, B. (2019) Determining thebiofilm forming gene profile ofStaphylococcus aureusclinical isolates via multiplex colony PCR method.RepBiochem Mol Biol7, 181.
54. Shirley, M. (2018). Ceftazidime-Avibactam: A Review in the Treatment of Serious Gram-Negative Bacterial Infections. Drugs 78 (6), 675–692. doi: 10.1007/s40265-018-0902-x
55. Shortridge, D., Pfaller, M. A., Castanheira, M., Flamm, R. K. (2018). Antimicrobial Activity of Ceftolozane-Tazobactam Tested Against Enterobacteriaceae and Pseudomonas Aeruginosa With Various Resistance Patterns Isolated in U.S. Hospitals, (2013-2016) as Part of the Surveillance Program: Program to Assess Ceftolozane-Tazobactam Susceptibility. Microb. Drug Resist. 24 (5), 563–577. doi: 10.1089/mdr.2017.0266
56. shukla, S.K. and Rao, T.S. (2017) An improved crystal violetassay for biofilm quantification in 96-well microtitre plate.Biorxiv100214, https://doi.org/10.1101/100214
57. singhal, N., Kumar, M., Kanaujia, P.K. and Virdi, J.S. (2015)MALDI-TOF mass spectrometry: an emerging technologyfor microbial identification and diagnosis.Front Microbiol6, https://doi.org/10.3389/fmicb.2015.00791.
58. Solano C, Echeverz M, Lasa I. Biofilm dispersion and quorum sensing. Current Opinion in Microbiology. 2014;18:96-104. DOI: 10.1016/j.mib.2014.02.008
59. Soriano, A., Carmeli, Y., Omrani, A. S., Moore, L. S. P., Tawadrous, M., Irani, P. (2021). Ceftazidime-Avibactam for the Treatment of Serious Gram-Negative Infections With Limited Treatment Options: A Systematic Literature Review. Infect. Dis. Ther. 10 (4), 1989–2034. doi: 10.1007/s40121-021-00507-6
60. Stewart PS, Franklin MJ. Physiological heterogeneity in biofilms. Nature Reviews. Microbiology. 2008;6:199-210. DOI: 10.1038/nrmicro1838
61. Stewart, N. K.; et al. "Kinetic and structural requirements for carbapenemase activity in GES-type β-lactamases." Biochemistry 54.2 (2014): 588-597.
62. Stoica, P., Chifiriuc, M.C., Rapa, M. and Lazar, V. (2017).Overview of biofilm-related problems in medical devices.In Deng, Y. and Lv, W., eds.Biofilms and ImplantableMedical Devices, pp. 3–23. Oxford, UK: WoodheadPublishing

63. Taylor PK, Yeung AT & Hancock RE (2014): Antibiotic resistance in Pseudomonas aeruginosa biofilms: towards the development of novel anti-biofilm therapies. Journal of biotechnology; 191: 121-130.
64. Torres J. A., Villegas M. V., Quinn J. P. 2007. Current concepts in antibiotic-resistant gram-negative bacteria. Expert Rev. Anti Infect. Ther. 5:833–843
65. Velasco R, Benito H, Mozun R, Trujillo JE, Merino PA, de la Torre M, Gomez B, Mintegi S; Group for the Study of Febrile Infant of the RiSEUP-SPERG Network. Importance of Urine Dipstick in Evaluation of Young Febrile Infants With Positive Urine Culture: A Spanish Pediatric Emergency Research Group Study. Pediatr Emerg Care. 2016 Dec;32(12):851-855. doi: 10.1097/PEC.0000000000000935. PMID: 27749810.
66. Verma S & Patil SS (2016): In Vitro Biofilm Formation by Uropathogenic Bacteria and their Antibiotic Susceptibility Pattern. Journal of Krishna Institute of Medical Sciences University; 5(3): 61-67.
67. Yadav, M.K., Vidal, J.E. and Song, J.J. (2020) Microbialbiofilms on medical indwelling devices. InNew and FutureDevelopments in Microbial Biotechnology andBioengineering: Microbial Biofilms, pp. 15–28. https://doi.org/10.1016/B978-0-444-64279-0.00002-5
68. Zowawi, H. M. et al. The emerging threat of multidrug-resistant Gram-negative bacteria in urology. Nat. Rev. Urol. 12, 570–584 (2015).