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High-throughput genomic technology in research of virulence and antimicrobial resistance in microorganisms causing nosocomial infections

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ABSTRACT

Most hospital-acquired infections are caused by organisms common in the general population and most are relatively harmless. Infection by nosocomial pathogenic bacteria is increasingly becoming a major threat to hospital patients. Molecular diagnosis of antibiotic resistant organisms such as *Clostridium difficile* Infections (CDI) Methicillin Resistant *Staphylococcus aureus* (MRSA), Extended-Spectrum β-lactamase (ESBL) *Escherichia coli*, Vancomycin Resistant *Enterococcus* (VRE), Carbapenem-Resistant *Klebsiella* (CRK), among others, is vital for prevention of healthcare-acquired infections in acute care facilities. DNA microarray besides being a promising diagnosis tool may also provide valuable information about the mechanisms of antimicrobial resistance and pathogenicity of these bacteria. This review aims to highlight the prominence of high-throughput genomic tools in research of virulence and antimicrobial resistance in microorganisms causing nosocomial infections.

Keywords: Microarrays; Nosocomial Infections; Virulence; Antimicrobial Resistance.

1. Introduction

1.1. *Nosocomial infections*

A nosocomial infection (from the Greek word for hospital "nosokomio"), also known as a "hospital-acquired infection", is an infection acquired in hospital, or other health care facility, by a patient who was admitted for a reason other than that infection. This includes infections acquired in the hospital but appearing after discharge, and also infections among hospital staff [1].

Nosocomial infections occur worldwide and affect both developed and underdeveloped countries. Although the current progress in public health and hospital care, nosocomial infections belong to the most common infections and complications in clinical medicine and continue to develop in hospitalized patients. Infections acquired in health care settings are among the major causes of death and increased morbidity among hospitalized patients [2]. It is estimated that at any one time, about one million and half people worldwide suffer from infectious complications acquired in health care units [3]. These infections prolong hospitalization, require more extensive diagnostics and treatment, and are associated with additional costs [4, 5].

The most frequent nosocomial infections are lower respiratory tract infections, surgical wounds, urinary tract infections and sepsis. Several studies have shown that these infections most commonly occur in intensive care units and in acute surgical and orthopedic wards. Infection rates are also higher in patients with increased susceptibility such as immunocompromised, age (infants and elderly), chronic hemodialysis patients, and those receiving chemotherapy treatments [6-9].

During the stay in health care facilities, patients are exposed to a variety of microorganisms. The contact between patients and the microorganism itself does not necessarily

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result in the development of clinical disease. There are other factors which influence nosocomial infections, such as the characteristics of microorganisms, including the virulence and resistance to antimicrobial agents [10].

Different bacteria may cause nosocomial infections. Infections may be caused by the transference of a microorganism from one patient to another person in the hospital (cross-infection) or may be caused by the patient own flora (endogenous infection). In addition, infection can be spread by environmental transfer, as from an inanimate object, through inhalation of aerosols, or from a substance recently contaminated by another human source.

Most nosocomial infections are caused by organisms common in the general population, in which are relatively harmless. They may not cause disease or a milder form of disease than in hospitalized patients. This group includes the anaerobic bacteria *Clostridium difficile*, facultative anaerobic such as *Staphylococcus aureus*, coagulase-negative staphylococci, *Escherichia coli*, *Enterococcus* spp, *Klebesiella* spp, and *Pseudomonas aeruginosa*, and the obligate aerobic *Acinetobacter baumannii* [11, 12].

Furthermore, the hospital environment supports the acquisition of resistance to antimicrobial agents by bacteria, complicating the treatment of infections due to drug-resistant pathogens. Antimicrobial-resistant bacteria are of particular importance, since impair or prevent the successful treatment of infections [13]. The increasing number of antimicrobial agent-resistant pathogens and high-risk patients in hospitals are challenges to progress in preventing and controlling these infections.

2. Antimicrobial resistance and virulence

The increasing resistance to antimicrobial agents has been a global problem in recent years in both developed and developing countries and it has rapidly become a leading public health concern. Shortly after the introduction of penicillin into general medical use in the 1940s, it was recognized that bacteria would develop resistance to antibacterial agents. By 1948, most of the *S. aureus* isolated in British hospitals were resistant to penicillin due to production of β-lactamases. [14].

As other antimicrobial agents were introduced, organisms resistant to them were isolated from infected patients or from the environment. This has developed into a cycle of antimicrobial agent development, introduction into clinical use, and the development of resistance-often to the point where these drugs becomes worthless in a few years [15].

The prevalence of antimicrobial resistance varies greatly between and within countries and between different pathogens. However, the fact that many patients receive antibiotics, even when its use is not indicated, can be the main reason for the emergence of multi-resistant strains, through the selection pressure and exchange of genetic resistance elements involved in resistance. While, microorganisms in the normal human flora sensitive to a particular antimicrobial agent are suppressed, the resistant strains persist and may become endemic in the hospital. The widespread use of antimicrobial agents not only for therapeutic purposes but also for prophylactic use has been identified as the main determinant for the emergence and spread of resistant microorganisms [16].

