Infectious diseases detection by microarray: An overview of clinical relevant infections

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ABSTRACT

Microarray technology is a powerful tool to investigate whole genome expression profiles to study the crosstalk between pathogens and associated hosts that cause illness. Microarrays have been used in several comparative genome hybridization studies of pathogens. In addition to detection and identification of pathogens, microarrays are ideal for characterizing genetic differences between bacteria isolates of the same species to the strain level. Furthermore, the use of microarrays has been gaining importance in the detection of viral agents. Here we explore the use of microarrays for simultaneous detection of viruses and modifications made over these techniques. Microarray technology has also been incorporated to compensate for time-consuming sequencing. The procedure of fungi identification based on sequences and studies reported the use of microarrays to identify pathogenic yeasts and molds by targeting the internal transcribed spacer regions in fungal rRNA genes. The remarkable advancement in genomics over the last decade has made it possible for microarray technology to evolve towards being the best option for clinical diagnostics because they have several advantages.

Keywords: DNA Microarray; Diagnosis; Bacteria; Virus; Fungi

1. INTRODUCTION

The complex interaction between a pathogen and its host is the molecular basis of infectious diseases. Microarray technology is a powerful tool to investigate the crosstalk between a pathogen and the host as it assesses whole genome expression profiles in response to disease [1].

Understanding the molecular details on both sides of the host-pathogen interaction will increase the knowledge of the pathogenesis of infectious diseases and offer improvements in their diagnosis, treatment, prognosis, and prevention. Usually, traditional experiments focus on a few genes as suspected virulent factors. However, microarrays have enabled the exploration of genome wide expression. They enable the discovery of groups of genes involved in the same biological process and virulent pathways that involve many genes that may not have been previously known. The microarray has made it possible to study multiple genes simultaneously. It is no longer necessary to study the expression of one gene at a time.

Pathogenic microorganisms, like bacteria, viruses and fungi, often employ complex mechanisms of virulence developed over millions of years of evolution, which have resulted in a variety of diverse ways for pathogens to successfully infect their host by avoiding host defense pathways. One of the most important applications of microarray technology is the ability to quickly identify an infecting pathogen that can cause a disease. Because pathogens have distinct genetic compositions and microarrays are able to examine all gene sequences, the array is an ideal tool for detecting pathogens [2].

Since the beginning of the century, several microarray systems have been developed that have the potential for simultaneous detection of many pathogens. This is of interest for homeland security, public health, medicine, and veterinary diagnostics. For example, Wilson and collaborators developed a Multi-Pathogen Identification (MPID) microarray that identifies eighteen pathogenic prokaryotes, eukaryotes, and viruses amplifying unique regions of DNA from each microorganism, using the microarray to detect the presence or absence of pathogen-specific DNA sequences [3,4].

Several methodologies for DNA microarray analysis

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have derived from the latest microarray technologies. Commonly, specialized software is applied to do the analysis by using tools to visualize array data, facilitate interpretation and to store results. Analysis has been improving throughout time, not only in assay capabilities (sensitivity and specificity), but also allowing high-throughput detection. Most importantly, the compatibility of microarrays with miniaturized devices could result in enhanced speed, sensitivity, and portability which are important factors in the field of diagnostics [5].

2. BACTERIA DETECTION

Microarrays have been used in a number of comparative bacteria genome hybridization studies. For example, it has been used to distinguish different strains of *Pseudomonas* [6] or *Mycobacterium tuberculosis* [7]. The microarray has even been used to characterize mutations in the *rpoB* and *katG* genes that confer rifampin and isoniazid resistance in *M. tuberculosis* [8]. Microarrays are also being incorporated in some clinical laboratories for the rapid detection and classification of methicillin-resistant *Staphylococcus aureus*, determination of antimicrobial drug resistance in several pathogens such as *Enterococcus* and *Mycobacterium tuberculosis*, as well in diagnosis of sepsis [8-10]. Besides detection and identification of pathogens, microarrays are a good option for characterizing genetic differences between isolates of the same species to the strain level.

