Facing new challenges in microbial resistance

M.-M. Azevedo [1,2,*], S. Costa-de-Oliveira[2,3,*], A.P. Silva[2,3,*], C. Pina-Vaz[2,3] and A.G. Rodrigues[2,3]

[1]School D. Maria II, V.N.Famalicão, Portugal
[2]Department of Microbiology, Faculty of Medicine, University of Porto, Portugal
[3]Cardiovascular Research and Development Unit, Faculty of Medicine, University of Porto, Portugal
*Author's contributing equally to this chapter

An increasing number of microorganisms causing disease are becoming resistant to antimicrobial agents commonly used for medical treatment. The ability of some bacteria and fungi to form biofilms is involved in severe infections, with the consequent emergence of resistance to the host immune system and to antimicrobial therapy. The search for novel molecules with antimicrobial proprieties or the rehabilitation of older molecules became imperative, as well as, the constant monitorization of the susceptibility pattern to antimicrobial drugs, using faster and more accurate methodologies. In this chapter we propose to i) explain microbial resistance, the emergence and re-emergence of multidrug resistant pathogens, ii) to update recent insights concerning biofilm-related infections in indwelling medical devices and novel biofilm inhibitors including antimicrobial materials/surfaces iii) to highlight new therapeutic strategies that include the use of natural products as antimicrobials, also as biofilm inhibitors, and in alternative the use of drugs that revert the antimicrobial resistance, and iv) novel strategies to identify and evaluate antimicrobial susceptible profile in clinical useful time.

Keywords antimicrobial resistance; biofilm-related infections; novel antimicrobial compounds; strategies to evaluate susceptibility profile

1. Microbial resistance, emerging and re-emerging of multidrug resistant pathogens

The most important factor in the evolution of microbial drug resistance in microorganisms is drug selection pressure. The extensive human use and misuse of antimicrobials in the clinic, community, animal husbandry and agriculture has resulted in a strong selection pressure for the emergence, enrichment and spread of various resistance mechanisms among pathogenic microorganisms. Additionally, bacteria are able to transfer genes from one bacterium to another by lateral and vertical gene transfer, which has played an integral role in evolution and diversification of antibiotic resistance [1, 2].

Microorganisms are classically considered intrinsically resistant in the basis of the clinical definition, in other words, if infections cannot be treated with a given antimicrobial. This is a common feature in bacteria, for which the three most relevant causes of this intrinsic resistance are (i) lack of the target, (ii) activity of chromosomally encoded antibiotic-inactivating enzymes and (iii) diminished uptake of the antibiotic. The later involves reduced permeability of the cellular envelopes and for the activity of efflux pumps [3]. Regarding fungi, intrinsic molecular changes in the target is responsible for naturally resistance to several drugs [4]. On the other hand, the process of resistance acquisition by the microbial cells can apparently involve: (i) reorganization of the membrane and its permeability, which includes change in lipopolysaccharide composition [5], decrease of porin content [6] and/or over expression of efflux pumps [7], and (ii) genetic changes. Other mechanisms may also determine the process of resistance acquisition, like horizontal gene transfer between organisms, especially among bacteria, or activation of cell signalling responses which are closely related to the behavior of microorganisms in the wildness; microbial communication (quorum sensing) [8, 9] and biofilm formation [10, 11]. Three main resistance mechanisms can be detailed:

1.1. Decrease in intracellular concentration

In order to cross bacteria cell membrane, drugs must have a low molecular weight, be hydrophilic and electric neutral. All antibiotics, with exception of beta-lactam need to cross the cell membrane to act. Overall, the membrane is impermeable to policationic antibiotics, which need the presence of oxidative transporters. Thus, anaerobic bacteria are intrinsically resistant to the class of antibiotics aminoglycosides [12]. Gram-negative bacteria are resistant to glycopeptides, due to its size and hydrophobicity, preventing the cross of porins (discussed below). Gram-negative bacteria have an extra "protection" provided by the outer membrane. For this reason some antibiotics that are active against Gram-positive are not active against Gram-negative. Lipopolysacharide (LPS) composition increases the asymmetry in the membrane architecture and the cross binding between LPS and divalent cations decreases permeability to hydrophilic agents [13].

