A prospect of current microbial diagnosis methods

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An accurate identification and characterization of pathogens is crucial in disease management. The appropriateness and effectiveness of the microbial diagnosis method influence the choice of the antimicrobial agent to be used in the treatment of infection. Traditionally, bacterial diagnosis is based on conventional and culturing-dependent approaches, such as culture and counting methods, generally coupled to morphological and physiological characterization. Currently, rapid technological advances in bacterial identification methods are occurring providing a bewildering wide range of techniques to detect, identify and differentiate bacteria. Molecular methods, such as ELISA and PCR, had introduced great improvements in bacterial identification as they contributed to speed up the analysis and the reduction of handling. However, it has been demonstrated that heterogeneous microbial communities are the main cause of several human infections. This genetic and phenotypic heterogeneity is crucial to microorganisms achieving adaptation to human host, and it might reflect distinct pathogenicity potential. The aforementioned molecular methods and new emergent methods, such as MALDI-TOF MS, have still limitations in full identification and differentiation of microbial heterogeneity. Therefore, a new generation of diagnosis methods able to detect and characterize microbial heterogeneity should be developed. Microbial infections are like dynamic systems and it is essential that diagnosis methods and technologies rapidly evolve to detect and measure changes occurring at individual and population level. This new kind of methods will allow a relevant shift about infection development understanding, as well about microbial mechanisms of resistance to antibiotics and human defences and persistence ability in human host that culminate in better medical decisions about antimicrobial therapy.

Keywords microbial diagnosis, diagnostic technologies, pathogen identification, microbial heterogeneity

1. Introduction

Microbial infections are a common cause or a recurrent complication of a panoply of diseases causing millions of deaths and demanding increased medical and social resources. A timely diagnosis is the most effective approach to prevent or control microbial infections or diseases. To perform a successful diagnosis and, consequently, to achieve an effective therapy, it is required an accurate and fast identification and characterization of the infecting pathogens, such as bacteria, fungi, viruses and parasites.

Apart from the food industry, the clinical diagnosis is the most interested field in microbial detection and identification since the implications of failing or delay in pathogen identification might be fatal to patients [1]. Therefore, methods for detection, identification and characterization are crucial for suitable antimicrobial therapy design. According pathogen identification and its biological traits, it is chosen the best antimicrobial approach to control or eradicate the microbial infection or disease. In addition, accurate identification is not only an issue of effective antimicrobial therapy but it also accounts for understanding the infection or disease development. Rapid pathogen detection, identification and characterization are, thus, the gold standard for diagnosis.

Over the years automated methods for microbial identification and characterization were continuously demanded attempting to respond to the clinical needs of fast and reliable diagnosis, as in cases of septicaemia or neonatal infections [2]. Automated tests have allowed increasing the number of clinical analysis and the accurate detection of several distinct microbial species at the same time. This more fast and accurate microbial profiling has significantly reduced patient mortality and morbidity rates.

In the context of microbial diagnosis, bacterial identification and characterization represents the largest interest of routine diagnosis, therefore this paper will focus essentially on bacterial diagnosis-identification methods. This paper will review the tools, technologies and methods currently available and applied in clinical field to perform bacterial diagnosis, and discuss technical procedures, advantages and drawbacks.

2. Microbial heterogeneity

Over the years, Koch's postulate, which states that a disease is caused by a single causative agent, was on the basis of microbial diagnosis-identification methods development [3]. However, a growing body of evidences has demonstrated that several human infections or diseases are caused by complex and mixed microbial communities [4]. Besides this polymicrobial nature of infections, the complex microbial communities might encompass a wide phenotypic heterogeneity fundamental to microorganism survival and adaptation to human host. For instance, oxygen-limited or anaerobic environments in cystic fibrosis (CF) lungs force *Pseudomonas aeruginosa* to trigger mechanisms of adaptation. Bacteria in CF lungs undergo genetic, morphological and physiological changes to survive for long periods

of time under the challenging selective pressure imposed by the typical CF growth conditions, the immune system action and antibiotic treatments [5]. Clonal diversification of *P. aeruginosa* after infection establishment differs remarkably from the early stages of infection in CF lungs, being *P. aeruginosa* conversion from non-mucoid to mucoid phenotype the hallmark of airway CF [6]. Mucoid colony morphology results from alginate overproduction, absence of flagellin and pilin and expression of other virulence factors. Within mucoid form, *P. aeruginosa* is more difficult to eradicate because their augmented resistant to antibiotics, as well as to action of host immune defences [6-8]. This resistance and protection is mainly due to alginate production that for this reason is considered a significant virulence factor [9, 10].