2.1. Tuberculosis

Approximately 1.4 million deaths were attributed to *M. tuberculosis* infections in 2012. When compared to other infectious agents, only HIV claims more lives than tuberculosis according to World Health Organization (WHO, 2012). Traditional evaluation of drug resistance in clinical samples of *M. tuberculosis* takes at least four weeks, causing either a delay in treatment or administration of often ineffective anti-mycobacterial drugs. Besides this complication, the number of individuals infected with drug-resistant isolates continues to increase. The resistance to two first-line antibiotics, isoniazid and rifampicin, may be caused by several different genetic changes which could be identified by molecular methods. In the evolution of microarrays, the most relevant system was designed by Gryadunov *et al*. They developed a biochip for detection of rifampicin-resistant and isoniazid-resistant strains of *M. tuberculosis* [11]. The biochip identifies over 95% of rifampicin-resistant and more than 80% of isoniazid-resistant *M. tuberculosis* strains in sputum samples. The biochip has 77 gel elements and detects the 27 most-common mutations in the *rpoB* gene responsible for rifampicin resistance as well as 11 mutations in the *katG* gene, five mutations in the promoter region of the *inhA* gene, and five mutations in the intergenic regulatory region of the *ahpC-oxyR* genes all of which can cause resistance to isoniazid.

The system demonstrates a sensitivity of 80% and a specificity of 100% compare to traditional testing for rifampicin resistance. All discrepancies between the microarray test and culture test were caused by either rare mutations not detectable by the microarray probes, or by unknown mutations. The newest generation of TB-biochips identifies mutations responsible for the emerging resistance of *M. tuberculosis* so the highly effective second-line fluoroquinolone antibiotics can be administered [12].

2.2. Meningitis

Human brain bacterial meningitis is a severe infection caused mainly by *Neisseria meningitidis*. Meningitis damages the outer membrane covering (meninges) of the brain which leads to fatality in those infected. The traditional methods of diagnosis are either time-consuming or have some limitations. Strain characterization using genotypic and phenotypic methods are necessary to develop new meningococcal diagnostic tools. Recently, the creation of a model of resequencing microarrays containing genomic sequences that vary between different meningitis subtypes to classify different isolates of *Neisseria meningitides* has been reported [13]. Using the resequencing array, the scientists were able to correctly classify 45 samples that were previously identified by conventional methods. But more importantly, the resequencing array was able to classify 12 previously unclassifiable samples into existing meningitis serotypes. Traditionally, meningitis has been classified by using immunoassays to identify serotypes in combination with capillary sequencing to identify sub-serotypes. In addition to being more accurate than the traditional serotyping methods, resequencing microarrays provide results in just 48 hours, much faster than traditional methods. The meningitis resequencing array can now be used to quickly identify new meningitis strains, as well as for epidemiological studies and vaccine research.

3. PARASITE DETECTION

Malaria remains to be one of the most common causes of death among infectious diseases. According to some estimates, up to 500 million people in the world are infected, approximately 1 million of which result in death, the majority of which are African children. The environmental ban on DDT resulted in a dramatic increase in the prevalence of malaria and the pathogen acquired resistance to chloroquine, quinine, and other common treatments. Arylaminie N-acetyltransferase (NAT) of *Plasmodium falciparum* is over-involved by the unusually

high AT content of its DNA. Direct testing of the pathogen for drug resistance is difficult enough. Sequencing and molecular mapping of the *Plasmodium* genome identified many single-nucleotide polymerphisms (SNPs) linked to or directly responsible for the resistance. For now, future tools for mapping malaria traits should be concentrated around developing methods to accurately characterize and measure phenotypic variation among individual parasites. Microarray based analysis of the resistant genes already reaches more than 94% specificity and can even be performed on asymptomatic patients at a highly effective cost [14]. In a recent review of molecular approaches, in particular, microarray-based methods are considered the most-promising tool for diagnostics and characterization of the malaria pathogen. High-throughput genotyping methods are now available for typing DNA from *Plasmodium falciparum* and for mapping parasite traits. Many more typing methods are under development, including those for other malaria species. Unfortunately, the malaria parasite is a single-cell organism, and it is thus challenging to detect or measure reproducible phenotypic variation between individual parasites [15].