In Gram-negative bacteria, the regulation of membrane permeability is also a function of membrane proteins. Regulation involves the joint action of porins and efflux pumps. Porins, found in Gram-negative and mycobacteria, form channels that transverse the outer membrane and end in the periplasm. They serve as the main entry for different classes of antibiotics such as beta-lactams or fluoroquinolones, as well as a large variety of small hydrophilic molecules

[14]. Indeed, some beta-lactam resistant strains of *E. coli* have shown a deficiency in the expression of the outer membrane protein (Omp) with alterations in its loop structure, caused by mutations. This can interfere with the interaction of the antibiotic with the surface of the channel, which determines its penetration inside the cell [15]. Moreover, porin-deficient mutants are also more resistant to quinolones, tetracyclines, chloramphenicol, nalidixic acid and trimethoprim [16]. *P. aeruginosa* has innate low susceptibility to beta-lactams due to its low porin content with distinct physicochemical properties as compared to other species [6, 17]. Fungi can develop resistance to antifungal drugs by similar mechanisms. Mutations that cause defects in the uptake of flucytosine or in its intracellular conversion are a frequent cause of resistance to this drug [18]. Failure to accumulate the drug intracellulary, may be caused by the changes in membrane lipids and sterols and result in lack of azole drug penetration [19].

Cellular efflux systems are responsible for the extrusion of both endogenous (e.g. toxic metabolites) and exogenous (e.g. bile salts) toxic compounds, playing an important role in the physiology and homeostasis of the cell. Efflux pumps are also useful tools for the cell to remove antimicrobials thus conferring resistance to a given drug or class of drugs. Although a variety of mechanisms account for distinct forms of resistance, the over-expression of efflux pumps extruding the antimicrobials is a major mechanism involved in the resistance of clinical isolates. The efflux pumps of Gram-negative bacteria, for reasons yet to be completely understood, have the capacity to recognize and extrude a wide variety of unrelated compounds such as antibiotics from different classes, biocides and other noxious agents like bile salts [20, 21]. Over-expression of these efflux pumps results in a multi-drug resistant (MDR) phenotype, raising serious therapeutic difficulties [22]. Other example is the resistance to tetracyclines mediated by the TetA protein, an antiporter embedded in the cytoplasmic membrane [23]. In fungi, the failure to accumulate the drug intracellularly, may also be caused by active efflux of drugs resulting particularly from overexpression of genes CDR1, CDR2 and MDR1 [24, 25]; the overexpression of efflux proteins and the associated increased activity is considered to be the most relevant mechanism of azole resistance, conferring cross-resistance to several azole compounds. Two types of efflux pumps, localized in the cytoplasmatic membrane: the ATP-binding cassette transporters, which use ATP as the energy source to drive transport, and the major facilitators, which are energized by the proton gradient across the cell membrane [26].

1.2. Enzymatic inactivation

The most important mechanism of resistance to beta-lactam antibiotics among Gram-negative involves the production of beta-lactamases. Extended-spectrum beta-lactamases (ESBLs) are generally acquired by horizontal gene transfer and confer resistance to oxyimino-cephalosporins, some being mutant derivatives of established plasmid-mediated beta-lactamases (e.g. TEM/SHV) or mobilized from environmental bacteria (e.g. CTX-M) [27]. AmpC cephalosporinases are species-specific chromosomally encoded beta-lactams, common but not ubiquitous among *Enterobacteriaceae* and *Pseudomonaceae*, which have also become mobilized onto transmissible plasmids [28]. The emergence of metallo-beta-lactamases (MBLs) with activity against carbapenems (e.g. the VIM and IMP families of enzymes) has compromised the clinical utility of this class of antibiotics [29]. MBLs can hydrolyse all clinical beta-lactam substrates, with the exception of aztreonam. The appearance and rapid spread of molecular class A carbapenem-hydrolysing enzymes (KPC-type b-lactamases) is the most recent development in the epidemiology of carbapenem resistance [30].