Bacteria use the generation of microbial heterogeneity in a population as alternative to gene regulation mechanism because the predetermined sensor-effect regulatory circuits, used to respond to external stimuli, are insufficient to face unpredictable stressors. Microbial heterogeneity might be caused from genetic changes arise from random, semirandom or programmed events, including deletion, duplication and acquisition of genetic material [11], increased the rates of spontaneous mutations [12, 13], random transcription, phage-related events, chromosomal duplications and gene amplification [14]. Moreover, microorganisms might create phenotypic variants through phenotypic switching that consists in reversible conversion of phenotypic states according environmental changes. This mechanism, recently reviewed by Sousa et. al. [13], provides a dynamic source of heterogeneity without the fitness costs of irreversible mutations. Stress-inducible mechanisms as phenotypic switching can greatly accelerate the adaptive evolution of microorganisms, which is of serious concern in the scope of microbial diagnosis and therapy design. In contrast to DNA replication or transcription, a general stress-inducible mechanism was not noticed until now; only similarities and differences among a series of this kind of mechanisms are been reported. Therefore, there are not identified specific molecules that can be target for antimicrobials action in order to block these stress-inducible mechanisms. So far, there are just some probable active components since the mechanisms of phenotypic switching activation are highly dependent of environment stresses.

All the above mentioned findings have challenged the suitability of the actual microbial diagnosis. Microbial heterogeneity, namely intra-strain variability, has great impact in pathogen diagnosis as it might disturb the medical decision about the antimicrobial agent and respective dosage to administrate to patient. The ability of the method in providing accurate information about microorganism is crucial to design effective treatment strategies. The current issue on microbial diagnosis-identification is the ability of the methods generating not just taxonomic results, but also characteristic features about the microorganisms isolated from patients. A conceptual advance in the scope of microbial diagnosis is required considering the actual knowledge about microbial adaptation, evolution and infection development.

In the following sections, it will be reviewed the current technologies regarding microbial identification and characterization and the methods available and applied in clinical field. Insights into their technical procedures, advantages and drawbacks will be also provided. Special focus will be given to the ability of those methods to detect microbial heterogeneity.

3. Microbial diagnosis methods

Classically, microbial identification is based on microscopy of specimens and culturing on solid media [15]. Over the last decades, rapid advances in microbial identification methods have occurred providing a bewildering wide range of techniques to detect, identify and differentiate microorganisms.

An ideal diagnostic method must be sensitive, specific, rapid, easy to perform (not labour intensive), and the resulting data must also be easily to interpret, widely available, cost effective and high-throughput. Additionally, it should be taken into account the incidence of the microbial infection, how contagious is the pathogenic agent and the health consequences for the patient and community. Obviously, there is not an optimal microbial diagnosis method. The selection of an adequate identification method must be supported by criteria such as sensitivity and specificity of analysis and time duration [16]. The choice of a microbial diagnosis method or technology should as well consider its ability to explore cellular differences beyond identification of conserved domains or characteristics shared by species. In fact, microbial diagnosis must include biological key factors such as susceptibility profiles and virulence factors expression. These biological characteristics are not only important for antimicrobial therapy design but also for monitoring and surveillance of routes of hospital and community infections [17].

Researchers are continuously searching for new tools that are fast, accurate, and ultrasensitive. It has been witnessed an increase research activity in the development of techniques for detecting and identifying pathogenic microorganisms [18]. The fast pace at which identification methods are being developed prompts the overview of the most predominant methodologies applied in clinical diagnosis of pathogens and their scientific principles emphasising bacterial identification techniques.