4. VIRUS DETECTION

For decades, virus isolation has been the gold standard method for virus identification but these techniques are time consuming [16]. Additionally, in virology, correct etiologic agent detection is mandatory especially in cases where clinical signs are very similar and can be confused among several viral infections. Likewise, obtaining a specific result for a certain etiologic agent leads physicians to provide adequate pharmacotherapy to patients. PCR has revolutionized the field of infectious disease diagnosis. Multiplex PCR, a variant of the test in which more than one target sequence is amplified using more than one pair of primers, has also been developed [17]. The use of microarrays has gained importance in the detection of viral agents, but what we are presenting is the use of microarrays for simultaneous detection of viruses and the modifications made over these techniques.

Based on publications, microarray approaches can be summarized into four viral infection groups to focus on: respiratory diseases, hemorrhagic fever (HF), neurotropic infection, and HIV with associated viruses.

4.1. Respiratory Diseases

Respiratory viral infections are a global problem mostly in the winter season. Just in the last 100 years there have been three pandemics with a high number of hospitalizations and deaths. The first pandemic occurred in 1918 by the influenza virus AH1N1. It caused 40 million deaths worldwide, followed by the 1957 pandemic influenza A

(H2N2) resulting in approximately 1 million deaths worldwide. The third pandemic was in Hong Kong caused by the influenza virus A (H3N2) in 1968 [18]. The last recorded human pandemic was caused by the H1N1 strain in 2009, where the virus was propagated in 214 countries [19].There are also other respiratory viruses capable to cause substantial outbreaks, like the coronaviruses that caused severe acute respiratory syndrome (SARS) in China in 2002 which spread by 2003 in all five continents [20].

Some influenza microarrays are designed to detect DNA. For instance, a universal microchip was developed for genotyping influenza A viruses with two sets of oligonucleotide probes allowing viruses to be classified by the subtype of hemagglutinin (H1 - H13, H15, H16) and neuraminidase (N1 - N9) [21].

Other researchers designed and evaluated a microarray (FluChip-55) for detection of influenza A and some subtypes (H1N1, H3N2, and H5N1) using a new approach based on the direct capture and detection of amplified RNA using a two-step hybridization process where viral RNA amplification is performed by rt-PCR, followed by a runoff transcription. The microarray assay consists of a 25-mer DNA oligonucleotides immobilized on a microarray surface, hybridization capture sequence of the influenza virus RNA is detected by the hybridization of a fluorophore-conjugated DNA oligonucleotide 25-mer to a second region on the target RNA. They tested 72 samples comprising of controls and isolates from human, avian, equine, and swine species. The results provided the correct types and subtypes for an average of 72% of the isolates, the correct type and partially correct subtype information for 13% of the isolates, the correct type only for 10% of the isolates, false-negative signals for 4% of the isolates, and false-positive signals for 1% of the isolates [22,23].

Not only have microarrays been designed to search for the presence of the etiologic agents, they have also been designed for the antibodies against them. In terms of SARS, a protein microarray approach was developed for this purpose with a total of 82 coronavirus expression proteins. They tested 399 serum samples from Canada which met the clinical and laboratory criteria for SARS-CoV infection during the 2003 Toronto SARS outbreak. The microarray classified 147 non-confirmed fever cases and 56 respiratory patients (36 confirmed SARS patients and 20 non-SARS individuals) from China. The microarray also identified patients with sera reactive against other coronavirus proteins. The main result was that this protein microarray was able to distinguish reactivity between human coronaviruses (HCoV-229E and SARS) [24].

