Chapter 1

Diagnostics Methods in Ocular Infections–From Microorganism Culture to Molecular Methods

Victor Manuel Bautista-de Lucio,
Mariana Ortiz-Casas,
Luis Antonio Bautista-Hernández,
Nadia Luz López-Espinosa, Carolina Gaona-Juárez,
Ángel Gustavo Salas-Lais,
Dulce Aurora Frausto-del Río and
Herlinda Mejía-López

Additional information is available at the end of the chapter

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1. Introduction

1.1. Conventional methods of microbiological diagnosis: Culture, isolation and phenotypic identification

Despite advances in the medical field, 4 of the 10 leading causes of death worldwide are due to infectious diseases [1]. At the eye, infections are one of the most common diseases, and bacterias are the first causative agent, followed by fungus and virus. Between these bacterias, *Staphylococcus* genus, *Streptococcus genus, Corinebacterium sp, Chlamydia sp, Pseudomonas aeruginosa, Escherichia coli, Enterococcus sp, Serratia sp*are frequent in keratitis, conjunctivitis, endophthalmitis and cornea ulcer [2]; *Fusarium sp, Aspergillus sp* and *Candida sp,* are the commonly fungus found in keratitis infections [3]; *Adenovirus, HSV-1 (Herpes Simplex Virus-1), HSV-2 (Herpes Simplex Virus-2), VZV (Varicella Zoster Virus), HPV (Human Papilloma Virus)* are important in conjunctivitis and keratitis [4].

In order to reduce complications from ocular infectious diseases is very important to provide appropriate early treatment. To make this possible, is essential microbiological identification of the causative agent of infection in the shortest time possible. However, the

microbiological diagnosis by conventional methods considered as gold standards, based on the culture followed by phenotypic identification of the microorganism once isolated, taken between 48 and 72 hours, depending on the requirements of the microorganism, and in the case of fungal infections, identifying and obtaining the antifungal susceptibility profile, come to take over a week. Identification time may be reduced by using automated equipment whose bases are the same as those used for manual identification, through biochemical profile of microorganisms. These tests are based on the ability to ferment, oxidize, degrade or hydrolyze different substrates or to grow on different carbon sources producing changes in pH that may be monitored using compounds that turn color depending on the pH. Automated systems work with cards containing dehydrated culture media with suitable substrates. Culture time elapses while the cards are automatically read and data are collected by a system confronts the data collected with a database through the microorganism is identified. Among the available automated identification systems are the VITEK 2 (bioMérieux, France) and BD Phoenix (USA), these Systems reduce time of identification to 6-12 h.

Although the main disadvantage is the time it takes the identification, cultivation allows the discovery of new or atypical strains, conservation of strains for further characterization and the ability to determine the antimicrobial susceptibility directly [5].

Given the urgency with which requires identification of microorganisms other strategies have been designed which further reduce the periods of time. These include the identification by endpoint PCR, real time PCR, microarrays and mass spectrometry directed to the detection of proteins or nucleic acids.

1.1.1. Endpoint PCR and real time PCR

Although the identification of microorganisms through culture is the gold standard this methodology presents some complications that are resolved using molecular biology techniques such as endpoint PCR and real time PCR, significantly reducing the outcome of days to a couple of hours. The identification of microorganisms using traditional microbiology is limited by slow growing organisms or poorly viable, besides giving false negative results due to treatment of patients with pre antimicrobial sampling [5]. Identification by culturing are required pure colonies, because in mixtures of microorganisms is impossible to identify the components of the mixture. All these constraints are solved by PCR. Using real time PCR is possible the detection of several microorganisms in the same assay [6]. This requires assembling multiple reactions in which the detection of microorganisms is carried out at the end of the amplification when by increasing the temperature gradually build dissociation curve (melt curve). Thus, if we know the temperature to which the DNA strands of amplicons are separated from each microorganism, then microorganisms present in the specimen can be identified [6,7].

Some of the limitations to the identification by PCR are that this reaction requires the use of specific oligonucleotides for each microorganism, then each PCR reaction is performed for one or a particular group of organisms suspected. The design of specific oligonucleotides requires knowledge of the genome of the microorganism as well as genomics variants, sometimes new strains or mutations cause that oligonucleotides designed for the identification

might not align correctly. Due to test sensitivity is possible to detect even one copy of the target sequence [6], so that contamination is one of the main problems, one reason for this is the inadequate ways of taking the samples and the presence of microorganisms from normal flora cause false positives [5].

Even with the disadvantages mentioned, there are reasons that justify the use of PCR for diagnosis of ocular infections, due to the importance of receiving prompt treatment to stop the infectious process because otherwise compromises the functionality of the eye. Ocular infections caused by fungi are those with more advantages in the detection by PCR, because fungi growth is very slowly, and detection by molecular means is very quickly, and it allows giving a specific treatment [8].