1.3. Target changes

Alterations in the drug target can occur both in bacteria and fungi. Several examples can be appointed. A very high prevalence of macrolide-lincosamide-streptogramin B (MLSB) resistant isolates has been found among Staphylococcal isolates [31] and the erythromycin ribosome methylase gene erm(C) is the most predominant MLSB resistance gene in clinical Staphylococcal isolates [32, 33]. The erm(C) gene has mostly been found on plasmids with mobilization and/or on plasmid recombination genes [34]. Methicillin resistance in Staphylococci is determined by mec, composed of 50 kb or more of DNA found only in methicillin-resistant strains [35]; mec contains mecA, the gene for penicillin-binding protein 2a (PBP 2a); PBP 2a confers cross-resistance to most currently available beta-lactam antibiotics [36]. Fluoroquinolones interact with DNA gyrase and topoisomerase IV, the enzymes that regulate conformational changes in bacterial DNA during replication and transcription. Resistance to fluoroquinolones arises through stepwise mutations in the coding regions of the gyrase subunits (gyrA and gyrB) and DNA topoisomerase IV (parC) [37].

Regarding fungi, in this perspective two distinct mechanisms have been described that confer azole resistance: (i) increased production of the azole target enzyme [38, 39]; (ii) point mutations in genes coding for the target enzyme, the *ERG* genes, thus reducing azole target affinity [40]. Mutations in the *FKS1* and *FKS2* genes, encoding a subunit of the β-1,3-glucan synthase complex (a main cell wall component), result in resistance to echinocandin drugs [41]; such mutations confer resistance to all clinically used echinocandins. Recently, the compensatory increase in the production of chitin, the second structural cell wall polysaccharide, has been proposed as a mechanism causing reduced efficacy of echinocandins.

Pressure, both clinical and commercial, to use antimicrobials both in humans and animals, the global mobility of populations and of food products, all ensure that the spread of MDR microorganism clones and of resistance genes will be a permanent phenomenon. A number of initiatives have been developed to encourage the wise use of antimicrobials. Even with the development of such programs and the heightened awareness of the interplay between resistance,

geography, treatment and gene transmission, it is likely that antimicrobials resistance will continue to develop more rapidly than novel drugs to treat infections may become available; maybe at best we can only hope to slow down a bit the spread of such organisms. The understanding of the mechanisms of resistance acquisition by microbial strains is therefore essential to prevent and overcome antimicrobial resistance.

2. New insights concerning biofilm-related infections in medical indwelling devices and novel biofilm inhibitors

Biofilms can be defined as communities of microorganisms attached to a surface embedded in a self-produced extracellular polymeric matrix [42]. These cellular associations are distinct from planktonic cells, due to the lack of interactions between microorganisms; association in biofilms is a particularly effective way to promoting its development, enhancing symbiotic relationships and allowing survival in hostile environments. The quorum sensing is a process of intra- and inter-species microbial communication, which allows microorganisms to present striking phenotypic changes when they are in high population densities. Typically biofilm are heterogeneous and derivatives of the metabolism of a specific species may be able to help the growth of other species and the adhesion of a given specie may provide ligands that promote the binding of another. Conversely, competition for nutrients and accumulation of toxic metabolites produced by colonizing species may limit the diversity of other species in a biofilm. Using microscopic techniques it has been possible to observe the large spatial heterogeneity of biofilms and of microbial cells that coexist in different physiological states. This heterogeneity is an important strategy, probably because such cells survive better to external attacks such as mechanical, chemical or pharmacological.

