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Review

Understanding bacterial resistance to antimicrobial peptides: From the surface to deep inside☆

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ABSTRACT

Resistant bacterial infections are a major health problem in many parts of the world. The major commercial antibiotic classes often fail to combat common bacteria. Although antimicrobial peptides are able to control bacterial infections by interfering with microbial metabolism and physiological processes in several ways, a large number of cases of resistance to antibiotic peptide classes have also been reported. To gain a better understanding of the resistance process various technologies have been applied. Here we discuss multiple strategies by which bacteria could develop enhanced antimicrobial peptide resistance, focusing on sub-cellular regions from the surface to deep inside, evaluating bacterial membranes, cell walls and cytoplasmic metabolism. Moreover, some high-throughput methods for antimicrobial resistance detection and discrimination are also examined. This article is part of a Special Issue entitled: Bacterial Resistance to Antimicrobial Peptides.

Contents

1. Introduction

In recent years, antibiotic resistance has increasingly become an uncontrollable health problem. Bacterial infections caused by resistant strains can be found in hospitals around the world, being extremely common in immune compromised patients [1]. Antibiotics are able to control bacterial infections, interfering with microbial metabolism and physiological processes, such as DNA replication and cell wall biosynthesis. Although multiple compounds are often used, cases of resistance to the majority of antibiotic classes used in hospitals have been reported [2].

The last report from the American Centers for Disease Control estimated that over two million illnesses and 23,000 deaths were caused by drug-resistant microbes in the USA in 2013 [3]. These numbers have encouraged health organizations to establish stricter policies for antibiotic use in order to curtail the emergence of resistance. These

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policies are unquestionably helping to protect patients in many countries. If, on the one hand, a reliable policy for the use of antibiotics is necessary, the development of new drugs with potential activity against these pathogens is also essential.

Antimicrobial peptides (AMPs) are effective antibiotic agents found in plants, animals and microorganisms. These molecules have a broad spectrum of action, often being active against bacteria, fungi and protozoans. The amphipathic structure, common to AMPs, facilitates their interactions and insertion into the anionic cell wall and phospholipid membranes of microorganisms [4]. Frequently, AMP activity results from the disturbance of cell membrane integrity. However, AMPs can act in different cell targets including DNA [5], RNA [6], regulatory enzymes [7] and other proteins [8], appearing as a promising alternative to classic antibiotics [9]. Nevertheless, once AMPs have been put into current clinical use, the development of AMP-resistant strains will be inevitable [10–12]. Thus, the understanding of bacterial resistance against these compounds is extremely necessary for a possible rational planning of the next antibiotic generation.

To shed some light on the bacterial resistance process, several technologies including mass spectrometry and high-throughput techniques have been applied to analyses of bacterial physiology in response to antibiotic stress [13]. In this review article we discuss different strategies by which bacteria can develop AMP resistance from the surface to deep inside, evaluating the bacterial resistance process layer by layer. Moreover, some technologies for detecting antimicrobial resistance are also discussed.

2. Conventional and high-throughput methods to discriminate bacterial resistance

Currently the increase in the development of bacterial resistance to available antimicrobial agents is a major health public problem in the 21st century. Therefore, it is necessary to investigate and monitor antibiotic resistance in order to discriminate the pattern of resistant bacterial strains and to propose the appropriate treatment. These efforts may be helpful to reduce medical expenses and treat patients effectively [14].

In order to measure the resistance of microorganisms to antimicrobial agents, a wide variety of different and conventional laboratory methods is available. Among these can be cited the disk diffusion assay, the broth dilution test and automated commercial systems based on classical biochemical analysis [14]. Either broth (macrodilution and microdilution) or agar dilution methods may be used to measure quantitatively the in vitro activity of an antimicrobial agent against a given bacterial isolate, reporting the minimal inhibitory concentration (MIC).

In order to perform these tests, a series of tubes or plates is prepared with a broth or agar medium, as appropriate to each test, in which various concentrations of the antimicrobial agents are added. The tubes or plates are then inoculated with a standardized suspension of the test organism and after incubation, the tests are examined and the MIC is determined [15]. In susceptibility testing methods using an agar-based medium, such as disk diffusion and Etest, the sizes of the zones of inhibition depend on many variables (i.e. the antimicrobial agent, disk content and inocula), which may represent a disadvantage of these methods [14,16]. These biases have been reduced using firmly established standardized interpretative breakpoints and automated systems.