Sensitivity of culture in cases of endophthalmitis is less than 70% and keratitis is not more than 80% due to the small inoculum [8]. If you have a sample that will uncover the presence fungal crop procedure and identification can take over a week delaying treatment. Therefore, the PCR for fungi provides the sensitivity needed in case of poor sample, even on the day of the sample obtaining [8]. Very few are currently available for clinical diagnostic kits for PCR. Roche provides kits for detection of *Chlamydia trachomatis*, CMV, EBV, Hepatitis A, B and C, HSV 1 and 2, VZV, HIV and *Neisseria gonorrhoeae*, meanwhile Bio-Rad have kits only for *Chlamydia trachomatis* and Mycoplasma. None company offers multiple trials or generic that can detect the presence of fungi and/or bacteria. Generic detection of bacteria is performed using oligonucleotides designed to bind to the conserved region of 16S ribosomal RNA gene, whereas for the detection of fungi, target is the 18S gene. While it is important to know the identity of the organism for appropriate treatment, discrimination between bacteria or fungi as causal agents of infection allows the introduction of generic treatment as early identification of the organism is carried out. The absence of commercial testing kitsallow that diagnostic laboratories use "home" methods at endpoint PCR and real time PCR designed and validated by themselves [8].

1.1.2. Identification by full genome sequencing

Time spent in genome sequencing has decreased. The first genome sequenced in 1995 (*Haemophilus influenzae*) took more than a year [9], whereas today technology is able to sequence hundreds of thousands of times faster. There is currently information around 3,144 complete genome deposited at the GenBank database [10]. All this information makes it possible to implement identification techniques based on sequencing. Through the comparison of sequences obtained from the analysis of clinical samples with the sequences contained in the databases, microbiological identification takes only a few hours with high certainty. These new technologies use PCR to amplify DNA, coupled to a parallel sequencing system using methods such as pyrosequencing, sequencing by ligation and sequencing by synthesis.

Although this technology is available from Roche 454 platforms, SOLID platform from Life Technologies and Illumina platform, plus the costs of technology, interpretation of results is perhaps the greatest barrier to the implementation of the sequencing genome as a routine identification technique in clinical laboratories [10].

In 2005, Yeo *et al.,* reported an outbreak of acute hemorrhagic conjunctivitis in Singapore [11]. Patients were diagnosed clinically with acute hemorrhagic conjunctivitis and it was identified by PCR the presence of an enterovirus and molecular typing confirmed a variant of coxsackievirus A24 (CA24v). Full-length genome sequencing results showed that CA24v virus was responsible for the outbreak and it was evolved from virus emerged 40 years ago.

1.1.3. PCR coupled to mass spectrometry using electrospray ionization (PCR / ESI-MS)

For identification by PCR/ESI-MS using oligonucleotides specific for bacterial groups rather than to a particular species, although variable regions are amplified between species and strains. Additionally, there are species-specific oligonucleotides used as primers that target genes for antibiotic resistance or some pathogenic characteristics [12]. Subsequent to amplification, amplicons are subjected to mass spectrometry and the pattern obtained is compared with those in the databases. The ability to identify an organism without prior knowledge of the Gram, or group of microorganisms is another advantage [12], since the stains are not required or previous isolates that provides fast trial and will always be possible to identify the microorganisms. This technology will be improving the identification of microorganism in ocular infections, it takes some advantages as certainty and specificity, and however the cost is the major disadvantage.

Kaleta *et al.,* designed a study to evaluate the feasibility of the use of PCR/ESI-MS to identify microorganisms directly from blood culture bottles in the clinical microbiology laboratory [12]. The high concordance of the results of this technique with those of standard methods, particularly at the genus level, demonstrates that PCR/ESI-MS technique is capable of rapidly evaluating clinically complex specimens providing information as to the selection and administration of targeted antibiotics.

About eye microorganisms, Pedreira *et al.,*evaluated the efficacy of a prophylactic regimen of daily topical 0.5% moxifloxacin and 5% povidone-iodine in patients with Boston type I. The patients with the prophylactic regimen were sampled and analyzed by standard culture methods and by PCR/ESI-MS [13]. The molecular diagnostic approach using PCR/ESI-MS yielded data comparable with those obtained using standard microbiologic techniques. Because of the high throughput nature and rapid results, the method might be a useful surveillance tool in patients with Boston type I.

2. Microarrays

As we described before, the PCR have several advantages over the culture of to identify microorganisms from infection. However, the disability to work with different genomes at the same time and the obtaining product with the same molecular size, make the PCR not the better method for diagnosis. Therefore, there have been developed new methods of diagnosis that not only reduce the time process; also they have more sensibility and specificity [14].

That is the case of the DNA microarrays, also called biochip, DNA chip, or gene array, which are defined as an orderly arrangement of samples gene for matching known and un-

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known DNA samples based on base-pairing rules and automating the process of identifying the unknowns and they were created by Brown P.O. y Botstein D. in 1999. An experiment with a single DNA chip can provide to researchers information on thousands of genes simultaneously, a dramatic increase in throughput. Microarray-based technology, with its advantage of highly parallel detection, has been applied to both population profiling and to functional studies of complex microbial communities in the environment [15, 16]. In addition, several studies have reported the use of PCR-amplified genomic fragment sequences as probes.

The gene arrays can be classified as macroarrays or microarrays, depending on the size of the sample spots. Macroarrays contain sample spot sizes of about 300 microns or larger and can be easily imaged by existing gel and blot scanners. The sample spot sizes in microarray are typically less than 200 microns in diameter and these arrays usually contain thousands of spots; microarrays require specialized robotics and imaging equipment.