Antimicrobial resistance bacteria are a major cause of nosocomial infections and are associated with increasing rates of mortality among hospitalised patients. Many strains of staphylococci, *Enterobacteriaceae*, and enterococci are currently resistant to most or all antimicrobials which were once effective [17, 18]. Moreover, multi-resistant *Klebsiella* spp, *P. aeruginosa*, *A. baumannii*, and *C. difficile* are prevalent in many hospitals [17, 19-23]. These pathogens are able to evade the effects of antibiotics through a significant number of mechanisms, compromising the effective treatment of infections. Health care units environment provides a selective antimicrobial pressure and a proper area for dissemination of resistance genes. The widespread use of antimicrobial drugs and the presence of sensitive microorganisms allow the acquisition and transfer of resistance genes and thus the emergence of highly pathogenic bacteria. This problem is particularly critical in developing countries where more expensive second-line antibiotics may not be available or affordable [24].

3. High-throughput genomic technology

High throughput genome-wide array based techniques such as Comparative Genomic Hybridization (CGH) and transcriptional profiling provide an opportunity to discover genes and/or pathways that are specifically activated in the different stages of bacterial infection or in response to antimicrobial drug exposure. In addition to helping to understand carriage and disease processes by pathogenic bacteria, such data provided by these tools may also contribute to antimicrobial and vaccine development through the identification of targets found in these organisms [25, 26].

3.1. *DNA microarrays*

In the era of "whole genome studies", the advent of genome sequencing and DNA microarray technology are increasingly gaining importance, as a high throughput technology to integrate gene information with biological function [26, 27].

The microarray is the most powerful genomics approach available for high through-put analysis. This tool is used to analyse both expression levels of genes in a given organism, and comparative hybridization of differentially labelled DNA from two samples. A DNA microarray is typically a glass slide on to which DNA molecules are immobilized in a random manner in specific locations called spots. A microarray may contain thousands of spots and each spot contain DNA probes that uniquely correspond to a gene. The DNA probes in a spot may either be a PCR product or

short stretch of oligonucleotide strands that correspond to a particular gene. The probes are printed on to the glass slide by a robot or using a DNA photolithography process (Affymetrix GeneChips) [28, 29]. On the other hand, the recent microfluidic hybridization method, combining the fields of microfluidics and DNA microarrays, shows several advantages such as less sample usage and reduced incubation time. These capabilities allow a rapidly and accurately detection of infectious pathogens and drug resistance markers [30, 31].

DNA Microarrays may be used to analyse gene expression in many ways, but one of the most common applications is to compare expression of a set of genes from a particular condition of bacterial cells (for example, a stress condition) to the same set of genes from a reference under normal conditions. This technology allows to analyse RNA preparations that were extracted from *in vitro*-cultivated as well as to determine the transcriptional status *in vivo*-derived bacteria at the level of the whole genome bacteria [32].

On the other hand, microarray CGH provides an estimate of the relative abundance of genomic DNA taken from test and reference organisms by hybridisation to a microarray containing probes that represent sequences from the reference organism [25]. Bacterial genome evolution is dominated by gene insertions/deletions and gene divergence [33, 34]. Genetic diversity of intra-species must be analysed if we are to gain a better understanding of the evolution of the genome of a given bacterial organism and use that information for instance for development of technical applications as vaccines or bacterial drug development [35]. [25, 35]. This will especially be valuable when particular virulence and antimicrobial resistance-associated genes are identified [25, 36-38]. Different studies on bacterial microarray CGH have demonstrated the power of the method in a comparative genomics context [34, 39].

A range of important bacterial pathogens, including multi-drug resistant isolates have been sequenced. These datasets have provided the opportunity to develop DNA microarray chips for comparative and gene expression studies, allowing the detection of antibiotic resistance and virulence genes. These microarrays are able to detect hundreds of resistance and virulence genes, and can be used to analyse a variety of diverse bacteria species including important pathogenic nosocomial organisms [40-42].

4. *Clostridium difficile*

The Gram-positive bacillus *C. difficile* is an obligate anaerobic and is the most frequent and clinically important cause of diarrhoea that has been strongly associated with the hospital setting. *C. difficile* differs from other nosocomial pathogens, since the emergence and prevalence of resistant strains is not directly linked to the treatment of the infection caused by this bacterium. The *C. difficile* infection (CDI) is nearly exclusively caused by antibiotic exposure in the treatment of other bacterial infections that disrupting the normal intestinal flora, allowing *C. difficile* to flourish. Many consider antimicrobial agents usage and poor hygiene within a clinical setting as key underlying factors of CDI outbreaks [43].

In the last decade the incidence of CDI has increased and significant outbreaks in several hospitals have been associated with a high number of cases of toxic megacolon, colectomy and mortality [44]. A recent review of mortality due to CDI, found attributable mortality of 8.03% in studies performed since the year 2000, compared with 3.64% in those before this date [45]. *C. difficile* is also associated with increased health care costs. CDI represents a significant problem to healthcare resources as treatment regimen requires spatial isolation of patients, intensified measures for infection control and the frequent use of disinfectants on surfaces and medical equipment. However, one of the main reasons by which *C. difficile* increases costs is by extending the length of time patients spend in hospital. Patients with CDI spend 7–21 days longer in the hospital and cost at least 50 % more, compared to patients who do not develop CDI during hospitalization [46].