3.1. Conventional or culture-based methods

Conventional or culture-based diagnosis methods are based on the ability to growth microorganisms *in vitro*, i.e., in artificial conditions. Culture and colony counting, Gram staining and morphological characterization are some examples of these methods and all of these rely on specific and biochemical identification [19, 20]. Generally, clinical samples are plated on a solid selective enrichment medium, or differential agar plates, to detect or isolate specific microbial species. To simply enumerate and identify the microbial population present in the sample, a general solid medium must be used. These methods are very sensitive, easily adaptable, quite inexpensive and they can return both qualitative and quantitative data about the bacterial population present in the clinical samples [20-22]. However, typical clinical samples are mixed cultures of microorganisms that can include several pathogenic species but also normal flora. In most cases, isolation procedures should be performed in order to obtain pure cultures. Therefore, the culture-based methods become labour-intensive and time-consuming because they have to include besides the usual culture medium preparation and inoculation of agar plates steps, a broad range of bacterial growth steps for species and/or strain isolation. Results are only obtained within 1–3 days and up to 7–10 days for confirmation [2, 19]. After the microbial isolation steps, pathogen characterization through phenotypic and susceptibility tests have to be performed [2, 22].

In the past, microbiologists and clinicians were trained to consider microorganisms as culturable. Therefore, the absent of microbial growth would meant absence of microorganisms in samples. However, infections may encompass within microbial population senescent or dormant cells that are viable but not able to growth *in vitro* conditions.

The detection of unculturable cells is particular relevant in biofilm-associated infections. Biofilms are microbial communities encased in self-produced matrix composed of exopolysaccharides, proteins and DNA. These sessile microbial forms provide an impressive source of genetic and phenotypic diversity [23, 24]. Moreover, biofilm populations might encompass increased number of dormant cells or persisters [25-27]. Persister cells are antibiotic-tolerant, not resistant cells, due to their non-growing state. They represent a small fraction (less than 1 %) of the population, being its detection very difficult and rare. As the majority of the antibiotics is only effective against growing cells, persisters are often out of the antibiotics action and thus a major obstacle of clinical diagnosis and infections treatment. After an antimicrobial action either of antibiotics or immune defences, persisters are able to restore the original population with similar susceptibility [28, 29].

In some cases, culture-based methods have additional limitations resulting from the great difficulties in growing specific pathogens in artificial media due to, for instance, particular growth and/or storage and transportation conditions of the clinical samples.

Concerning staining methods, Gram staining is the most applied as it can provides results within minutes. However, complete microbial identification usually takes 1 or more days because staining methods may be difficult to interpret or be inconclusive, requiring often specialized staff [30].

Despite the technological advances, culture-based methods are fundamental to clinical diagnosis and its usage still remains in clinical practice because, conversely to other methods, they can detect acute infections in early stages with selectivity and sensitivity [1, 17, 19, 22].

As conventional methods require several days to give results, as they depend on bacteria ablity to multiply and form visible colonies, the accuracy and reliability of these methods are strongly dependent on skilled taxonomical expertise [16]. Therefore, any modification on these methods reducing the time of analysis is welcome.

Currently, there are available some automated identification systems, such as the blood culture systems BacTec (Becton Dickinson, Franklin Lakes, NJ, USA), BacT/Alert (bioMérieux, Marcy l'Etoile, France) and the systems VITEK (bioMérieux), PHOENIX (Becton Dickinson), or Microscan WalkAway (Siemens Healthcare Diagnostics, Munich, Germany), that give reliable results in shorter periods of time and allow, in addition, the antimicrobial susceptibility testing. However, the analysis must be performed from pure cultures [17].

3.1.1. Colony morphology characterization

Identification of a microorganism may follow two analyses: 1) phenotypic characterization, i.e. the study of the observable traits resulted from gene expression, or 2) genotypic characterization, i.e. the study of the traits encoded within its genome [31]. Colony morphology relies on the characterization of the phenotypic traits exhibited not by an individual microorganism but by a group of microorganisms-colonies. This technique consists in plating clinical samples on solid growth medium and observed to the naked eye the colonies formed by those bacteria. Analysis of clinical pure isolated cultures from agar plates is routinely performed with simple protocols [22].

Despite the fact that several authors described it as old-fashioned [2, 17], colony morphology characterization is commonly used to complement conventional microbial identification (e.g. staining and biochemical tests) [32-34]. The simple procedure, no specific technical skills to perform and analyse colony morphology formed by bacteria, as well as its low cost, are definitely its main advantages over the actual advanced techniques. Its major drawback is the long interval of time to obtain results that take normally 1-3 days [33].