There are several steps in the design and implementation of a DNA microarray experiment, as is shown in Figure 1.

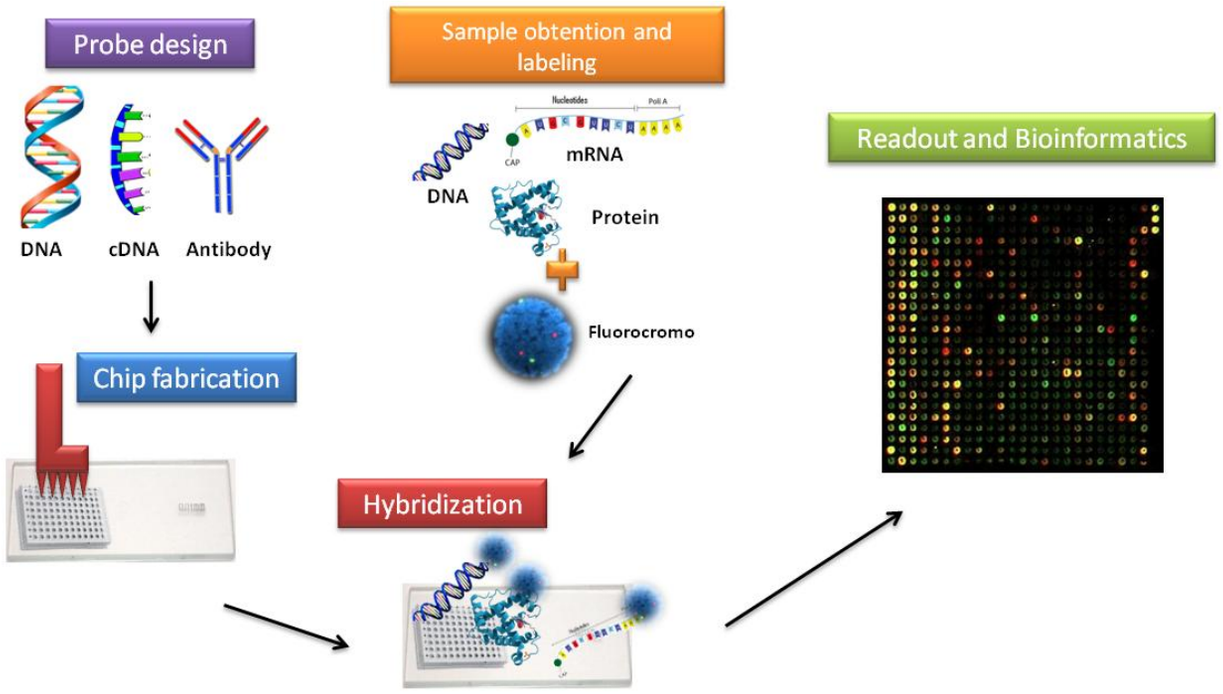

Figure 1. Design and implementation of DNA microarray experiment

It is important to mention that the microarrays made with cDNA are called spotted arrays because the probes are created *in vitro* and the robot put the spots on the microplate. Instead of the microarrays with oligonucleotides which are created *in situ* [17]. Recent studies have used synthesized oligonucleotides as probes because of their flexibility in design and preparation; with intensive specificity evaluation applied to the probe design criteria [18].

Both macroarrays and microarrays can have two application forms for the DNA microarray technology: the identification of sequence (gene/gene mutation) and determination of expression level of genes.

The determination of expression level of genes the microarrays can study the transcriptome or the proteome. For the transcriptome microarrays the probes consist on cDNA that hybridize with the mRNA of the cell. By the other hand, the proteome microarrays can use proteins as probe or the antibody making the antigen-antibody reaction. One example of this microarray is the peptide microarray analysis of *in silico*-predicted epitopes for serological diagnosis of *Toxoplasma gondii* infection in humans [19].

About the identification of gene sequence, microarray should have genomic DNA as probe of a specific chromosome, specifically all the genes that compose the chromosome. Or when a microarray only has a gene with one or more different nucleotides called Single Nucleotide Polymorphism (SNP) can detect a gene mutation [20].

These microarrays are used to determinate the cancer progression; all the changes on these gene are important to establish a clinical forecast [20]. Such microarrays have been used for the detection of specific bacteria [22, 23], species determination [24], and screening of environmental sequences related to a certain function within a community [25, 26].

Chin-I *et al.,* coupled 16S rDNA PCR and DNA hybridization technology to construct a microarray for simultaneous detection and discrimination of eight fish pathogens (*Aeromonas hydrophila, Edwardsiella tarda, Flavobacterium columnare, Lactococcus garvieae, Photobacterium damselae, Pseudomonas anguilliseptica, Streptococcus iniae* and *Vibrio anguillarum*) commonly encountered in aquaculture. The array comprised short oligonucleotide probes complementary to the polymorphic regions of 16S rRNA genes from the target pathogens. The results showed that each probe consistently identified its corresponding target strain with 100% specificity [27].