The emergence of CDI is believed to be associated with ageing population, and due to the dissemination of hypervirulent clones. In particular, the mutant hypervirulent strain PCR ribotype 027 (North American) has been found to produce 16-fold more toxin A and 23-fold more toxin B in addition to the binary toxin. This global epidemic strain has also been reported to cause outbreaks in Europe with increased morbidity and mortality [47-49].

Resistance to erythromycin, clindamycin and fluoroquinolones is exhibited among most *C. difficile* pathogenic strains. Ribosomal methylation mediated by *erm* genes confers high level resistance to erythromycin and clindamycin. In addition, two main mechanisms of fluoroquinolone resistance have been identified, such as amino acid substitutions in the quinolone-resistant determining region of target enzymes, widely spread in many bacteria; and decreased antibiotic accumulation inside the bacterium due to an overexpression of efflux pump systems [50]. Vancomycin and metronidazole are the antibiotics of choice to treat CDI, although vancomycin has been shown to be more effective in patients with severe CDI. However, CDI is an ongoing challenge since about 20% of the treatments with metronidazole or vancomycin fail [51, 52].

Fidaxomicin is a first-in-class macrocyclic antibiotic being developed as a therapy for CDI and presents advantages over other antimicrobial agents used to treat CD. Fidaxomicin is clearly more potent *in vitro* than vancomycin against clinical isolates of *C. difficile*. In addition, this compound is minimally absorbed after oral administration, achieves high concentrations in the intestinal tract, long post-antibiotic effect, and restricted activity against normal gut flora, providing active and selective therapy for CDI, has little activity for inhibiting other bowel flora species [53, 54].

Several techniques are used to understand the

epidemiology and pathogenicity of *C. difficile* strains. Microarray CGH comparison studies between *C. difficile* strains revealed that only 16-19.7% genes were shared by all strains [55, 56]. The common core gene set containing conserved genes in all tested *C. difficile* strains is unusually lower than the core genome estimates of other bacterial species [57]. One contributing cause to the emergence of the PCR ribotype 027 strain is its increased resistance to antibiotics, including fluoroquinolones. A Microarray CGH study across *C. difficile* strains, from different origins, showed different levels of divergence of coding sequences involved in antibiotic resistance [55].

Under conditions that are not favourable for growth, *C. difficile* produces metabolically dormant endospores via asymmetric cell division. The use of Genome-wide microarray approach in *C. difficile* 630, an epidemic, virulent and multi-drug-resistant strain, showed a highly dynamic gene expression during germination and outgrowth [58].

CodY, a global regulatory protein that monitors the nutrient sufficiency of the environment has shown to be a potent repressor of toxin gene expression in *C. Difficile*. DNA microarray analysis, using a *codY* null mutation strain showed a overexpression of 146 genes, including metabolic and a major group of virulence genes. The coregulation of these genes by CodY underscores the connection between nutritional sufficiency and pathogenesis in this bacterium [59].

5. *Staphylococcus aureus*

Most of the medical literature shows that *S. aureus*, is certainly the most notorious and prevalent Gram-positive nosocomial pathogen found in clinical samples, being a leading cause of both skin structure infections and blood stream infections with considerable morbidity and mortality [60-62].

The seriousness of the infections caused by *S. aureus* is linked to the different potential infected tissues, ranging from skin and soft tissues to lower respiratory tracts and bloodstream. Moreover, these infections are intensified by the bacterial potential to develop multiple antimicrobial resistances. The most striking example is those conferring resistance to methicillin and other β-lactam antibiotics, known as methicillin-resistant *S. aureus* (MRSA). In many countries, most hospital associated *S. aureus* strains are resistant to methicillin, with MRSA rates upper 50% [63]. However, there are considerable geographical variations in MRSA rates when infections are grouped by continent of origin. MRSA rates are especially high in North America and Asia, and are significantly lower in western Europe [61].

Hospitalized patients whose immune systems are weak show a high frequency of *S. aureus* infections. Besides, infections by *S. aureus* in these kind of patients may develop into extremely dangerous and life-threatening diseases such as osteomyelitis, meningitis, necrotizing pneumonia and infective endocarditis [61, 64].

The antimicrobial agent vancomycin has been first-line antibiotic treatment for serious infections caused by MRSA, including complicated skin-structure infections, bloodstream infection, and pneumonia [64]. However, despite being the criterion standard therapy, the susceptibility of MRSA to this antimicrobial agent may be decreasing, and reports of clinical failure are increasing [65, 66]. Moreover, antimicrobial drug requires intravenous administration, and occasionally patients experience unacceptable side effects. Linezolid, a member of the new oxazolidone class of antibiotics, is highly active *in vitro* against MRSA and has excellent oral bioavailability. However, the emergence of linezolid -resistant *S. aureus* has been reported in recent studies [67, 68]. The emerging potential of vancomycin and linezolid resistant *S. aureus*, provides a serious concern for the future treatment of hospital-acquired infections.