With exception of colony morphology characterization, microbial heterogeneity is usually masked in some conventional methods, such as biochemical tests, because they rely on an average of millions of cells of a clinical

sample, Through colony morphology characterization, individual microbial diversity, both derived from genetic changes or reversible changes as those occurred in phenotypic switching, can be indirectly evaluated [35].

Colony morphology characterization has been used for different purposes as an indicator of biological changes in bacteria caused by mutations, plasmid insertions, environmental effects, antimicrobial agents action and virulence potential. Applied to clinical diagnosis, colony morphology characterization has undoubtedly advanced the knowledge about microbial diversity, adaptation and evolution. For instance, as abovementioned *P. aeruginosa* properties change according to CF lung disease development. The clonal diversification of *P. aeruginosa* in CF lungs was evident when plating bacteria on agar plates and observed their different colony appearance among non- and mucoid phenotypes. Another interesting colony variant frequently exhibited by *Staphylococcus aureus* isolated from chronic bacterial infections, such as osteomyelitis and device-related infections, is the small colony variant (SCV). Its designation comes from their small-colony size, typically 10 times smaller than the usual *S. aureus* colonies, after 24-48 h of growth on agar media [36, 37]. SCV are normally hyperpiliated, hyperadherent, auxotrophic, excellent biofilm formers and exhibit autoaggregative behaviour [36]. In addition, SCV also show augment resistance to several classes of antibiotics [38, 39], reason by that they persist more efficiently in the host. SCV have been observed for other bacterial species that not only *S. aureus* [40-43].

Altered colony appearance was demonstrated for several other bacteria species such as *Burkholderia pseudomallei* [44], *Streptococcus pneumonia* [42], *Enterococcus faecalis* [45] and *Haemophilus influenza* [46]. Any modification in colony morphology, such as colour, opacity, size and texture, may be a sign of altered expression of one or more bacterial traits. The correlation between bacterial features, such as antimicrobial resistance, virulence factors expression and persistence ability, and colony morphologies is just about unknown, however extremely important. The current knowledge about these relationships is scarce due to the existence of several barriers of the colony morphology characterization, in addition to those inherent of being a culturing dependent method. In fact, the lack of standard experimental guidelines, universal morphological criteria, unequivocal concepts and terms to perform colony morphology evaluations are the main handicaps of this method. Colony morphology descriptions have been performed without consensus of experimental parameters, such as time of colony growth, solid media usage and colony density *per* plate. Moreover, the colony descriptions have been performed according authors assumptions using their own criteria, vocabulary and concepts about morphological traits. Therefore, the knowledge gained about bacterial diversity is somewhat inconsistent and unstructured.

The methods of bacterial isolation and identification remain almost unchanged since their first utilization. Currently, it is demanded high-throughput analysis and automated systems that can return rapid and reliable results. The authors consider colony morphology characterization with great potential in clinical diagnosis and crucial improvements must be performed. Therefore, some efforts have been performed to become colony morphology characterization an advanced method [47, 48]. REF JMM

3.2. Molecular methods

Molecular methods, such as immunological and nucleic acid-based techniques, seriously improved microbial diagnosis. Compared to traditional culture-based methods, molecular techniques have some advantage because they use more stable genotypic characteristics than reversible phenotypic characteristics. Molecular methods are more adequate for routine analysis since they are generally faster, more specific, sensitive and accurate. Moreover, these methods can be performed and results interpreted by staff with no taxonomical expertise [17, 19]. Results can be obtained in a expedite way because molecular methods are based on the detection of molecules that can be extracted directly from bacteria without the need of culturing This means that the microbial community can be elucidated through its molecular composition [3, 49].

3.2.1. Immunological Techniques

Almost 30 years ago, immunological (or serological or antibody-based) techniques arose originally to detect viruses. The development of immunoassays introduced great improvements in microbial diagnosis because they were the first technique able to perform rapid identification and characterization without culturing steps [17].

