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James V. Rogers
Editor

Microarrays

Principles, Applications and Technologies

Genetics - Research and Issues

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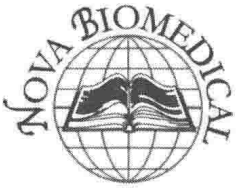
GENETICS - RESEARCH AND ISSUES

MICROARRAYS

PRINCIPLES, APPLICATIONS AND TECHNOLOGIES

JAMES V. ROGERS
EDITOR



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GENETICS - RESEARCH AND ISSUES

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Preface

In April 2003, the completion of the Human Genome Project was the capstone to a scientific journey that began decades earlier when Francis Crick and James Watson unraveled the mystery of the human genetic code. The sequencing of the human genome opened the flood gates of ideas and imagination to explore the structure and function of specific genes. Empowered researchers applied the fundamental principles of biology's central dogma to advance areas of scientific investigation ranging from genetics and evolution to molecular medicine, health, and disease.

The rapid advance of science and technology yielded the development of the microarray technique. Deciphering genetic sequences propelled the use of microarrays from a new fad in genome research to a ubiquitous tool in biological investigations. Turning genes on or off by the thousands critically orchestrates everyday biological processes and the amount of data generated from a single microarray experiment can be overwhelming, time-consuming, and tedious. By combining technological advances in microarrays with statistical and bioinformatics software, investigators have become armed with some of the most powerful means of biological problem-solving. Statistical reduction enables researchers to sort through the transcriptome and generate useable lists of significantly-modulated genes highlighting changes that chance alone cannot explain.

The underlying theme of this book is the use and application of microarray technology throughout the life sciences. This compilation of chapters provides an overview of how microarrays can be used to address diverse complex biological questions. In this collection, authors present information on using microarrays to unlock molecular mechanisms and gene expression patterns associated with research areas ranging from biodefense, pathological changes, and monitoring antimicrobial resistance genes to diagnostics, marine biodiversity, and dermal toxicology.

By using microarray technologies and specialized software, gene-expression profiles can provide insight into the development or evaluation of prophylactic and/or therapeutic treatments that target specific molecular pathways or genes. These data can ultimately lead to effective analyses using a molecular-systems biology approach that can feed into translational and predictive research. Due to the abundance of microarray data available in the open scientific literature, researchers can apply statistical approaches to identify biomarkers and perform risk assessments without the need of experimentation. In this book, authors utilized previously published data for the functional prediction and understanding of molecular

processes, the identification of reference genes using meta-analysis, and the development of a phenotypic anchoring model for drug-induced liver injury.

Biomarker-based discovery and risk assessment are important to provide mechanistic insight into factors as such as genetic background, diet, and lifestyle modulate the risk of disease. The continual development and refinement of improved molecular techniques to predict, treat, and prevent disease is critical to improving human health. Researchers must identify key components indicative of individuals or populations that pose an increased risk of infection or disease in order to achieve the goal of highly-individualized medical interventions (e.g., personalized medicine). For example, one chapter identifies an 18-gene signature that differentiates treatment responders and non-responders to interferon therapy. Based on the expression levels of this gene signature, a positive prediction value higher than 96% was observed for accurately predicting whether an HCV patient will or will not respond to interferon therapy. Moreover, another chapter describes the use microarrays to uncover mechanistic details underlying Alzheimer's disease pathology that track to cholesterol and lipid metabolism while highlighting the interactions between cholesterol and amyloid-β peptide.

Although using microarrays for monitoring changes in mRNA transcripts has dominated the application of this cutting-edge tool, other approaches for using microarrays include expression analysis of non-coding RNAs. Both microRNAs and long non-coding RNAs are involved in many biological processes by regulating transcriptional activity and subsequent processes. In this book, three chapters discuss the expression, annotation, and function of microRNAs and long non-coding RNAs to characterize macrophage microvesicle function, cutaneous inflammatory disorders, and elucidate their potential involvement in pathophysiological roles.

Almost every cell type within an organism contains the same genetic information locked up within DNA, but what makes these cells different from one another is based on the number, type, and magnitude of genes that are activated. The transcription of DNA to messenger RNA and subsequent translation into proteins is what leads to the unique phenotype exhibited by each cell, which could ultimately be normal or abnormal. To help differentiate one cell type from another, or differences among tissues, DNA microarrays have advanced the understanding of the genome with respect to structure and function. The implementation of microarrays and companion statistical and informatics tools is continually evolving and helping scientists discover new and exciting aspects of life science that will advance systems biology to a new level of understanding of how biological organisms function and respond to internal and external stimuli as well as provide insight into personalized or predictive medicine.