An atypical MRSA strain was isolated during an epidemiological survey of *S. aureus* in cystic fibrosis patients, in France. Genome analysis of this MRSA isolate using high throughput sequencing method and microarray CGH analyses revealed the presence of a new antibiotic inducible phage [69]. Antibiotic-mediated phage induction may result in high-frequency transfer and the unintended consequence of promoting the spread of bacterial virulence and/or antibiotic resistance determinants. Expression microarrays, showed that the genes differentially expressed between strains from cystic fibrosis patient and non-cystic fibrosis patient, involve phage elements or resistance determinants [69]. In addition, CGH comparisons between hospital- and community-associated isolates in Canada have revealed genetic differences which included open reading frame encoding potential virulence factors [39]. Moreover, in Romania, one of the countries with the highest prevalence of MRSA, several *S. aureus* clinical isolates from different infections were recently characterized by microarray hybridisation [70].

Rhodomyrtone, is a natural antibacterial drug displayed significant antibacterial activities against MRSA. Microarray analysis showed a significant modulation of gene expression, in MRSA exposed to subinhibitory concentrations of rhodomyrtone. Genes up-regulated included genes involved in metabolism of amino acids, which can be related with the antimicrobial mechanism of rhodomyrtone. In addition, overexpression of virulence factors was also detected [71]. Moreover, a recent microarray analysis was used to investigate changes in gene expression in the EMRSA-15 strain (NCTC 13142), exposed to manuka honey, a broad -spectrum antimicrobial agent. In this analysis, the microarray data indicated notable changes in expression of several genes, with potential clinical significance, including important MRSA virulence determinants [72].

6. Coagulase-negative staphylococci

Coagulase-negative staphylococci (CoNS) are a heterogeneous group of opportunistic pathogens whose adaptability to persist and multiply in a variety of environments causes a wide spectrum of diseases in humans. Species such as *Staphylococcus epidermidis* and *Staphylococcus haemolyticus* are commonly found on the skin and mucous membranes of humans and are therefore often found in clinical specimens [73]. *S. epidermidis* is the most frequently isolated member of the group of CoNS has emerged as a major cause of nosocomial infections. It has been associated with bacteremia, wound-related infections, postoperative infections, and most important intravascular catheter–related infections.

Usually an innocuous commensal microorganism on human skin, CoNS can cause severe infection after penetration of epidermal and mucosal barriers, which frequently occurs in patients during the insertion of indwelling medical devices [74]. In these patients, the host defence mechanisms often seem unable to handle the infection and, in particular, to eliminate the staphylococci from the infected device because of the development of a biofilm on the foreign body surface [73, 75, 76].

S. epidermidis differs from *S. aureus*, by its inability to produce coagulase and usually not cause pyogenic infections in non-compromised patients, with the exception of native valve endocarditis [77]. Because of the lack of severely toxins tissue-damaging exoenzymes, compared to *S. aureus*, *S. epidermidis* infections are usually subacute or chronic. [74]. The success of *S. epidermidis* infection, is usually attributed to its ability to adhere to surfaces and to remain there, being the biofilm formation considered to be the main virulence factor [78].

Treatment of CoNS infections is generally difficult due to the ability of these bacteria to develop resistance to all described antibiotics, and because the slime capsule of staphylococci represents an almost impermeable barrier to many antibiotics [79]. Moreover, methicillin-resistant CoNS (MRCoNS) are currently a common finding among hospitalized patients [80, 81]. Therefore, glycopeptide antibiotics (vancomycin and teicoplanin) are usually the antimicrobial drug of choice for treatment of infections by MRCoNS [82, 83]. However, in the last decade decreased susceptibility of CoNS isolates to glycopeptides has been reported from different parts of the world, and thus representing an emergent challenge to the clinicians [84-86].

Comparative genomics has been used as an alternative approach to identify bacteria virulence determinants. A study performed between clinical and benign *S. epidermidis* strains revealed a high genetic variability of this bacterium as a species. Several markers were identified for *S. epidermidis* invasiveness, which included proposed virulence factors and potential targets for drug development against *S. epidermidis* infections [87].

Biofilm formation leads to a non-aggressive and protected form of bacterial growth with low metabolic activity, which is optimally suited to guarantee long-term survival during chronic infection. The development of a biofilm is accomplished through a series of sequential steps, each of which is characterized by changes in gene expression in response to environmental signals and cell-cell signalling [88]. The gene expression profile of a *S. epidermidis* during biofilm development was analysed by [89] using a DNA microarray representing its entire transcriptome. In this study, the pattern of gene expression in *S. epidermidis* biofilms is characterized by a distinct physiological state which presumably results in increased protection from antibiotics and the immune defense of the host and allows bacteria to persist during infection.

Moreover, a microarray developed to detect of up to 90 antibiotic resistance genes in Gram-positive bacteria, was recently used to identified antibiotic resistance genes in MRCoNS strains isolated from pets and horses [90].

7. *Escherichia coli*

E. coli is the head of the large bacterial family, *Enterobacteriaceae*, the enteric bacteria, which are facultatively anaerobic Gram-negative, and is commonly found in the intestinal tract of humans. The emergence of multi-resistant *E. coli* has been observed, identifying it as a major threat to public health. European studies, showed that *E. coli* exhibited a Europe-wide increase of resistance to fluoroquinolones, third generation cephalosporins and aminoglycosides [91].