Microarrays have also served for the discovery of new

respiratory viruses. A DNA microarray was designed to detect a wide range of known viruses containing the most highly conserved 70 mer sequences. During an outbreak of SARS, this microarray showed the presence of an unknown coronavirus in a viral isolate from a patient, further characterized this virus, and resulted in a new member of the coronavirus family [25].

4.2. Hemorrhagic Fever

Viruses associated with hemorrhagic fever (HF) are mainly found in the families *Arenaviridae*, *Bunyaviridae*, *Flaviviridae and Filoviridae*. This condition presents an acute febrile syndrome characterized by generalized bleeding in severe infections. General symptoms are malaise, prostration increase in vascular permeability, and coagulation abnormalities [20]. Derived from the enormous number of viral agents that can cause HF, some molecular methods have been used for diagnostic, for instance rtPCR and real time PCR [26]. Based on microarrays, a detection and identification approach was designed for seven agents of the *Flaviviridae* family: yellow fever, West Nile virus (WNV), Japanese encephalitis, and the dengue 1 - 4 viruses, which are causing severe human disease in tropical and subtropical areas all over the world [27].

4.3. Neurotropic Infections

Some viruses in the *Flaviviridae* family, and also Bunyavirus and Togavirus families, affect the central nervous system (CNS) producing meningitis and encephalitis syndromes, for instance WNV. Acute encephalitis can be due to many causes, although most of them are viral. The most frequent cause of encephalitis worldwide is herpes simplex virus type 1 (HSV-1) but this is not the only etiological agent [28].

A DNA microarray for the detection of 13 specific pathogens in meningitis and encephalitis cases was developed for the most common neurotropic viruses including HSV-1, varicella-zoster virus [VZV], and enteroviruses. Also, a microarray comprising of 38 gene targets was developed for the detection of several other viruses capable of causing CNS syndromes. The array was used for the detection of multiplex PCR-amplified viruses. A total of 41 clinical specimens were positive for echoviruses (23 samples), HSV-2 (4 samples), VZV (4 samples), human HSV-7 7 (1 sample), HSV-6A (1 sample) and HSV-6B (2 samples), Epstein-Barr virus (3 samples), polyomavirus JC (1 sample), and cytomegalovirus (2 samples). This concluded that clinical sensitivity, specificity, and negative and positive predictive values of the assay were 93%, 100%, 100%, and 83% respectively, comparing microarray to the single-virus PCR [29].

4.4. HIV

In a blood transfusion there is always a potential risk to transmit pathogens such as hepatitis B and C viruses (HBV and HCV respectively) or the human immunodeficiency virus type 1 (HIV-1) [30]. Since detection methods like RT-PCR not only detect the pathogen but also can measure the amount of virus, a microarray was developed by combining both methodologies. The study described an original approach for simultaneous quantitative identification of these viruses in blood plasma specimens using real-time PCR with primers immobilized on a microarray [31].

Another novel use of microarrays with HIV has been the identification of resistance biomarkers on HIV-1, including pathways that may be critical in anti-HIV-1 vaccine design. The microarrays used gene expression analysis on blood samples obtained from HIV-1 highly exposed commercial sex worker seronegatives to investigate possible mechanisms of the observed reversal of HIV-1 susceptibility in activated CD4 and T cells, performed in genome-wide expression analysis using Agilent microarrays [32,33].

4.5. Perspectives: Viral Identification Platforms

The most complex microarrays are comprised of three kinds of platforms for massive virus identification. The first one is called Virochip which is a panviral platform able to detect all known viruses and novel variants of them. The current version of this approach has been developed using an Agilent microarray platform and consists of about 36,000 probes derived from over 1500 viruses in GenBank [34].