Contents

Preface		**vii**
Chapter 1	Microarray Technology for Category 'A' Bioterrorism Agent Research: Current Status and Perspectives *Lauren Huettner and Eric M. Vela*	**1**
Chapter 2	Similarity Threshold Selection Tools for Gene Co-Expression Networks *Liliana López-Kleine and Luis Guillermo Leal*	**23**
Chapter 3	Mining Gene Expression Microarrays for Long Non-Coding RNAs *L. Zhang, D. R. Wagner and Y. Devaux*	**37**
Chapter 4	An *In Situ* Electroporation and Flow Device for Mechanotransduction Studies *Zoltán Kis, Leila Towhidi, Henry Ip, Emmanuel Drakakis, Anil Bharath and Rob Krams*	**49**
Chapter 5	A New Application for Microarrays: Analysis of Global MicroRNA Expression Profiles in the Extracellular Microvesicles of Human Macrophage-Like Cells *Tadayoshi Kagiya and Masayuki Taira*	**69**
Chapter 6	Identification of Reference Genes by Meta-Microarray Analyses *Issac H. K. Too, Sean S. J. Heng, Oliver Y. W. Chan, Bryan M. H. Keng, Ching-Yee Chia, Claudia W. X. Lim, Wan-Ting Leong, Qinghao Chu, Ernest J. G. Ang, Yongjie Lin and Maurice H.T. Ling*	**81**
Chapter 7	Invertebrate DNA Chip: Opportunities and Challenges in the Development and Application of Microarrays for Marine Biodiversity Studies *Srujana Chitipothu, Alessia Cariani, Fabio Bertasi, Marco Stagioni, Marc Kochzius, Dietmar Blohm, Fausto Tinti and Monica Landi*	**101**

Chapter 8 Using Microarrays to Decipher the Virus-Host Interactions in HCV Infected Patients: Implications for Viral Resistance, Drug Development and Personalized Medicine **135**
Jing Sun, Jianguo Yuan, Bing Liu, Shilin Li, Yujia Li, Xiaoqiong Duan, Chunhui Yang, Peibing Zeng and Limin Chen

Chapter 9 Microarray Diagnostics for Orthopoxviruses, Filoviruses, Arenaviruses and Type A Influenza Virus **147**
Alexander N. Sinyakov and Vladimir A. Ryabinin

Chapter 10 Using DNA Microarray Technology to Monitor the Distribution of Antimicrobial Resistance Genes in *Escherichia Coli* Isolated from Animal Colibacillosis Outbreaks **187**
Pietro Badagliacca, Luke Masson, Alfreda Tonelli, Giacomo Migliorati and Josée Harel

Chapter 11 Phenotypic Anchoring of Liver Injury in Rats by Gene Expression Profiling **211**
Minjun Chen, Reagan Kelly, Lillian Tong, Jürgen Borlak and Weida Tong

Chapter 12 Application of Microarray Technology in Toxicology **231**
Abir T. El-Alfy

Chapter 13 Microarray Analysis of Gene Transcription Profiles in Chronic Rhinosinusitis **251**
Marcin Fraczek, Beata Rostkowska-Nadolska and Malgorzata Kapral

Chapter 14 Transcriptomic Analysis of Oligomeric Amyloid-β Treated Primary Cortical Neurons Reveals Dysregulation of Genes Involved in the Biosynthesis of Steroids and Cholesterol **269**
Bilal Malik, Cathy Fernandes, Richard Killick, Alessia Usardi, Ritchie Williamson, Stuart Kellie, Brian H. Anderton and C. Hugh Reynolds

Chapter 15 Application of Microarrays to Cutaneous Chemical Exposures **297**
James V. Rogers and Jennifer A. Price

Index **315**

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Chapter 1

Microarray Technology for Category 'A' Bioterrorism Agent Research: Current Status and Perspectives

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Abstract

Since the terrorist attacks and subsequent anthrax letters in the early 2000s, the United States has been on heightened alert for potential bioterrorist attacks, or the deliberate release of viruses, bacteria, or other agents used to cause morbidity and mortality in people, animals, or plants. Although many infectious pathogens could be employed in the event of an attack, certain pathogens known as category A bioterrorism agents pose the greatest risk to the public and national security, and could be potentially life-threatening if weaponized. Current pathogen detection and diagnostic methods are not ideal, providing results with poor speed and accuracy, and host-pathogen interaction molecular profiles have yet to be fully characterized. Microarray technology provides unique resources for the rapid and accurate detection and diagnosis of these pathogens, as well as a platform to allow researchers to create genetic profiles for epidemiological studies and study host-pathogen interactions for the development and testing of vaccines, therapeutics and prophylaxis.