Yu-Cheng *et al.,* designed the DNA probes and PCR primers for the detection of *Listeria monocytogens, Staphylococcus aureus, Enterobacter sakazakii, Escherichia coli* O157:H7, *Salmonella spp., Vibrio parahaemolyticus, Streptococcus agalactiae*and *Pseudomonas fluorescens* by using two sets of multiplex PCR, followed by a chromogenic macroarray system, these organisms in milk or other food products could be simultaneously detected [28].

An example of microarray designed for infection diagnosis is a microarray developed by Uchida *et al.,* for the direct detection of pathogens in osteoarticular infections by polymerase chain reaction amplification and microarray hybridization [29].

And finally, and the most interesting DNA microarray used for the endophthalmitis diagnosis is the one developed by Tsutomu *et al.* They used 13 samples of vitreous fluid (VF) obtained from 13 patients during vitrectomy. Vitreous fluids from three patients with suspected endophthalmitis and ten controls without infection were subjected to testing for the presence of bacteria and fungi in culture tests, polymerase chain reaction (PCR) analysis, and DNA microarray analysis. The DNA microarray contained the spots for 16S rDNA, variable and conserved areas for bacteria, and the 18S rDNA for fungi. No control sample was positive for bacteria or fungi in the culture test, PCR, or microarray analysis. Specimens from two patients (Cases 1 and 2) with suspected endophthalmitis were positive for bacteria

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in PCR, and a specimen from one patient (Case 3) was positive for fungi in PCR. *Klebsiella pneumonia* (Case 1), *Streptococcus agalactiae* (Case 2), and *Candida parapsilosis* (Case 3) in the PCR-positive specimens were identified by DNA microarray analysis within 24 hours. Culture results were also positive for *K. pneumonia* in Case 1, *S. agalactiae* in Case 2, and *C. parapsilosis*in Case 3, but required 3 to 4 days to obtain [30].

For infection diagnosis, microarray analysis is complementary to routine cultures for identifying causative microorganisms and is likely to be a useful tool in patients who require rapid diagnosis and early treatment.

3. Aptamers

The term aptamers derives from the Latin *aptus*, it means to adapt [31]. Aptamers are synthetic nucleic acids (DNA or RNA) that bind specifically to a wide variety of molecules including metal ions K^{2+}, Hg^{2+}, Pb^{2+}, ATP, antibiotics, amino acids, vitamins, organic dyes, peptides and proteins, additionally aptamers are not immunogenic and non-toxic, superior to antibodies [32, 33, 34, 35]. Thirty and 60 nucleotides usually comprise the length of the central region, so that the total length of the aptamer is 70 to 100 nucleotides. For selection of aptamers with higher affinity the SELEX method is used (Systematic Evolution of Ligands by Exponential Enrichment). Wherein the target molecules are incubated with a population of aptamers, which interact with the target molecule by affinity, non-interacting target molecules are removed and the oligonucleotides are amplified by PCR and characterized by sequencing, being able to maintain a stock by its introduction to bacteria using plasmids. After obtaining the individual aptamers were characterized by their interaction with the target molecule by techniques as SPR, ELISA, Western blot or slot blot [36].

3.1. Applications aptamers

The aptamers can be used in different areas of study; some of its applications are reviewed below.

Biotechnology: the aptamers can be used for protein purification [37] and also for the development of techniques such as western blot or chromatin immunoprecipitation [38], also to monitor the status of phosphorylation of proteins *in vivo* [35]. There is an aptamer with activity of inhibitor of coagulation factor IXa by the addition of antisense RNA, this is an important method to control bleeding in patients who are intolerant of heparin, the aptamer is of interest for therapeutic and diagnostic [40].

Therapy: The therapeutic targets can be divided into two classes, the intracellular targets such as transcription factors, and extracellular targets such as viruses. The aptamers can be administered intravenously or subcutaneously; there is also topical application to prevent pathogens interaction with their receptors on mucosal surfaces. The release of intracelular-aptamers to bind their targets has been made mainly by the incorporation into liposomes or by systems of viral-based expression. A technique using a liposome to release viral vector fusigenicaptamer DNA in target cells, showed sequestration of E2F (transcription factor)

leading to a reduction in the growth of abnormal vascular tissue that is typically seen after angioplasty [41]. Some research groups have studied the expression of aptamers in cells. An example of this is the expression of a chimeric transcript initiating sequence, consisting of a human tRNA-Met and anti-HIV reverse transcriptase-pseudoknotaptamer under control of RNA polymerase III promoter in human 293 cells. The chimeric RNA resulted in a reduction of over 75% in viral replication, similar results were observed when carrying out the transfection in Jurkat cells [41]. The FDA (Food and Drug Administration, USA) approved the system Eyetech /Pfizer's aptamer (Macugen) for treatment of related macular degeneration. The target of Macugen is VEGF (Vascular Endothelial Growth Factor), preventing choroidal neovascularization [43, 44]. There are aptamers against amyloidogenic proteins such as peptide Aß associated with Alzheimer disease [45] and against abnormal proteins in prion diseasesand scrapie, and Creutzfeldt-Jakob disease [46, 47].