A major concern about biofilm developement is the emergence of microbial resistant strains [43]. Recent estimates show that over 65% of all the hospital infections originate from biofilms [44], such as indwelling device related infections. Central venous catheters are a particularly high risk category of devices [45]. Catheter-related bloodstream infection (CRBSIs) result in increased healthcare costs, in longer hospital stays and increased mortality [46, 47, 48]. In US more than 200 000 health-care-related bloodstream infections occur each year and most of them are associated to intravascular devices [49, 50]. In Scotland, around 250 isolates of meticillin-resistent and meticillin-sensitive strains of *Staphylococcus aureus* (MRSA and MSSA respectively), are collected from the bloodstream of patients with bacteremia every trimester [51]. A study conducted at a Portuguese University Hospital demonstrated that patients admitted at ICUs with central venous catheterization showed colonization mainly with *S. epidermidis* (40.2%), *S. aureus* (12.8%) and *Candida* spp (7.0%) [52]. A large number of studies suggested that many, if not all, episodes of Candidaemia are also catheter correlated [53, 54]. *Candida albicans* is the fourth principal cause of vascular catheter-related infections and the third most important cause of urinary catheter-related infections [55,56]. A correlation between *Candida* spp biofilm formation and resistance to antimicrobial agents was already well documented [57].

Biofilms are much more resistant to antimicrobials than planktonic cells and hence treatment of biofilm coated surfaces with conventional antimicrobials may fail, as it takes >1000 times more antibiotics to kill biofilm cells than to kill planktonic cells [58]. Thus, it would be preferable to prevent biofilm formation than killing its cells after its establishement. In this perspective novel biofilm inhibitors are highly needed; its discovery remains an important challenge to the scientific community and has a very interesting potential to be used in medical devices.

Cerium is a rare earth element of the lanthanide group and is used in the management of burn wounds, with a significant reduction in patient morbidity and mortality [59]. Data from the literature showed the bacteriostactic effect of cerium nitrate against bacteria [59]. A recent study corroborated this result and additionally revealed the microbicidal effect of cerium nitrate against other bacteria and yeast [60]. Furthermore, it was documented that lower concentrations of cerium nitrate were effective against *C. albicans* biofilm formation [60]. This compound reduces the activity of microbial pathogens due to their action at different cellular targets [61] such as disruption of cell membrane, inhibition of cellular respiration and inhibition of glucose metabolism. Cerium nitrate could represent an alternative in such medical applications since toxicity is rare within lanthanide compounds.

Chitosans are biocompatible polyaminosaccharides obtained by deacetylation of naturally occurring chitin, isolated from crustacean exoskeletons, chitosans are credited with a broad spectrum antimicrobial activity. A recent study revealed a significant reduction in biofilm formation by *S. epidermidis* and *C. albicans* in presence of chitosan [60]. Chitosans could induce cell wall leakage by ionic surface interaction or by teichoic acid binding and extraction of membrane lipids, mRNA and protein synthesis inhibition, suppression of microbial growth through external barrier formation and metal chelation [62]. Recent results demonstrated that chitosan-coated catheters inhibit *C. albicans* biofilm formation in vivo [63]. Furthermore the same study showed a significant decrease, (~70%) in the metabolic activity of biofilms of *C. albicans* and *C. parapsilosis* in vitro. Other study revealed that chitosan-coated surfaces exhibit antibiofilm properties against bacteria and fungi in vitro [64]. This fact could be attributed to the ability of cationic chitosan to disrupt negatively charged cell membranes as microbes settle on the surface [65].

Polyethyleneimine (PEI) is a synthetic polymer, weakly basic, aliphatic and nontoxic, and also polycationic due to the presence of primary, secondary and tertiary amino groups. According to the literature PEI nanoparticles exhibited a strong antibacterial effect [66, 67]. The high activity of polycationic agents is reflected by the absortion of positively

charged polymers onto negatively charged bacterial surfaces [67]. This process is thought to be responsible for the increase of cell permeability and disruption of cell membranes. A recent study demonstrated that covalently bound quaternized polyDMA-EMA and PEI inhibited biofilm growth of *C. albicans* with reductions up to 92% [68]. Other authors revealed that alkylated PEIs attached to flat macroscopic surfaces and to those of nanoparticles make these materials highly bactericidal upon Gram-positive and Gram-negative pathogenic bacteria [69]. As described by Beyth et al., 2006, PEInanoparticles exhibited a strong antibacterial effect against *Streptoccocus mutans* [66]. QA-PEI nanoparticles completely inhibited bacterial growth, including both Gram-positive (*S. aureus*) and Gram-negative (*E. coli*) bacteria. The study above described confirms that polycations bearing quaternarium ammonium moieties inhibit bacterial growth in vitro and have potential use as additives in medical devices. [70].