The emergence and wide dissemination of extended spectrum β-lactamases (ESBLs) among clinical *E. coli* isolates in hospitals, has caused a major concern in several countries, being frequently implicated in human infections. These infections have a great impact on public health due to an increased incidence of treatment failure and severity of disease. ESBLs mainly include TEM, SHV, and CTX-M enzymes. Among them, the highest number of variants described during the last years corresponds to the CTX-M family [92]. The presence of CTX-M enzymes render *E. coli* resistant to a variety of β-lactams, and are transferred via plasmids that can also include resistance genes to several unrelated classes of antimicrobial agents [93].

The dissemination of CTX-M enzymes around the world has been referred as the "CTX-M pandemic" mostly because of the increase of reports worldwide. One of the most interesting issues in the dispersion of CTX-M enzymes from *E. coli* is the participation of specific clones. New approaches based on MLST typing have demonstrated that despite a high diversity among CTX-M producers, a few clones or sequence types grouped in clonal complexes have been repeatedly found linked to CTX-M enzymes. This suggests that they are involved in the dissemination of these enzymes and that the adaptive success of some CTX-M enzymes could also depend on specific sequence types or clonal complexes where they are frequently present [94].

Although *E. coli* is not traditionally associated with

nosocomial infections, due to the acquisition of resistance determinants, it has emerged as the leading Gram-negative pathogen responsible for bloodstream and urinary tract infections [95, 96]. The invasive *E. coli* infections are mainly due to extraintestinal pathogenic *E. coli* (ExPEC) which often originate from the urinary tract (uropathogenic *E. coli*, UPEC) [97]. ExPEC strains are genetically distinct from commensal *E. coli* found in the intestinal flora. They are usually characterized by a predominance of phylogenetic group B2, and encode a large number of virulence factors responsible for pathogenesis outside of the gastrointestinal tract. The virulence factors belong to various functional groups among which adhesins, toxins, iron sequestration systems, and polysaccharide coatings. These virulence factors are necessary for bacteria to overcome innate host defences, invade host tissues, and to trigger a local inflammatory response [98].

Several studies using DNA microarrays have been conducted in *E. coli* isolates to detect antimicrobial resistance genes and virulence genes [99-102]. In studies performed in *E. coli* strains from different sources, CGH approach has shown to be a valuable tool for understanding the clonality of pathogenic *E. coli*, by defining the core genome, identifying regions of variation, and identifying antimicrobial and virulence-associated genes [34, 38, 103, 104].

cDNA microarray screening for the gene expression analysis in fluoroquinolone-resistant and sensitive *E. coli* were performed recently in clinical isolates from UTI patients. The upregulation of phage shock protein operons, *pspC* and deoxyribonucleic acid adenine methyltransferase were suggested to contribute to acquiring fluoroquinolone resistance [105].

8. *Enterococcus spp*

Enterococci are Gram-positive bacteria that typically colonise the gastrointestinal tract of humans and animals, and may also colonize the upper respiratory tract, biliary tracts and vagina of otherwise healthy persons [106, 107]. Most enterococci are not virulent and are considered relatively harmless, with little potential for human infection. However, they have also been identified as nosocomial opportunistic pathogens with increased resistance to antimicrobial approved agents causing infections, most commonly, urinary tract infection, but also cholecystitis, cholangitis, peritonitis, septicemia, endocarditis, meningitis, and surgical site infections in hospitalised patients [108, 109].

The genus Enterococcus includes more than 17 species, but only two species, *Enterococcus faecalis* and *Enterococcus faecium*, account for most clinical infections in humans. Other enterococcus species, *E. gallinarum*, *E. casseliflavus*, *E durans*, *E. avium*, *E. hirae*, *E. malodoratus*, *E. mundtii*, *E. raffinosus*, and *E. solitarius* are isolated much less frequently and represent less than 5% of clinical isolates. However, frequent misidentification by classical biochemical or microbiological methods suggests that their importance might be underestimated [110-113]. Accurate species identification of enterococci has become important, in particular because some species have been recognized as human pathogens following the wide prevalence of acquired antibiotic resistance [114].

Enterococci are intrinsically resistant to many antimicrobials and have the ability to acquire resistance to glycopeptides and aminoglycosides, among others, posing a challenge to therapeutic options [115]. Multidrug-resistant enterococci have been increasingly identified as the most important pathogens responsible for nosocomial infections in man [109]. The acquisition of vancomycin resistance by enterococci (VRE) has seriously affected the treatment and infection control of these organisms. VRE, particularly *E. faecium* strains, are frequently resistant to most antimicrobial agents that are effective in the treatment of enterococcal infections, which leaves clinicians with limited therapeutic options. VRE colonization and infection occurs predominantly in patients with severe underlying illness, extended length of hospital stay, and previous antibiotic exposure. The development of newer antimicrobial drugs, such as quinupristin-dalfopristin, linezolid, daptomycin, and tigecycline with activity against many VRE strains has improved this situation; however resistance to these agents has already been described [116].

Clonal spread is the dominant factor in the dissemination of multidrug-resistant enterococci in North America and Europe [117]. Virulence and pathogenicity factors have been described using molecular techniques. Several genes isolated from resistant enterococci encoding for the virulence factors such as aggregation substance, surface adhesins, sex pheromones, lipoteichoic acid, extracellular superoxide, gelatinase, hyaluronidase, and cytolysin (hemolysin). Each of them may be associated with various stages of an endodontic infection as well as with periapical inflammation [118].