3.2. Diagnostics and biosensors

The high affinity and specificity of aptamers make them ideal as reagents for diagnosis. And also aptamers can be detected by differential staining fluorescence that results in a high sensitivity. There are an aptamers called "beacons" that have many uses ranging from detection of environmental pollutants and thus also to monitor the levels of carcinogens or drugs in the blood [48]. The development of quantum dot aptamers also could help to establish the role of aptamers as biosensors [49, 50]. The quantum dots are novel fluorophores with a different emission profile, but all they are excited in the same wavelength. In this system multiple copies of an aptamer is attached to a single quantum dot, and each aptamer base is binding to a complementary strand. The plug moves on ligand binding, leading to large increases in fluorescence emission. If different aptamers are immobilized each on a single quantum dot, multiple ligands can be detected in a single assay. The aptamers have great potential as early warning systems to detect cell surface binding to damaged or diseased cells.

3.3. Aptamers: An approach to diagnostic microbiology

It has previously been addressed different approaches to the application of aptamers. The use of aptamers in microbiology is interesting, in order to have new tools for the diagnosis of infections. Today, several research groups are involved in aptamers development aimed at the detection of microorganisms. Duan *et al.,* by means of the system evolution of ligands by exponential enrichment (SELEX) developed a DNA aptamer labeled with carboxyfluorescein (FAM) that binds specifically to *Vibrio parahaemolyticus* [51]; Zelada *et al.,* through aptamer system potentiometric biosensors based on carbon nanotubes attached to a single wall (SWCNT) were able to identify and detect *Escherichia coli* with a linear response [52]. Aptamers represent a very flexible technique for the detection of microorganisms such is the case of the determination of *E. coli* based on immunomagnetic separation and real time PCR aptamers, this technique consists of three steps, first the binding of *E. coli* to an antibody conjugated to a magnetic bead, the second RNA aptamer is captured on the surface of *E. coli* forming a sandwich and then a heat process release the aptamers and these are amplified using real time PCR. The sensitivity of this method allowed the detection of 10 *E. coli* in 1 mL of sample [53]. Aptamers have also been developed for quantum dot fluorescence assays

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against *Bacillus thuringiensis*, detecting up to 1000 CFU/ml [54]. Application of aptamers in the microbiological diagnosis and the advantages respect to other diagnostic techniques must be analyzed; however there is not much information about the application of aptamers in the microbiological field.

3.4. SOMAmers

SOMAmers (Slow Off-rate Modified Aptamers) are single-stranded deoxynucleotides type aptamers selected *in vitro* from large random libraries, for their ability to bind small molecules, peptides or proteins [55, 56]. SOMAmers are aptamers carrying dU residues in position 5 that are involved in interactions with target molecules [57].

SOMAmers have been created for more than 1000 protein targets of different molecular functions, including known diseases and physiological associations. The target families broadly include receptors, kinases, growth factors and hormones, and also include a diverse array of intracellular and extracellular proteins.

The core of the reagents is a SOMAmer coupled to biotin, via a photocleaveable linker allowing binding to streptavidin of the complex in the washing steps (Figure 2). A fluorophore Cy3 incorporated into the capture reagents allows quantification of protein available commercially available systems based microarray systems but not necessary for all assay formats (SomaLogic ®).

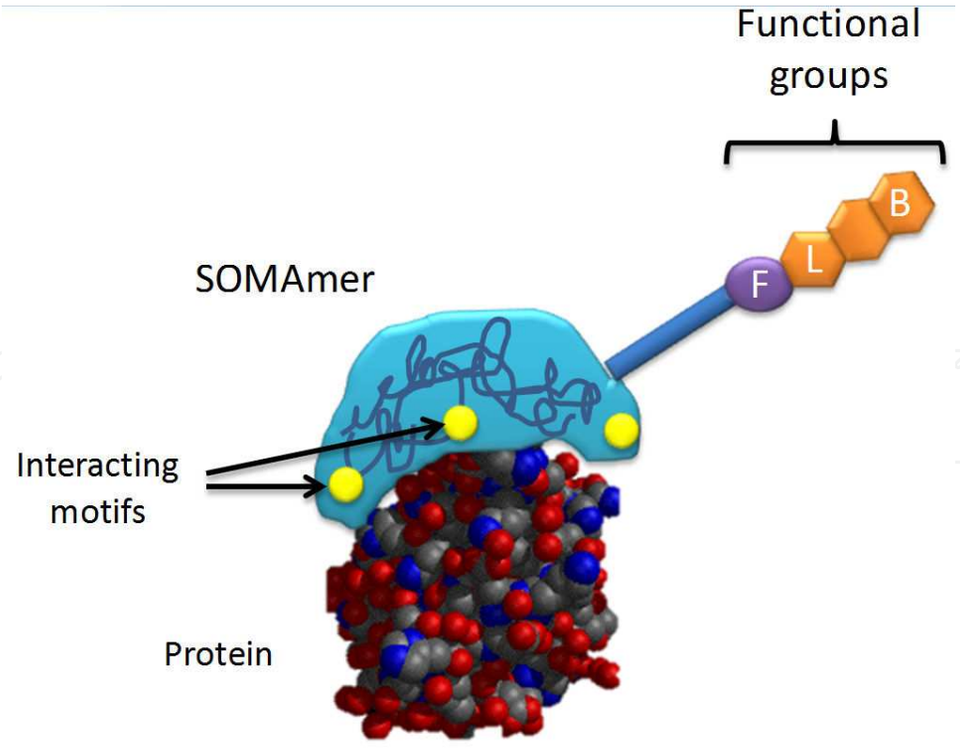

Figure 2. SOMAmer-protein complex. SOMAmer binds specifically to protein target through interacting motifs. Functional groups are: B= biotin for capture; F=Cy3 for detection and L=photocleavable linker.