3. New therapeutic strategies that include the use of natural products as antimicrobials, as biofilm inhibitors and the use of drugs that revert the antimicrobial resistance

Antibiotics and analogous compounds, together called antimicrobial agents, have been used for the last 70 years to treat infectious diseases. These compounds greatly reduced morbidity and mortality from infectious diseases, providing the current basis for infectious disease control. However, the spread of antibiotic resistance is a global public health problem and a challenging question. Resistance involves agents used in the treatment of bacterial, fungal, parasitic and viral infections. At present, the search and improvement of new compounds extracted from natural resources became a global trend. Natural products account for more than 30% of the therapeutic agents prescribed today [71].

Plants are important sources to explore in order to unveil new antimicrobial agents. A long-standing work showed that phenolic compounds (predominant active compounds in some family plant) were effective against Gram-positive bacteria [72]. Likewise, an herb named *Urtica dioica* that has been used for many years in numerous parts of the world to treat stomachache, rheumatic pain, colds and cough, exhibits bactericidal and antimycotical activities [73]. This plant showed stronger antimicrobial activity against Gram-positive bacteria than Gram-negative, which could be related to lipopolysaccharides content in the outer membrane of Gram-negative bacteria [73]. According to another study, infections such as triggered by *Vibrio parahaemolyticus*, *Bacillus cereus*, *S. aureus*, including MRSA, can be treated with *U. dioica* [74]. Several species of the *Hyptis* genus are an important source of bioactive compounds, with a wide range of antimicrobial activity. Crude extracts and phytochemicals isolated from *Hyptis verticillata*, demonstrated antimicrobial properties against a great number of pathogenic organisms [75]. It is also known that leaves and latex of *Calotropis procera*, a member of the family Asclepiadaceae disply bactericidal and fungicidal effects upon pathogenic organisms [76]. Another study documented that *Quercus infectoria* extract impaired staphylococcal biofilm formation. This evidence supports the traditional use of this plant [77]. Regarding a natural compound such as honey, it has been used as a medicine since ancient times [78]. In the literature several reports describe the efficacy of honey in the treatment of ulcers and wounds [79, 80, 81, 82]; furthermore its antibacterial properties are well documented [83, 84, 85]. A recent study revealed that honey is effective against bacteria and fungi [86].

Concerning “honey-water” (similar to honey, excluding the content in hydrogen peroxide due to the heat treatment) [87], is a honey-based compound produced in Portugal a long time ago; its antibacterial and antifungal activity was also confirmed [88]. Furthermore, the same authors observed the decrease in the infectivity of Qß bacteriophage, suggesting the potential use of “honey-water” to combat enteric viruses. The antiviral activity of “honey-water” will undoubtedly result from the polyphenol and propolis content, since it was attributed to these compounds antiviral properties. Recent studies document the antiviral activity of honey [89, 90, 91]; nevertheless its mode of action remains unknown.