GreeneChipPm is an even more ambitious platform, that has been designed for panmicrobial detection. This platform is comprised of 29,455 60-mer oligonucleotide probes for the detection of vertebrate viruses, bacteria, fungi, and parasites. The system has been tested using blood, respiration, urine, and tissue samples containing bacterial and viral pathogens. GreeneChipVr (only for viruses) version 1.0 contained 9477 probes to address all vertebrate viruses (1710 species, including all reported isolates). GreeneChipPm version 1.0 contained 29,495 probes that included probes comprising GreeneChipVr, as well as 11,479 16S rRNA bacterial probes, 1120 18S rRNA fungal probes, and 848 18S rRNA parasite probes [35].

The third approached is Lawrence Livermore Microbial Detection Array (LLMDA).This platform contains probes fitted onto a glass slide. Each probe tests for a particular sequence of DNA and small groups of probes and can be used to check for specific bacteria or viruses up to the species level. The LLMDA can test for over 2000 viruses and 900 bacteria. The newer version of this

platform will expand to a capability to nearly 6000 viruses and 15,000 bacteria as well as fungi and protozoa organisms [36].

5. FUNGI DETECTION

Currently, invasive fungal infections (IFIs) have emerged as major causes of morbidity and mortality in immunocompromised patients. Candidiasis and aspergillosis are the most common IFIs in patients receiving immunosuppressive treatment for cancer or organ transplantation. *Aspergillus*, *Rhizopus*, and *Mucor* fungi species can be fatal in oncology patients causing cutaneous fungal infections [37]. Also, aspergillosis has showed a high mortality rate, up to 90%, for patients with hematological malignancies [38]. Different kinds of malignancies and autoimmune diseases, *Aspergillus spp*, *Fusarium spp*, and *Penicillium spp*, are important sources of infection. Moreover, a systemic infection with *Penicillium marneffei* is the third most common opportunistic infection in HIV co-infected patients in South East Asia [39].

Diagnosis of fungal infections can be done by a) cultivation and isolation of the infective organism. Fungi culture may require several weeks to grow and conventional identification of pathogenic fungi is time consuming. Therefore, it is often imperfect for early initiation of an adequate antifungal therapy; b) serologic assays. However this test may not be conclusive with one single serum sample that detects circulating antifungal antibodies (especially in immunosuppressed subjects). Analysis a of sera sample in the acute and convalescent stage is necessary for a definitive serologic diagnosis which could take 3 to 4 weeks [40]; or c) histopathological assays. Although the histopathological test is relatively rapid and available to diagnosis invasive fungal disease, atypical morphological features cause problems in accurate identification [41]. Nowadays, the increasing incidence of IFIs in immunocompromised patients emphasizes the need to improve diagnostic tools [42].

Currently, a relatively new way of fungi identification is being performed using probes designed from the most commonly used gene targets as 28S fungal and gene regions of rRNA such as internal transcribed spacer (ITS). Morrison and colleagues had reported the oligonucleotide probes for detecting Aspergillus species and other filamentous fungi. The unique ITS regions allow for the design of specific nucleic acid probes for five *Aspergillus*, three *Fusarium*, four *Mucor*, two *Penicillium* species, and medically important infectious diseases in humans [43,44].

During the past two decades, techniques based on DNA microarrays have been used for the identification of pathogens, and disease-causative agents.

Microarray technology has been incorporated to compensate for time-consuming sequencing. The procedure of fungi identification based on ITS sequences and studies reported the use of microarrays to identify pathogenic yeasts and molds by targeting the ITS regions in fungal rRNA genes [45-48].