Immunological techniques are based on the binding between diagnostic antibodies and specific antigenic determinants of the target bacteria. The enzyme-linked immunosorbent assay (ELISA) is the most common used technique. Despite the different types of ELISA actually available, such as immunofluorescence assays, latex agglutination assays, line immunoassays or lateral-flow immunoassays, all involve an enzyme-mediated colour change reaction to detect and often also to quantify antibody binding as a measure of pathogen presence [1, 18, 21]. ELISA techniques have improved the clinical diagnosis due to their high-throughput capacity, speed, relatively low-cost and simple nature, as well as because the possibility to quantify the target pathogen [17, 19].

Another immunological approach is the serological assays. Since the human body produces specific antibodies in response to microbial infection, it is possible to use those antibodies to detect an acute or past infection. Antigen microarrays introduced great improvements in diagnosis enabling testing several pathogens at the same time. However,

serological assays are usually ineffective due to its long duration before seroconversion. Some automated advances were already performed to short the assay duration [17, 50].

Similar to all techniques, immunology-based methods have also some limitations. Lack of selectivity and sensitivity are their major limitations due to the difficulty to generate selective antibodies and the requirement of large amounts of the respective antigen to quantify bacteria. The development of antibodies with the required degree of specificity is difficulty for complex organisms as bacteria. Polyclonal antibodies were introduced to overcome this limitation and they are used successfully in bacteria detection because of the recognition of multiple epitopes. However, not always they exhibit the sufficient specificity to detect bacteria. Such limitation can be overcome through monoclonal or recombinant antibodies, but often epitopes are ubiquitously shared within bacterial species. Therefore, antibody-based assays are relatively unsophisticated for the detection of bacteria [16-18].

3.2.2. Nucleic acid-based techniques

The use of nucleic acid-based techniques for the detection and identification of bacteria was initiated when polymerase chain reaction (PCR) was developed in the early 1980s. Through the advent of PCR, it was possible to amplify DNA sequences and make their presence into a detectable signal. The "boom" of information availability started just with the access to the pathogen gene sequence. As result, public databases like GenBank (http://www.ncbi.nlm.nih.gov/Genbank/) started growing [50].

Nucleic acid-based techniques encompass DNA- and RNA-based technologies. DNA-based technologies include PCR, and its variations, and DNA microarrays. RNA-based technologies include reverse transcriptase PCR (RT-PCR) and transcription mediated amplification (TMA) [49].

The main characteristic of all nucleic acid-based techniques is the selection of genetic sequences through which it is possible to identify pathogens. Generally, the pathogen identification is performed, choosing ubiquitously conserved genes, called housekeeping genes, or through screening of random parts of the its genome [16]. Targeting genes encoding resistance and/or virulence factors may be useful to obtain information about microbial pathogenicity [17].

PCR techniques have the ability to produce millions of copies of a specific portion of DNA sequence, that may be a gene or gene clusters, with high fidelity in 3 to 4 h. Through the use of nucleotide sequences from 16S ribosomal RNA genes, it is now possible to identify or, at least, to infer about the bacterial species [3, 21, 22, 51]. For the detection of bacteria, PCR uses primers that are 20-30 nucleotides sequences homologous to the ends of genomic DNA region to be amplified. The conventional PCR procedure consists in a thermocyclic process of repetitive cycles of DNA denaturation, primer annealing and extension through a termostable DNA polymerase. The detection of the amplified sequence is performed through agarose gel electrophoresis using fragment length as indicator for identification [1, 17]. If particular pathogen identification is intended, a unique DNA sequence is determined and specific primers are designed enabling the determination of pathogen presence or absence. PCR-based methods are very sensitive being the results obtained within few hours, 5 to 24 h, with increased identification accuracy [1, 3, 21, 49].

Despite PCR had provided great improvements in microbial diagnosis, the use of species-specific sequences to detect and identify pathogens requires the prediction of the species presence in the clinical samples. This limitation is similar to that of culture-based methods and equivalent to the need of selective culture media to determine species identification. In addition, there is a limited number of PCR assays that can be perform per clinical sample and infections or diseases are not monocultures being frequently caused by species-species interactions or even strain-strain interactions [3]. In attempt to overcome these limitations, a multiparameter approach, the multiplex PCR that allows multispecies analysis, was developed. However, similar to other PCR approaches, multiplex PCR need some prediction of the bacterial species present in the clinical samples [1, 3, 17].