3.4.1. SOMAmers applications

Comparison between proteome of healthy and diseased tissues from human using Somascan, can provide major knowledge of the biology of the disease and may lead to the discovery of new highly specific biomarkers for diagnosis, prognosis and therapeutic targets for the development of new drug treatment and it will improve personalized medicine.

Somascan premium has been used for the discovery of biomarkers for the detection of mesothelioma in the population exposed to asbestos. SOMAmers reagent showed better performance with respect to the ELISA test. Also the system is used to discover biomarkers for the detection of non-small cell lung cancer [57]. Moreover the system can be applied in tumor tissue lysate to obtain biomarkers associated with the disease as well SOMAmers same reagents can be used for histochemical evidence [58]. The SOMAmers represents an effective tool for biomarker discovery in different areas such as oncology, neurology, cardiovascular and metabolic diseases. To microbiological purposes as those related to the detection of agents in microbial infections SOMAmers represent a good alternative tool that may be applied for microbiological diagnosis in the future.

4. Mass spectrometry in microbiological diagnosis

Since its discovery, over a hundred years, mass spectrometry has been a useful tool to understanding the chemistry of proteins and biological processes involved. However, until the discovery of soft ionization techniques such as MALDI (Matrix-assisted laser desorption/Ionization) and ESI (electrospray ionization) this methodology could be used as a routine tool in laboratories. [59].

Recently, mass spectrometry (MS) has entered to microbiology laboratories, offering a fast and reliable identification of microorganisms based on proteomic analysis.

4.1. Mass spectrometer

The mass spectrometer, in summary, is supported on the fragmentation of proteins to small peptides or other biomolecules to smaller molecules and then be ionized, these molecules are separated by the acceleration of ions in an electric field and then detected in based on their charge/mass ratio, in a gas phase state to produce a corresponding electrical signal to detect ions [60, 61].

A mass spectrometer is mainly composed of three elements in a vacuum atmosphere: an ionization source, a mass analyzer and detector.

4.1.1. Ionization source

The result of applying a source of ionization in a sample was the production of electrically charged ionized particles that gain or loss electrons in a gas phase.

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There are several ionization processes that can be employed for the same purpose, to produce ionized peptides [62] among these processes are MALDI (Matrix -assisted laser desorption/Ionization) and ESI (electrospray ionization) that are most known.

4.1.2. MALDI (desorption / ionization matrix-assisted laser)

In this method, the sample is soaked in an organic matrix which is crystallized with air and is irradiated by a laser, matrix most used are the acid α-cyano-4-hydroxy-trans cinnamic acid, 2,5-dihidrobenzoic acid or sinapinic acid [59].

In MALDI, the protein or peptide of interest is coprecipitated with the organic compound which is capable of absorbing laser light of an appropriate wavelength. The laser allows to prepare the compound fragmentation and disruption of the crystalline matrix generating a cloud of particles, these particles capture electrons and therefore remain as negatively charged ions in most cases (Figure 3A) [59, 62].