Leinberger, DM *et al*. 2005, designed and developed oligonucleotide probes based on the sequence variations of the ITS regions of the rRNA gene cassette are used to identify *Candida albicans*, *Candida dubliniensis*, *Candida krusei*, *Candida glabrata*, *Candida tropicalis*, *Candida parapsilosis*, *Candida guilliermondii*, *Candida lusitaniae*, *Aspergillus fumigatus*, *Aspergillus flavus*, *Aspergillus niger*, and *Aspergillus terreus*. The diagnostic microarray was developed for the rapid and simultaneous identification of the 12 most common pathogenic *Candida* and *Aspergillus* species. By using universal fungal primers (ITS1 and ITS4) directed toward conserved regions of the 18S and 28S rRNA genes respectively, the fungal ITS target regions were simultaneously amplified and fluorescently labeled. This method was validated by using 21 clinical isolates samples. The microarray was able to rapidly and reliably detect and clearly identify the fungal pathogens within four hours after DNA extraction [48].

In 2007, Birgit Spiess *et al*. reported a sensitive DNA microarray to detect and identify DNA from 14 fungal pathogens: *Aspergillus fumigatus*, *Aspergillus flavus*, *Aspergillus terreus*, *Candida albicans*, *Candida dubliniensis*, *Candida glabrata*, *Candida lusitaniae*, *Candida tropicalis*, *Fusariumoxy sporum*, *Fusarium solani*, *Mucor racemosus*, *Rhizopus microsporus*, *Scedosporium prolificans*, and *Trichosporon asahii* in blood, bronchoalveolar lavage, and tissue samples from high-risk patients. The results in clinical samples from neutropenic patients showed the specific detection of the 14 fungal pathogens by using a combination of multiplex PCR and consecutive DNA microarray hybridization. The capture probes were derived from unique sequences of the 18S, 5.8S, and internal transcribed spacer one region of the fungal rRNA genes which contribute significantly in improving the diagnosis of IFI [49].

Candida Albicans

Candida albicans (*C. albicans*) is the most common human fungal pathogen which causes candidiasis, a common nosocomial infection that causes serious problems to both immunosuppressed and postoperative patients. *Candida* species are the fourth leading cause of nosocomial bloodstream infections in the United States. They are a leading cause of invasive fungal infections and are an emerging problem in hospital medicine.

The diagnosis of invasive candidiasis is difficult. Over the last 50 years, different strategies have been devel-

oped including detection of antibodies, antigens, and no-nantigenic fungal components such as DNA, D-arabinitol, and b-1,3-D glucan [50-52]. Once the genome of *C. albicans* was sequenced and microarrays had been developed, the technology was used to study different aspects of its biology. In recent years, several reports have described the use of DNA arrays technique to study different aspects of fungal pathogenesis, the responses to different environmental conditions, biofilm formation, and antifungal drug resistance [53-56]. The development of new DNA microarrays systems for fungal detection is relevant for clinical diagnostics of fungal infection diseases [57].

6. CONCLUSIONS

Even though there has been tremendous progress in genomic tools in the last few years, microarray technology has made strong progresses over the past decade and continues to be a great option for clinical diagnostics, where it has several advantages. Currently, applications of microarrays include DNA and protein estimation, antigen, and enzymatic activities ensuring their continuing presence in clinical diagnostics.

Early diagnosis of infection is critical to provide an effective treatment. Unfortunately, sometimes multiple tests are required to identify the pathogen responsible. However, microarray technology is available for identification of pathogens and is ideal for characterizing genetic differences between bacteria isolates of the same species to the strain level. Furthermore, the use of microarrays has been gaining importance in the detection of viral agents. Microarray approaches can be useful for groups with frequent viral infections like respiratory diseases, hemorrhagic fever, neurotropic infection, and HIV with associated viruses. The continuously increasing incidences of invasive fungal infections in immunocompromised patients emphasize the need to improve diagnostic tools. Currently, a relatively new way of fungi identification is performed using probes designed from the most commonly used gene targets as 28S fungal and regions of the rRNA genes such as the internal transcribed spacer.

Currently the development of microarray systems with DNA, proteins, antibodies, peptide, or aptamer molecules as probes has significant advancements in spite of some technical problems and now they have an impact not only in research but also in the clinic and in biomarker development.

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