Enterococci species show significant differences in the incidence of virulence factors. Generally, *E. faecalis* appears to harbour more virulence determinants than *E. faecium* strains which are generally free of virulence factors [119]. On the other hand, considering the distribution of the antibiotic resistance according to the species, the *E. faecium* shows a higher level of resistance than *E. faecalis* [120, 121].

Due to the versatile nature of enterococci as a commensal and as a pathogen and its impact on human health, it is of great interest to study the mechanisms that are related to the pathogenic condition. Considerable information can be obtained by studies of the genetic diversity of the species. The use of microarrays can allow the analysis of genomic diversity in detail, obtaining indications regarding the evolution of the strains within a species.

CGH Microarrays compared *E. faecalis* from different sources against the hospital V583 strain showed considerable diversity in gene content. The percentage of divergent genes in these test strains varied from 15% to 23%, and the main variation was found in regions corresponding to

exogenously acquired or mobile DNA in V583. Virulence factors, antibiotic resistance genes, and integrated plasmid genes dominated among the divergent genes [36].

Oxidative stress works as an important host/ environmental signal that generate a wide range of responses in bacteria. A recent transcriptome study in *E. faecium*, using DNA microarray showed that the AsrR regulon (antibiotic and stress response regulator) was composed by 181 genes, including diverse groups involved in pathogenesis, antibiotic and antimicrobial peptide resistance [122]. DNA microarrays also provides an opportunity to combine the principles of transposon mutagenesis and microarray-based screening technology to identify potentially important bacterial virulence or resistance genes. A microarray-based transposon mapping was developed by [123] to identify *E. faecium* genes that contribute to ampicillin resistance. In this study, several novel mechanisms that contribute to ampicillin resistance in *E. faecium* were identified.

Rapid organism identification via molecular diagnostic assays can help to decrease the time to appropriate antimicrobial therapy. The Verigene Gram-Positive Blood Culture (BC-GP) Test utilizes microarray technology to detect specific bacterial DNA from positive patient blood cultures. This approach allows identifying genus, species, and genetic resistance determinants for a broad panel of Gram-positive bacteria directly from positive blood culture bottles. This microarray technology seems to be useful in optimizing antimicrobial therapy in bloodstream infections caused by *Enterococcus* species [124].

9. *Klebsiella spp*

Klebsiella species are important pathogens, responsible for causing a spectrum of nosocomial-acquired infections, particularly in intensive care units. *Klebsiella* spp, as opportunistic pathogens mostly cause infections in immunocompromised patients who are hospitalized and suffer from severe diseases such as diabetes mellitus or chronic pulmonary obstruction. Nosocomial *Klebsiella* infections are caused mainly by *Klebsiella pneumoniae*, clinically the most important species of the genus. *Klebsiella oxytoca* also has been isolated from clinical specimens of patients, however to a much lesser extent [125]. It has been estimated that *Klebsiella* spp is responsible for 3 to 8% of all nosocomial bacterial infections, with urinary tract infection, pneumonia, and primary bacteremia being the most common manifestations. Strains of *K. pneumoniae* are the second most common cause of Gram-negative bacteremia [126]. *Klebsiella* spp can survive and multiply in nutritionally poor, humid environments at room temperature. Subsequently, *Klebsiella* species may contaminate food, enteral feedings, and infusion fluids, leading to common-source outbreaks [127].

Klebsiella species show extensive antimicrobial agents resistance profiles, including third generation cephalosporins, aminoglycosides and quinolones. This is especially true for ESBL-producing *Klebsiella* spp. The emergence of *K. pneumoniae* producing ESBL has been reported as an important cause of nosocomial infection in the Europe and United States. The prevalence of ESBL-producing *K. pneumoniae* strains in hospital environment ranges from 5 to 25% in several places of the world [128]. Since ESBL production frequently is accompanied by multi-resistance to antimicrobial agents, therapeutic options become limited.

Carbapenems such as imipenem or meropenem possess the most consistent activity against ESBL-producing *Klebsiella* strains. Both antibiotics are considered the agents of choice in the treatment of infections due to ESBL-producing organisms [129]. However, *Klebsiella*-producing carbapenemases have rapidly emerged and disseminated worldwide. The carbapenemases hydrolyze all β-lactam antibiotics, including carbapenems, and their high potential for rapid, wide dissemination constitutes a major clinical and public health threat [129, 130].

Although several virulence factors are described as being involved in the infective potency of the community-acquired strains, data concerning for virulence determinants expressed by nosocomial strains of *Klebsiella* species are scarce. However, most clinical isolates possess a well-defined polysaccharide capsule that appears to be a critical virulence factor [131]. The capsular material forms thick bundles of fibrillous structures covering the bacterial surface in massive layers, preventing the bacterium from phagocytosis by polymorphonuclear leukocytes [132].