The commercial systems available are based primarily, or in part, on some of these standardized manual methods and may provide results essentially equivalent to these methods [15]. However, the use of automated or semi-automated systems, i.e., VITEK® 2, BD Phoenix® and MicroScan® WalkAway® [17] in microbiology labs, which also expose bacteria to graduated dilutions of antibiotic drugs, can give a result in fewer hours than the manual methods. They provide an advantage in detecting resistance, ordering fewer laboratory tests during the diagnostic process, completing the diagnostic workup using fewer sample collections, reducing laboratory costs and preventing resistant strains from spreading rapidly [18–20]. Automated systems performing identification of reduced antimicrobial susceptibility strains are increasingly being used [15,20]. But in some cases a diversity of screening non-standardized methods plus confirmatory testing by more elaborate techniques have to be used to detect different levels of antimicrobial resistance between clinical isolates with a heterogeneous population of cells. This was observed in the GISA strain (GISA, glycopeptides intermediately susceptible to *Staphylococcus aureus*) and their heterogeneous variant hGISA (hGISA, heterogeneous glycopeptides intermediately susceptible to *S. aureus*), whose isolates probably represent the extremes of a common phenotype that confer a variable level of reduced susceptibility to glycopeptides [21,22]. In addition, Lo-Ten-Foe and co-workers [20] showed by comparison between testing different types of antimicrobial susceptibility that the automated system used was a reliable and easy-to-use tool to determine *Enterobacter cloacae* and *Acinetobacter baumannii* colistin resistance, but that it cannot detect antimicrobial resistance in hetero-resistant isolates [20].

Although resistance has usually been analyzed at the phenotypic level by monitoring bacterial growth in the presence of various antibiotics, molecular high-throughput subtyping methods are available and their use seems to be necessary in order to discriminate distinct levels of bacterial resistance and to overcome the difficulties encountered in conventional tests [23]. Moreover, in general, genotypic tests may be advantageous over phenotypic assays, being much faster and capable of circumventing problems associated with a sometimes low resistance phenotypic expression [24]. Hence, the high-throughput methods based on DNA-assays (genomic and transcriptomic tools) and on proteomic-assays have been used to discriminate bacterial resistance and to assist in the management of infections.

Studies carried out in pathogenic bacteria have revealed that genes across diverse functional categories participate in determining the level of intrinsic and acquired susceptibility/resistance to antibacterial agents, known as the resistome, and the ongoing delineation of this resistome may provide fundamental insights both into antimicrobials' mode of action and into the bacterial response to inhibition and resistance [14,25]. Thus, the use of these tools can be advantageous due to their sensitivity and rapid turnaround times, which may provide clinical benefits that offset the cost [26]. The molecular methods to detect antibiotic resistance based on genomic analysis, such as gene sequencing [25,27,28], have been increasingly implemented in clinical laboratories to complement diagnosis and treatment.

Some reports have described the development of variations on techniques as demonstrated by Zimenkov and co-workers [29], in a study which showed an uncomplicated and easily implemented microarray technique. This was capable of detecting mutations in the *gyrA* and *gyrB* genes responsible for fluoroquinolone resistance and mutations in the *rrs* gene and the *eis* promoter locus that are associated with the aminoglycosides and capreomycin resistance in *Mycobacterium tuberculosis*.

Another genomic approach, Scalar Analysis of Library Enrichments (SCALEs), was applied to map the effect of gene overexpression onto Bac8c (an 8 amino acid AMP) resistance in parallel for all genes and gene combination in the *Escherichia coli* genome, being capable of successfully identifying an elaborate network of genes for which overexpression leads to low-level resistance to this specific AMP [30]. In addition, a molecular test in association with conventional screening tests could provide valuable antibiotic resistance information to facilitate the management of patient therapy and the prevention of transmission [28].

Other DNA-based techniques, especially PCR, are often used to examine bacterial resistance genes [31]. Besides, real-time PCR (Q-PCR) assays have also been used to detect and quantify genes correlated with resistance, as demonstrated in *S. aureus* to achieve more accurate and rapid detection of macrolide–lincosamide–streptogramin B resistance genes (i.e., the *erm* genes). These genes are commonly observed in Gram-positive bacteria, such as the genera *Enterococcus*, *Bacillus*, *Streptococcus* and *Staphylococcus* correlated to bacteria 23S rRNA