Introduction

Microarray technology enables the study of interactions between hundreds to thousands of molecules simultaneously through the analysis of a very small amount of analytes and

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poses a unique way of characterizing how cells and organisms adapt to changes in the external environment [1]. Molecules are differentially expressed during various cellular pathways and mechanisms constantly occurring within an organism, and changes in the environment result in changes in molecular expression. Microarrays are used to analyze differences in gene expression levels, small nucleotide polymorphisms, protein and carbohydrate levels, as well as antibody expression. This method is not considerably different from other hybridization techniques, such as Northern and Southern blotting, but the unique aspect of microarray technology is the incredible amount of information that is obtained at one time. Historically, technology only allowed researchers to focus on a minimal number of analytes, but microarray technology allows for the concurrent study of a myriad of genes. The simultaneous and parallel determination of the expression of numerous analytes that microarray technology allows renders it a powerful laboratory tool [2]. Although microarray technology is still developing as a technique and must overcome many barriers, microarrays are quickly becoming successful, reliable and mature genomic tools for both biomedical and clinical research, along with epidemiological and clinical practices. Microarrays have revolutionized the current scientific research approaches involving various diseases, including heart disease, metabolic disease, mental illness, cancer biology, infectious disease, and bio- or agroterrorism by introducing a new platform for disease detection, diagnostics, the effects of pathogen infection, and vaccine and therapeutic drug development.

Microarray technology consists of a compilation of microscopic features affixed onto a small surface support, where probes with target molecules produce qualitative or quantitative data after being hybridized with test material. The type of array can be distinguished by characteristics including the nature of the probe, the solid-surface support used, and target detection [3]. For example, DNA microarrays are created by arranging minuscule amounts of thousands of gene sequences on a single microscope slide (a database of over 40,000 gene sequences is available to researchers). Once a gene is activated, the cell begins to copy specific segments of that gene and generate messenger RNA (mRNA) that is present in various cells and can be collected and labeled by using reverse transcriptase (RT) enzyme and fluorescent nucleotides. The process allows for the generation and labeling of complementary DNA (cDNA) to the respective mRNA. Once the cDNA is generated, it can be collected and placed on DNA microarray slides for hybridization commonly involving red or green fluorescent dyes. Specialized scanners can be used to measure the fluorescent intensity for each area on the microarray slide. An active gene produces more molecules of mRNA, resulting in more labeled-cDNA, which can in turn be hybridized to the DNA on the microarray slide to generate various levels of fluorescent intensity. Thus, less active genes produce fewer mRNA resulting in a lower level of fluorescent intensity. Measurement of fluorescence intensity signifies expression levels of a particular gene when compared to controls. For host-pathogen interactions, changes in gene expression occurs in various cells and tissues in response to a disease state, and the changes in gene expression are consistent when the host organism has been exposed to a specific infectious disease pathogen. These changes in gene expression may be specific to the pathogen and can be utilized as potential biomarkers to identify a clinical profile associated with the infectious disease, otherwise known as a "disease signature." Microarray technology offers a unique method to study host-pathogen interactions at a global level and can be used as a tool to delineate molecular mechanisms of tissue responses in host organisms infected with various pathogens. These

technologies may also offer the possibility of identifying new approaches to diagnostics, detection of disease, and development of new vaccines, therapeutics, and prophylaxis.