Significant genomic diversity, using genomic shotgun array was shown among *K. pneumonia* pathogenic isolates obtained from nosocomial infections and community-acquired in Taiwan hospitals. This genomic microarray was conducted using probes from NTUH-K2044 genome, a strain isolate from a patient with liver abscess and meningitis. Hierarchical cluster analysis of these clinical isolates showed three major groups of genomic insertion-deletion patterns that correlate with the strains clinical features, antimicrobial susceptibilities, and virulence phenotypes with mice [133]. Moreover, [134] examined the genetic diversity among *K. pneumoniae* clinical isolates, using a genomic microarray containing probe sequences from multiple *K. pneumoniae* strains. That study has shown that a genomic region containing the citrate fermentation genes was not universally present in all strains. This region contains genes that contribute to the adaptation of bacteria to different nutritional conditions.

The DNA microarray is also commonly used in transcriptome analysis. A study to evaluate differential gene expression was performed amongst susceptible *K. pneumoniae* isolate and a resistant clinical derivative. A different pattern of gene expression profile was observed in resistant isolate when compared with the susceptible isolate [135]. Also, microarray analyses were performed to determine the RarA regulon

RarA is a newly identified AraC-type regulator that is associated with the multidrug resistance phenotype of

various unrelated classes of antibiotics (chloramphenicol, ciprofloxacin, norfloxacin, olaquindox, tetracycline, and tigecycline). Transcriptome analysis, using bespoke microarray slides demonstrated the role of RarA in the MDR phenotype of *K. pneumoniae* [136].

10. *Pseudomonas aeruginosa*

Although much of the medical and scientific attention has been focused on Gram-positive multidrug-resistant bacteria, such as MRSA and VRE, resistance within Gram-negative bacilli continues to increase, creating situations in which few or no antibiotics that retain activity are available.

P. aeruginosa is a Gram-negative bacterial opportunistic pathogen that is able to cause a wide range of invasive diseases and nosocomial outbreaks. It is a common pathogen in hospitals and particularly in intensive care units, affecting mainly critically ill and immunocompromised patients [137, 138]. The infections caused by *P. aeruginosa* have been considered to be polyclonal endemic infections that follow secondary endogenous intestinal and primary respiratory tract colonization in patients who have previously received antimicrobial drug therapy [139]. This bacterium is the most common Gram-negative organism associated with nosocomial pneumonia [140], and has the potential to become extremely harmful especially for cystic fibrosis patients, who are easily affected by chronic lung infections.

Patients infected by this species are more likely to develop multiple organ failure and to die than patients with other types of pneumonia. Nevertheless, outbreaks caused by some particularly multi-resistance strains have also been reported. Usually these outbreaks are normally circumscribed in time and space and are assigned to a point source of infection which can be identified in the environment [141, 142].

P. aeruginosa has become increasingly resistant to various antimicrobial agents and frequent multi-drug resistance are associated with nosocomial strains [143]. Previous reports showed that infections in patients by *P. aeruginosa* were related to empirically treatment with inappropriate antimicrobial agents and thus to a significantly higher mortality rate [144, 145]. Broad-spectrum β-lactams such as carbapenems, are potential antimicrobial agents for the therapy of infections caused by *P. aeruginosa*. However, the emergence of carbapenem-resistant *P. aeruginosa* isolates has increased due to the intensified use of these compounds, limiting treatment options [146]. Fluoroquinolones also show potency against *P. aeruginosa* responsible for hospital-acquired infections. Ciprofloxacin, due to its potent activity against *P. aeruginosa* is most frequently quinolone for treatment of infections cause by this bacterium. Levofloxacin, a respiratory quinolone has also been widely used in recent years. However, the use of Levofloxacin was associated with an increased incidence of fluoroquinolone resistant *P. aeruginosa*, while the use of ciprofloxacin is not described as relating with this association [147].

In an infection cause by an individual *P. aeruginosa*, the virulence factors have a primary importance to establish and maintain the infection, and the expression of a particular virulence determinate depends of the infection type. *P. aeruginosa* is capable to encode an impressive range of virulence determinants responsible for pathogenesis, and are described as belonging to adhesins and other secreted toxins [148].

For epidemiological purposes, discriminating *P. aeruginosa* isolates is essential to define distribution of clones among hospital environments, and to correlate clones to their source. A genotyping study, using species-specific oligonucleotide-microarray with clinical *P. aeruginosa* strains isolated in Italian hospitals have shown that microarray typing provides a genotype definition which is particularly suitable for epidemiological studies [149].

P. aeruginosa is highly resistant to antibiotic treatment, largely due to its ability to form biofilms. Bacterial communication via quorum sensing (QS) has been reported to be essential for the creation of mature and differentiated biofilms in this organism. High-density oligonucleotide microarrays used to analyse global gene expression patterns, modulated by QS regulons in *P. aeruginosa*, have shown that several genes, including genes involved in resistance and virulence were upregulated by QS [150]. In addition, data from cDNA microarray showed that expression of 382 genes was significantly different in *P. aeruginosa* treated with C2, a novel QS inhibitor. Forty-four of these genes are involved in transcriptional regulation, including a significantly upregulation of the *qscR*, which encodes the LuxR-type receptor QscR (quorum sensing control repressor) in *P. aeruginosa* [151].