Propolis, another natural bee product, has also been extensively investigated; it was demonstrated that this compound has bacteriostatic and bactericidal activity [92, 93]. Recently it was revealed that propolis could inhibit multi-resistant bacteria and pathogenic yeast. As described by Shokri and collaborators (2011), propolis can inhibit fluconazole-resistant *C. glabrata* [94]; studies performed by Choudhari and co-workers, (2012) [95], and Vera and co-workers, (2011) [96], demonstrate the effectiveness of propolis against multi-resistant bacteria, MRSA, *Enterococcus* spp. and *Pseudomonas aeruginosa*. Another study developed in Portugal with bacteria and yeasts showed that *C. albicans* was the most resistant species to propolis and *S. aureus* the most sensitive between the tested species [97]. A work carried out by AL-Waili and colaboratours, (2012) investigated the single and combined effect of propolis with honey in order to prevent the growth of microorganisms [98]. These authors conclude that propolis inhibited antibiotic resistant *E. coli* and *S. aureus* and *C. albicans*, in single and polymicrobial cultures; in addition, a synergistic effect between propolis and honey [98] was documented. In another study, propolis exhibited significant antimicrobial activities against resistant and multi-resistant microorganisms to antibiotics; however Gram-negative bacteria were less susceptible than Gram-positive [99]. The mechanism underlying propolis antimicrobial activity might be attributed to the effect of several compounds, such as phenolic and flavonoid and their synergistic effect [100, 101]. Propolis may act against the cytoplasmic membrane, compromising bacterial motility, enzyme activity, cell division and protein synthesis [102,103].

Hamamelitannin is a polyphenol extracted from the bark of *Hamamelis virginiana*. This natural biocompatible and inexpensive compound belongs to the family of tannins. Hamamelitannin could inhibit infections associated with

medical devices in vivo [43]. Furthermore, in vitro activity against *A. baumannii* has also been recognized [60]. Hamamelitannin seems to inhibit the quorum sensing system of such bacteria, reducing their virulence [43]. The mechanism of action of this compound is not yet elucidate.

Concerning biofilm, new agents that inhibit its formation would be highly desirable and urgent as previously mentioned. Recently, it was shown that natural honey inhibited growth, viability and biofilm formation by *S. mutans* [104]. This organism, together with other oral bacteria is responsible for the formation of dental biofilms [105]. Regarding biofilm metabolic activity it was also documented that hamamelitannin decreased bacterial biofilm activity at subinhibitory concentrations [106].

Further efforts are needed aiming the identification of additional bioactive compounds and also the *in vivo* evaluation of its respective antimicrobial activity. Recently, the European Commission decided to connect pharmaceutical industry, research capacities (Universities) and small companies in order to search for novel antibiotics [107].

The knowledge of the mechanisms involved in antimicrobial resistance brought by the genomic era supports the development of therapeutic strategies in order to bypass drug resistance. An important cell mechanism of antimicrobial resistance is the active transport of drugs out of the cell by efflux pumps [108-112], expressed not only by bacteria and yeasts but also by humans cells [113, 114]. The main strategy to reduce efflux impact involves the maintenance of a high antimicrobial concentration inside the cell, at its site of action. This could be achieved by the use of antimicrobials that are not substrate of efflux pumps. The second approach would be the development of inhibitors or chemosensitizers of efflux, affecting its target, the activity, by blocking access to the binding site, or even the efflux pump transcription. In humans, one of the factors that is responsible for the failure of cancer therapy are ATP-dependent drug efflux pumps, such as P-glycoprotein (P-gp) [115]. P-gp substrates such as FK506 [116] or cyclosporine A (CsA) [117] are immunosuppressors that are able to inhibit the efflux mechanism in human cells. They act similarly in *C. albicans* strains, inhibiting the calcineurin-mediated azole tolerance by binding to small, abundant, conserved binding proteins called immunophilins. CsA binds with cyclophilin A (Cyp1p) and FK506 with FKBP12 [108, 118, 119]. By inhibiting calcineurin these compounds act synergistically with azoles [119-121]. Ibuprofen ([2-(4-isobutylphenyl)-propionic acid has been described to act synergistically with pyrazinamide [122], fluconazole [110, 123, 124] and amphotericin B [125]. In *C. albicans* expressing CDR efflux pumps, the presence of ibuprofen increased azole intracellular accumulation, changing the resistant phenotype to susceptible [110, 124]. This potent anti-inflammatory, non-steroidal drug might play an important role in future antifungal therapeutic strategy.