Measurements based on DNA species-specific sequences do not allow detecting heterogeneity on bacterial population and determining the metabolic state of cells. Nucleic acid-based technologies equally detect metabolic active, senescent and dead cells because they just detect intact DNA. From the point of view of infection, the discrimination of the metabolic state of cells is fundamental [17]. As abovementioned, the presence of persisters cells is one of the main causes of chronic infections [25] as they are able to grow and restore the microbial population even after aggressive antimicrobial therapies. The diagnosis of persisters is crucial to design effective approaches to eradicate all microbial population and to avoid repeated exacerbations, antibiotic resistance episodes and, consequently, the establishment of chronic infections.

In addition, the presence of extracellular DNA might influence the DNA-based signals detected. In CF lungs, for instance, significant amounts of extracellular DNA released by bacterial cells destroyed by immune defences have been detected. Therefore, DNA-based signals obtained during diagnosis will be influenced by the presence of extracellular DNA that might lead to misidentification. This measurement artefact can be minimized adding propidium monoazide (PM) prior to nucleic acids extraction. PM does not enter in cells that have structural integrity, connecting just with extracellular DNA or with DNA of dead cells. PM intercalates into double-stranded nucleic acids with chemical cross-links formed with bright light exposure. Therefore, the DNA linked with PM is unable to be used as template for PCR reactions [52]. The PM application is crucial in airway sputum samples.

Taxonomic identification, as abovementioned, is not sufficient to design an efficient antimicrobial therapy. More biological details about bacteria must be provided including resistance profiles and virulence factor expression. PCR

approaches are able to provide such information, however PCR must be performed targeting resistance and virulence coding factors genes to determine biological profiles.

In summary, pathogen identification in clinical context remains a challenging task for all assays and technologies. Effective identification requires methods of analysis that have to meet a number of challenging criteria, being time to obtain results and sensitivity the most important (Table 2). Clinical diagnosis needs more rapid and sensitive methods able to provide the most complete results possible.

Table 1 Characteristics of conventional (culture-based), immunological and nucleic acid based-assays. Adapted from [16, 19].

Method	Assay duration (hours)	Detection limit (cfu/mL or g)	Time before result	Specificity
Plating technique	> 72	1	1-3 days	Good
Immunological technique	1-3	10^4	1-2 hours	Moderate/good
Nucleic acid-based technique	1-3	10^3	6-12 hours	Excellent

3.3. New emergent methods: MALDI-TOF MS

Few developments in microbial diagnosis have had so impact in identification of pathogens as matrix-assisted laser desorption/ionization time of flight mass spectrometry (MALDI-TOF MS) [31, 53]. Indeed, among the new emergent methods, MALDI-TOF MS is the most used to identify microorganisms and has already replaced conventional methods in some laboratories [2].

MALDI-TOF MS approaches are able to perform species identification and even strain differentiation based on molecular signatures. It is based on the ionization of cocrystallized sample material by short laser pulses. The ions are accelerated and their time of flight is measured in a vacuum flight tube [15]. MALDI-TOF MS has seriously improved clinical diagnosis not just of bacterial pathogens, as well as in detection of tumors, rheumatoid arthritis, Alzheimer's disease, and allergies through the identification of specific biochemical markers [54]. The successful implementation of this technique in the routine of laboratories is due to its fast, easy and high-throughput characteristics. Moreover, it generates simple and easily interpretable spectra.

To identify bacteria by MALDI-TOF MS, samples are dissolved in a matrix solution and placed on the steel surface of the target plate to dry. After matrix and sample cocrystallization on the target plate, the mixture is then transported to the measuring chamber [15]. Once a sufficient vacuum has been created, the individual samples are exposed to short laser pulses. Bacteria and matrix are vaporized by the laser energy, leading to the ionization of the analytes, typically (ribosomal) proteins. The ions are vaporized in an electromagnetic field, created by a potential of about 20 kV, before they enter the flight tube [15, 55]. The time of flight (TOF) of the proteins to reach the detector at the end of the flight tube is precisely measured. The degree of ionization and the mass of the proteins determine their individual TOF. Based on this TOF information, a characteristic spectrum is recorded constituting a specific sample fingerprint, which is unique for a given species. Increased m/z (mass and ion charge ratio) corresponds to lower speed and longer time need to reach the detector [55, 56].