ParA and ParB proteins in *P. aeruginosa* are important for optimal growth, nucleoid segregation, cell division and motility. In a recent study, microarray analysis showed that ParA and ParB besides their role in accurate chromosome segregation may act as modulators of genes expression, including antibiotic resistance and susceptibility factors and genes involved in virulence [152].

11. *Acinetobacter baumannii*

Although the organisms previously described have been subject of much attention as causing nosocomial infections, some other bacteria, such as *A. baumannii* has, in recent years, emerged as one of the most troublesome pathogens related with hospital-acquired infections. This Gram-negative non-fermentative coccobacillus can utilize a variety of both carbon and energy sources and is able to grow in a range of temperatures and pH conditions [153]. These properties may explain the ability of *A. baumannii* strains to persist in either wet or dry conditions in a hospital environment, thereby contributing to transmission. This hardiness, associated with multidrug-resistance, contributes to the *A. baumannii* has been increasingly reported, in the 15 years as a prevalent cause of nosocomial infections in intensive care units [154, 155].

A. baumannii it has intrinsic resistance to certain antimicrobial agents and has acquired resistance to many others including carbapenems which are drugs of choice in the treatment of severe infections, leaving few therapeutic options [156]. As a consequence of this, treatment of infections attributed to *A. baumannii* is challenging, and has been shown to increase mortality and length of hospital stay. The increase in carbapenem resistance among *A. baumannii* is mediated by two groups of β-lactamases, carbapenem-hydrolysing class D beta-lactamases and class B metallo-beta -lactamases [157]. However, the most widespread β-lactamases with carbapenemase activity are carbapenem-hydrolysing oxacillinases belonging to molecular class D. These enzymes belong to three unrelated groups of clavulanic acid-resistant beta-lactamases, represented by OXA-23, OXA-24 and OXA-58, that can be either plasmid or chromosomally encoded [158].

The bacteria commonly target the most vulnerable hospitalized patients; those who are critically ill with breaches in skin integrity and airway protection. *A. baumannii* has been implicated in a wide range of severe nosocomial infections including pneumonia, bacteraemia, meningitis, urinary tract and wound infections. Nosocomial pneumonia is the most common infection caused by *A. baumannii*, and nosocomial post-neurosurgical meningitis is much less common [159]. As multidrug-resistant *A. baumannii* infection usually occurs in severely ill patients in the intensive care unit, the associated crude mortality rate is high [160].

Despite a reputation for relatively low virulence, multidrug -resistant *A. baumannii* infection represents a very high threat to patients. The cause of many outbreaks by this organism is becoming endemic in hospital environments.

Comparative genomics of multidrug resistance *A. baumannii* analysis showed that this bacterium is a diverse and genomically variable pathogen. In addition, these studies also demonstrate the usefulness of comparative genome sequencing for analysis of putative resistance mechanisms in *A. baumannii* [161, 162].

DNA microarray is a useful tool for performing gene expression studies in bacteria. Coyne and colleagues (2010), developed an oligonucleotide-based DNA microarray to evaluate expression of genes for efflux pumps in *A. baumannii* and to detect acquired antibiotic resistance determinants. In this study has also been described a new efflux pump involved in the antibiotic resistance of *A. baumannii* [163]. More recently, a DNA microarray was designed to detect 91 target sequences associated with antibiotic resistance [164].

12. Conclusion

The issue of nosocomial infections caused by antimicrobial resistant bacteria should not be underestimated. Research efforts to clarify the mechanisms of antimicrobial resistance and microbial pathogenicity have provided valuable information. However, further development in this domain is required in order to obtain a comprehensive understanding and thus allowing the development of new alternative treatments and predict more accurately the evolution of resistance.

Microarray technology is a powerful tool that can be designed for analysis of comparative genomics hybridization (presence or absence of a gene) or for transcriptomics studies (level of gene expression). Although bacterial strains may appear identical based on current typing methods such as serotyping or even multilocus sequence typing (MLST), they may present potentially important genetic and phenotypic differences. Microarray CGH based on bacterial genome-sequenced strains has shown to have a high potential to determine the overall genetic similarity between strains, and therefore be essential in providing higher-resolution typing. Moreover, the availability of gene expression profiles for different bacterial pathogens, obtained by microarray technology has proved to be an essential resource in the study of bacterial infections.

Additionally, this technology as demonstrated to be useful for performing functional genomic analysis in nosocomial bacterial infections in order to gain a global view of the multifactorial phenomenon of antimicrobial resistance and virulence and to identify novel genes involved in pathogenesis and resistance. Much of the research using DNA microarrays aimed at identifying novel therapeutic targets for the treatment of bacterial infections and has been particularly directed at new drug development and identification of specific virulence factors and regulatory pathways that are relevant to the disease process.

Diagnostic DNA microarrays have also been developed, including for nosocomial pathogenic bacteria detection. Although, from a clinical perspective, the utility of DNA microarrays either for an isolate typing or for transcriptomic analysis is uncertain, genomics studies can provide insights that may impact in clinical decisions. Moreover, whole genome analysis tools can be necessary as a diagnostic tool, because it provides more detailed information than other typing methods, and it offers additional data about the mechanisms responsible for antimicrobial resistance phenotype or the genetic machinery necessary for bacterial pathogenesis.

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