Another helpful strategy to overcome antimicrobial resistance would be the design of inhibitors that could act indirectly on efflux, de-energizing the ATP or H^+ dependent transporter, by lowering the cytoplasmic ATP concentration or depleting the electrochemical potential of the plasma membrane, respectively [126, 127]. However, by altering ATP and membrane potential, other cellular metabolic activities could be compromised. Alternatively, the promotion of drug antimicrobial uptake could be a strategy to overcome resistance. Recently, sugars such as mannitol, glucose, fructose and pyruvate have been shown to generate proton-motive force and promote the uptake of aminoglycosides by *E. coli* and *S. aureus*, resulting in enhanced susceptibility to this class of antibiotics [128]. However, this approach is limited to aminoglycosides because it requires relatively high concentrations of sugar (e.g. 10 mM), but not the β-lactam antibiotic ampicillin and the fluoroquinolone ofloxacin. Recently Pan and co-workers showed that (Z)-4-bromo-5-(bromomethylene)-3-methylfuran-2(5H)-one (BF8) can revert antibiotic tolerance of *Pseudomonas aeruginosa* [129]. The medical complexity of patients taken together with the intricate cellular mechanism involved in drug resistance demands such alternative strategies to be explored.

4. New strategies to identify and evaluate susceptible profile in useful clinical time

Infection diagnosis is based upon the isolation, identification and antimicrobial susceptibility evaluation of the involved microorganisms. Despite all the recent scientific progress, microbiology diagnosis is still slow needing at least 48 hours. Automation or semi-automation now allows the simultaneous analysis of a great number of strains but didn't speedup the time result. Identification and evaluation of the antimicrobial susceptibility profile are both based on the ability of the microorganism to grow on different subtracts or in the presence of different drugs. The consequences of this delay are obvious: clinicians have often to start empiric treatment, using broad-spectrum antimicrobials, with a high risk of failure and of formation of antimicrobial resistance; antimicrobial detection is too late to prevent its dissemination. Thus hospital related infection is a plague even in high quality health units over the worth.

Regarding identification, immunological techniques and the emergence of molecular biology assays, specially 16S ribosomal RNA sequencing or real-time PCR detection of selected genes, have created a revolution, providing speed and specificity, especially regarding identification. Nevertheless, such techniques remain complicated and costly, and are not suited for use on the vast majority of routine samples. They had the additional advantage on identification of uncultured pathogens [130]. In the last 2 years the availability of matrix-assisted laser desorption ionization time-of-flight mass spectrometry (MALDI-TOF MS) techniques, until then confined to research laboratories, has changed the laboratory workflows for the identification of pathogens. MALDI-TOF MS is now a mature technique for bacterial and fungi identification with great promise [131,132]. All data show that MALDI-TOF MS correctly identifies the great majority of bacteria and fungi processed routinely, in a few minutes. It also has the potential to directly identify

pathogens in biological fluids, such as urine samples and blood cultures. For this application however, further well-designed prospective studies are warranted.

However, regarding antimicrobial susceptibility few progresses were made. Molecular techniques and MALDI-TOF MS can both punctually reveal certain mechanisms of resistance like the presence of *mecA* gene define a *S. aureus* resistant strain to methicillin (MRSA) [133], the presence of degradation products of a carbapenem due to the presence of a carbapenemases [134]. It should be stressed that a lot of mechanisms are not well characterized and several mechanisms could be present at the same time. In addition, a broad spectrum antimicrobial phenotype is necessary in clinical routine. Flow cytometry seams a real promising tool to be used with that proposes. The method is based on the analysis of morpho-funtional characteristics of a great number of microbial cells; by using suitable dyes it is possible to perform a rapid detection and to examine the nature of drug-induced damaged to microorganisms [135]. Pina-Vaz and co-workers has been developing flow cytometry applications both using monoclonal antibodies in order to identify microorganisms [136] but mainly for susceptibility evaluation, with considerable advantages over conventional methods [137, 138, 139]. It represents a real breakthrough in microbiology diagnosis.

However susceptibility tests are not always accurate predictors, since they do not take into account the dynamic and complex biology of microorganisms exposed to an antimicrobial drug in vivo.

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