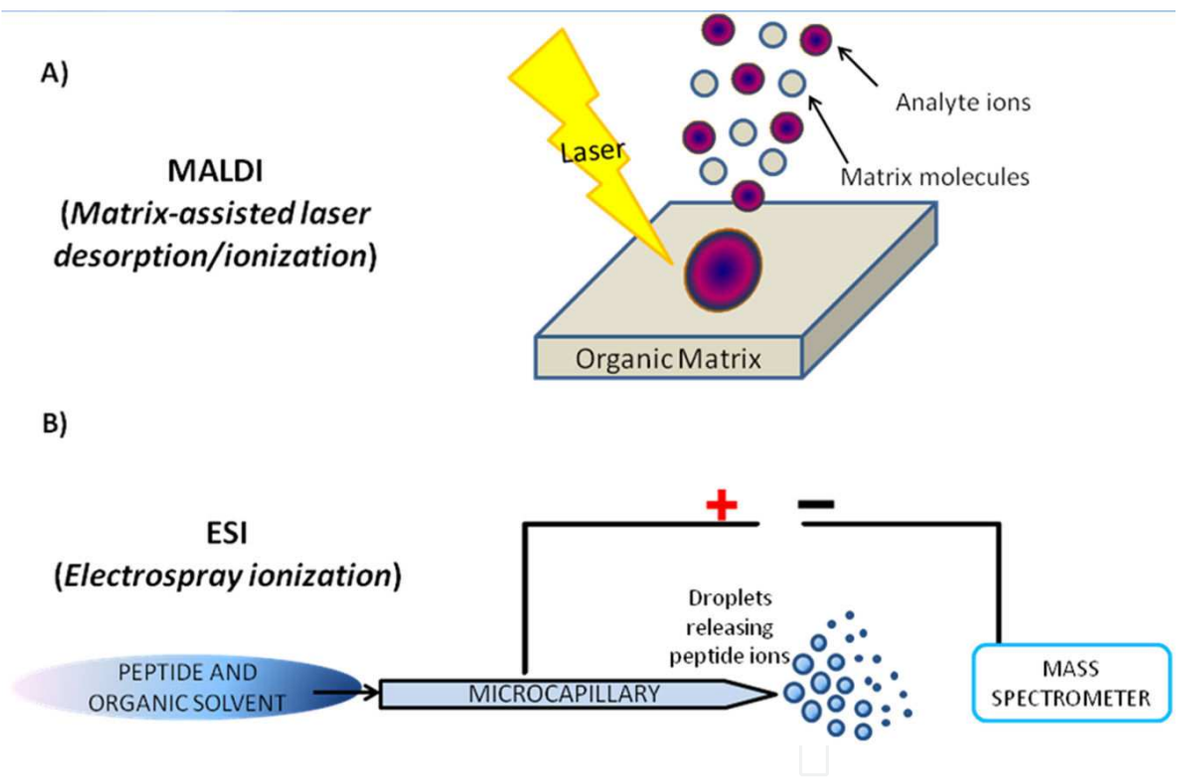

Figure 3. Schematic representation of A) MALDI (Matrix-assisted laser desorption/ionization) and B) ESI (Electrospray ionization) method. Protein samples must be ionized before they pass through mass spectrometer.

4.1.3. ESI (electrospray ionization)

In this process, the sample is dissolved in an organic solvent, this mixture passes through a fine capillary tube that is maintained in an electric field produced by an electrode near to the capillary and other on the detector, this mixture is sprayed to form high load of tiny droplets of the solvent that evaporates quickly, thus produce a series of gaseous ions that result

from protonation of side chains such as Arginine and Lysine, these ions fragmented by electric field are then detected (Figure 3B) [63].

4.1.4. Mass analyzer

Is the main component of the mass spectrometer, the charged fragments (ions and radical ions), are accelerated and deflected by a strong magnetic field that affects their travel resulting in a curvilinear path. Ions and radical ions are collected, detected and quantified with high accuracy and sensitivity, depending on the mass/charge ratio (m/z). [64].

There are several analyzers; however the most common type is the TOF (Time of flight). This analyzer defines a flight zone through which the ions are accelerated by acquiring a high kinetic energy, and during this trip will be separated according to their ratio mass/charge (m/z). Most of ions generated have a single charge (z = 1), so that the ratio m/z is equal to m. The length of time for each ion in reaching the detector is called flight of time and depends on this ratio (Figure 4) [59].

4.1.5. Ion detector

At the end of the fragmentation and separation of ions from a sample by MALDI or ESI, ions impact on the detector. The fragments after flowing through the pipe in electric field (TOF) are deflected and detected, not-charged fragments are not deflected by the field and lost in the pipe walls, but the charged fragments are recorded by the detector, and mass are calculated from the flight of time. In many cases, before the detector is the reflector, which increase the resolution of the technique (Figure 4) [65, 69].

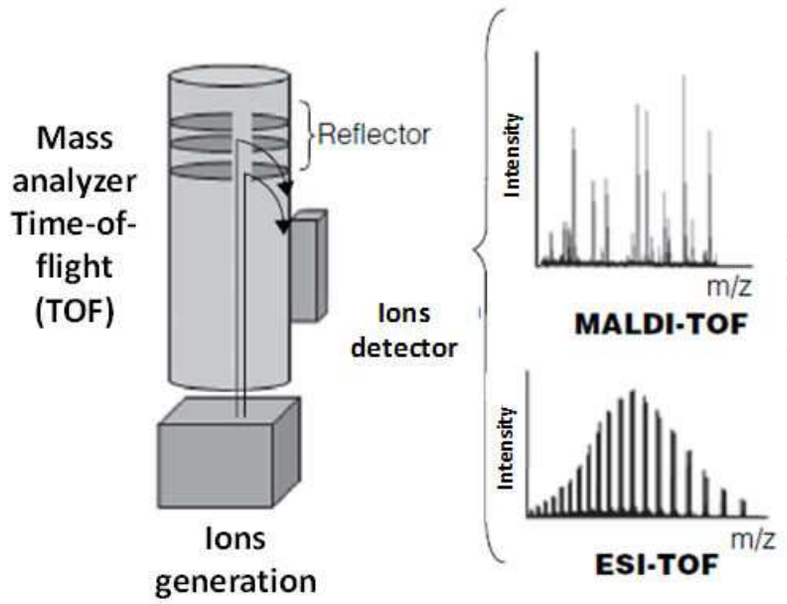

Figure 4. Scheme of Mass Spectrometer. Mass analyzer characterized and separated ions according to their mass/charge ratio (m/z). Ions detector generate mass spectrum for every ion detected. MALDI and ESI mass spectrum obtained are shown.