It was demonstrated that MALDI-TOF MS and conventional identification methods are both highly accurate for the identification and differentiation of isolated bacteria and yeast [53, 57, 58]. Minor discrepancies between biochemical, molecular, and MALDI-TOF MS-based identification and differentiation results have been observed [59]. Differences are seen mainly because MALDI-TOF MS pathogen identification is based on the analysis of ribosomal protein spectra. Species that do not differ sufficiently in their ribosomal protein sequences, such as *E. coli* or *S. pneumonia*, are still hard to be differentiated by MALDI-TOF MS [32, 60].

MALDI-TOF MS has been used as new strategy for directly bacterial pathogen identification in clinical samples such as blood [61] and urine [62]. The fast and reliable results obtained coupled with simple procedure, without a cultural enrichment step, have made this method popular in clinical diagnosis context [2, 59, 63]. Furthermore, it is a high-throughput technique that returns increased amount of detailed data about microbial community composition, including non- and culturable bacteria at the same time [2, 3]. However, some clinical sample material is most often rich in host proteins and normal flora, which might occur overlapping of mass spectra [15]. So, in these cases, additional protocol steps are required to accurately perform MALDI-TOF MS analysis. This approach has been as well applied to differentiate genotypes detecting the presence of genetic elements coding the expression of antibiotic resistance and virulence factors. Several authors have reported the ability of MALDI-TOF MS to discriminate isolates in methicillin-resistant *Staphylococcus aureus* (MRSA) and methicillin-sensitive *Staphylococcus aureus* (MSSA) [64-66]. Despite the progressive advances demonstrated in microbial diagnosis field, the Food and Drug Administration, in the United States, does still not approved the function of MALDI-MS systems for pathogen identification [2].

Similar with epidemiological typing of bacterial isolates, i.e., differentiation of strains through specific mass peaks detection, typing of bacterial colony morphology variants using MALDI-TOF MS had received the interest from the authors. Authors intended to test other application of MALDI-TOF MS in microbial diagnosis field. Recent work revealed that MALDI-TOF MS exhibited distinct results comparing with colony morphology profiling [67].

MALDI-TOF MS, similar to other techniques, it has still limited sensitivity since it required 10^4-10^5 cells per assay that is rarely found in clinical samples, with exception of some urinary samples [22]. It has also as well limited ability to detect resistant bacteria due to the most of the genes encoding antibiotic resistance are mobile genetic elements acquired by horizontal gene transfer and are not necessarily linked to systemic taxonomic relationships [15, 31]. Unculturable microorganisms are also a limitation of MALDI-TOF MS. Because those microorganisms are typically less than 10% of the population, mass spectra obtained are prone to be lost in background noise [31]. Costs are also an important factor when considering integrating this technique into a laboratory. The initial investments and maintenance costs are extremely high in contrast to low overall operating costs. High throughput sample analysis and the low amount of consumables required, dependent of MALDI-TOF MS approach applied, makes this method competitive [2, 15]. Although just one instrument usually is needed to process all number of samples per day of a hospital or clinical laboratory, technical problems with it might stop samples analysis and might have great impact on hospital and clinical routine [2].

4. Future trends

In addition to the technical development that actual methodologies offer, a conceptual advance in microbial diagnosis reflecting the information available about microbial adaptation and evolution is essential. Methods or technologies able to detect and profile microbial communities certainly will have impact in microbial diagnosis and, consequently, in antimicrobial therapy. Through those methods, it will be possible to determine the population heterogeneity and target-specific antimicrobial therapies, avoiding by this way antibiotic resistance episodes and acute and repeated exacerbations of infections [14]. As previously mentioned, microbial heterogeneity is usually masked in conventional methods with exception of colony morphology characterization. Despite the great potential of colony morphology characterization, only few improvements have been noticed in this method. Therefore, the authors of this present work already performed some improvements in colony morphology characterization technique in order to standardize and become a semi-automated technique (data unpublished).

Other techniques and methods are also available, able to detect heterogeneity at single-cell level. In fact, some microscopy methods, such as light microscopy (bright or dark field, fluorescence and phase control) and electronic microscopy (SEM, TEM) have also potential to detect microbial heterogeneity, however they are less frequent used in clinical laboratories and more applied in research centres [2]. However, promising advances were already performed in this area.

Fluorescent in situ hybridization (FISH) have been revealed an attractive and promising tool for rapid and reliable detection and identification of bacteria. FISH assays combine microscopic and molecular methods being so rapid and ease-of-use, as typical in staining methods, and specific like molecular methods. Assays consist in direct hybridization using labelled oligonucleotides probes (15 to 25 nucleotides in length) covalently labelled at 5' end with fluorescent labels that are species-specific [17, 68, 69]. Further hybridization, stained cells are detected using epifluorescent or confocal laser microscopy. As a DNA-based method, the use of species-specific DNA sequences has the advantage to detect and identify unculturable and slow-growing bacteria that other methods cannot perform it. However, it is need isolation and pre-culture in liquid and solid media to produce a significant amount of target cells to obtain a detectable signal, which makes this method lacked of sensitivity. This limitation can be overcome targeting rRNA that exists in higher amount in bacterial cell and facilitates the description of the complex microbial communities associated to infections. In addition, there are a limit number of probes that can be used in one experiment and background fluorescence might be a problem in some clinical samples. Moreover, the use alone of FISH does not provide information about cellular metabolic state [17, 68, 69].

In recent years, the application of "omics" technologies, including genomics, transcriptomics, proteomics and metabolomics, for discriminate the microbial diversity of the infection community has definitely gave rise to improvements in the scope of microbial diagnosis [70]. They have allowed the determination of gene expression, as well as the respective timing, stimuli-dependent and molecular coding expression. However, the proprieties of a microbial community result from interactions between multi-phenotypes and/or interactions between differential biochemical niches, which can be metabolic, predatory, competitive or cooperative. The whole interactions that happen within the community define its interaction with the human host environment [71]. The current methods of cultivation, biochemical and molecular characterization allow to associate bacterial species and their activities but not understand their network. "Omic" technology alone is not truly useful to determine and characterize complex microbial communities.

The microbial diagnosis has already advanced to metagenomic where it is possible to catalogue the complex mixtures of genomic components of the bacteria species analyzing the microbial DNA of the community directly from natural samples. The early environmental gene sequencing of the clones allows obtaining a natural profile of the diversity in the samples. The advantage of this approach is the absence of clones cultivation and PCR steps, avoiding by this way missing the detection of unculturable microorganisms, as well as to reduce the potential of bias introduced by amplification [71, 72]. Metagenomics is truly important, for instance, to discriminate differences between the normal flora and unhealthy microbial composition in order to link specific microbial populations with environmental alterations

or processes. In addition, it also allows determining the effect of antimicrobial agents in the treatment of microbial infections and diseases. Such information is fundamental to implement in real-time effective and custom antimicrobial therapies to prevent or control microbial infections, as well as to assist microbial surveillance [22]. So far, metagenomic is not applied in clinical diagnosis routine.

5. Conclusions

The growth conditions in the human host impose to bacteria physiological changes that they undergo to adapt and survive. Current evidences have demonstrated that microorganisms are able to diversify expressing degrees of resistance and virulence at different times in order to persist in human body. Microbial adaptation is relevant to the infection or disease progression, thus it is need to be taken into account in microbial diagnosis. Over the last years, clinical laboratories have undergone important changes with the introduction of molecular and automated techniques. Today, the most applied diagnosis methods in clinical field are those based on culture and counting methods and PCR, mainly due to their selectivity and reliability. Although culture-based methods are more time-consuming and labour-intensive than PCR, both provide conclusive and unambiguous information. However, the limitations of the current microbial diagnosis methods and technologies and the introduction of novel systems to deal with the unpredictable microbial heterogeneity must be recognized. Instruments equipped with automated systems, such as databases search functions, for pathogens identification and mainly for pathogens profiling, including antimicrobial resistance and virulence factors expression, are urgently needed.

Despite this paper only had focused on bacterial infections and diseases, microbial diagnosis is even more complicated. Clinical samples are composed not just by multi bacterial species but also by fungal and viruses. Therefore, microbial diagnosis-identification methods should be adequate to this polymicrobial nature. These advances will benefit not just microbial diagnosis, but also the elucidation of the mechanisms underlying infection development and bacterial adaptation to human host. These inputs will help the discovery of new therapeutic approaches to control or eradicate microbial-associated infections.

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