The mass spectrum of a compound is typically represented as a bar graph with the masses in the X axis, and the intensity or relative abundance of the ions of the m/z reaching the detector in the Y axis. The highest peak is assigned as 100% of intensity known as the base peak, and the main peak or molecular ion is the peak corresponding to the unfragmented radical cation (Figure 4) [65].

4.2. Fingerprinter obtaining and analysis

The actual data acquisition with MALDI-TOF-MS is nowadays generally performed in an automated manner. That is, the laser focus scans the sample in a predefined pattern and accumulates a mass spectrum from a defined number of laser pulse cycles, generally several hundreds to yield a representative average mass spectrum. The raw spectrum is generally processed to yield a mass fingerprinter that contains the information about peak apex *m*/*z* values, thus reducing the size of individual files considerably. The essential step for species identification is the comparison of the mass fingerprinter of the sample, to be identified to a database containing reference mass fingerprints, for example MASCOT, SWISS-PROT [66].

4.3. Spectrometry in the diagnosis of microbiological specimens

The first application of this technique was the study of the chemical structures of organic compounds in the area of Structural Chemistry and also the identification of compounds in the field of organic chemistry, eventually, the use of mass spectrometry spread to biology, geology and recently the clinical and medical area.

In recent years, this technique has been applied as a routine method in the microbiology laboratory, as a useful tool for the identification of microorganisms using the bacterial colony directly and proteins extracted from the microorganism.

Once obtained mass spectrum and it is compared with a database, software assigns identification and a reliable value of such identification.

MALDI-TOF has been the most used technique in the microbiological diagnosis for bacterial identification; some databases are used for identification profiles. This technique has been useful to obtain the profile of microorganisms for diagnosis using colonies directly from the culture media [67].

One advantage of the technique results from the culture obtained from a sample or sample directly, in this connection, trials have been performed to determine the functionality of the art regarding the identification, has been performed identification of bacteria and yeast using MALDI-TOF, allows for quick low-cost diagnosis compared to conventional techniques as Vitek-II, API and biochemical tests, and it is known that these technologies are validated by comparing the technique by identifying the microorganisms from the samples at the species level and it must be matched [68].

Recently, the mass spectrometer has taken a major challenge to modernize and facilitate its use by the coupling of other techniques such as Vitek, an automated method of identifying microorganisms. In recent years several studies have been performed in routine clinical laboratory using this new technology: Vitek ® MS Biomeriuex.

This mass spectrometric technique is based on MALDI-TOF coupled to a Vitek-II and Myla, a database which receives the fingerprint of the sample from culture or sample directly and then identifies the organism. This technique has been used identifying fungi from clinical samples. Results from this study identifying 18 fungi with a quick and inexpensive strategy, since it does not require a prior extraction of proteins. These tests were perfomerd on clinical isolates from 20 patients, which were also evaluated using Vitek 2. Comparison between results from Vitek-MS and Vitek 2 correlated in 93% [69].

Ferreira *et al.,* analyzed 294 facultative anaerobic and aerobic isolates obtained from different clinical samples, using conventional microbiological methods compared to conventional microbiological methods. In the analysis they concluded that bacterial clinical isolates identification obtained by MS MALDI-TOF shows excellent correlation with identification obtained by conventional microbiological methods. Moreover, MS MALDI-TOF allows the identification of bacteria from colonies grown on agar culture plates in just a few minutes with a very simple methodology and hardly any consumable cost [70].

5. Conclusions

Ocular infections are one of the most frequent events in ophthalmology, and the treatment for these diseases must be fast, precise and effective, in order to get this goal is important to identify and characterized microorganisms involved. Clinical diagnosis of ocular infections can be confirmed by several techniques based on microbiological test of ocular samples. These techniques includes classic microbiological test, where is necessary isolate microorganisms to characterize them by biochemical analysis; molecular biology techniques, endpoint PCR, real time PCR, microarrays and aptamers (e. SOMAmers) can obtain results in a short period time, as well as high sensitivity and specificity.

However, in the last years, mass spectrometry approach has dramatically changed the microbiological field. Microbiological identification by mass spectrometry has great advantages: 1) Culture and isolation of the microorganism is not necessary, so that fastidious microorganisms can be identified, 2) High sensitivity and accuracy for the microorganism identification results in a reduction of sample amount, that is common in ocular samples, and 3) Resistance markers and resistance profile can be determined at the same time of identification analysis.

In summary, the evolution of microbiological identification methods has improved treatments that impact in the prognosis of ocular infection, reducing complications and avoiding blindness cases, and as a consequence life quality of patients will be better.

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Author details

Victor Manuel Bautista-de Lucio[1], Mariana Ortiz-Casas[1], Luis Antonio Bautista-Hernández[1], Nadia Luz López-Espinosa[1], Carolina Gaona-Juárez[1], Ángel Gustavo Salas-Lais[1,2], Dulce Aurora Frausto-del Río[1] and Herlinda Mejía-López[1]

1 Microbiology and Ocular Proteomics Department, Research Unit, Institute of Ophthalmology "Fundación de Asistencia Privada Conde de Valenciana I.A.P.", Mexico City, Mexico

2 Immunoparasitology Laboratory, Parasitology Department, Escuela Nacional de Ciencias Biológicas, Instituto Politécnico Nacional, Mexico City, Mexico

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