With the looming threats of infectious agents being used as bioweapons, it is clear that there is a strong need for better detection, diagnostic, and drug development tools. Since the terrorist attacks and subsequent anthrax letters in the early 2000s, the United States has been on heightened alert for potential bioterrorist attacks. Bioterrorism is defined by the Centers for Disease Control and Prevention (CDC) as the deliberate release of viruses, bacteria, or other agents used to cause illness or death in people, animals, or plants [4]. Bioterrorism agents are classified into categories, A, B, or C agents, each defined by how easily the pathogen can spread and the severity of morbidity and mortality that they may cause. Category A agents, the highest priority agents, pose the greatest risk to the public and national security due to four main factors: (1) the ease of person to person transmissibility; (2) infection may result in high mortality rates; (3) outbreaks of the pathogen may cause public panic; and (4) the pathogens require special action for public health preparedness [5]. These pathogenic agents include, but are not limited to *Bacillus anthracis* (anthrax), *Clostridium botulinum* toxin (botulism), *Yersinia pestis* (plague), Variola major (smallpox), *Francisella tularensis* (tularemia), and certain viral hemorrhagic fevers including Ebola virus, Marburg virus, Lassa virus and Machupo virus (Table 1) (6-9). Studies show that The United States is inadequately prepared to face further biological attacks (10). This can be partially attributed to the lack of government organization, but can also be due to the lack of appropriate vaccines, prophylaxis, therapeutics, and unperfected diagnostic tools. Although fairly new technology, microarrays have been increasingly utilized in scientific studies and the use of the technology in studies pertaining to category A bioterrorism agents has increased greatly since the 2001 attacks (Figure 1). Microarrays can help bridge the gap in the insufficient scientific knowledge of these deadly pathogens and have the potential to be key contributors in the development of new detection and diagnostic methods, and technologies aimed at developing new drugs, along with the determination of drug efficacy.

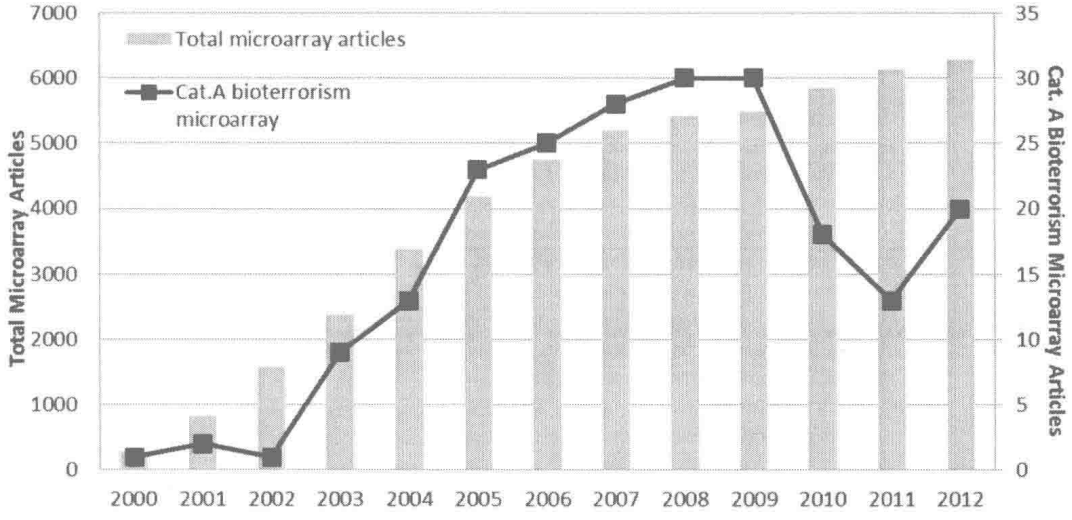

Figure 1. Microarray publications. The number of manuscripts published utilizing microarray technology (bars) and the number of microarray publications that have category A bioterrorism applications (line) are pictured.

Detection and Diagnostic Tools

The US Homeland Security Presidential Directive 21 defines biosurveillance as "the process of active data-gathering with appropriate analysis and interpretation of biosphere data that might relate to disease activity and threats to human or animal health—whether infectious, toxic, metabolic, or otherwise, and regardless of intentional or natural origin—in order to achieve early warning of health threats, early detection of health events, and overall situational awareness of disease activity"[5]. Conventional biosurveillance relies on the combined use of an automated system to evaluate case and suspect case reporting, biosensors, statistical surveillance, and data visualization of pre-diagnostic and diagnostic data for the earliest possible detection of biological events that may be indicative of a public health emergency. Alongside being able to rapidly and efficiently detect a potentially dangerous agent in the environment and various samples, the accurate diagnosis of individuals that have possibly been exposed to such agents is equally, if not more important. Being able to quickly distinguish an infection of a bioterrorism agent from any other pathogen is critical due to the fact that these agents have the ability to cause major disease, even death in a short period of time, and lead to a presentation of non-specific flu-like clinical symptoms, mostly clinically indistinguishable from the majority of diseases in the stages of early infection. Until recently, the use of traditional pathogen detection and diagnostic methods, such as biochemical assays, polymerase chain reaction (PCR), enrichment culture, serology, and microscopy, have been limiting due to their specific laboratory and time requirements, making them inefficient in addressing potential bioterrorism concerns [11]. Novel, more technologically advanced molecular detection and diagnostic methods, such as microarrays, provide the potential for rapid, highly sensitive and specific pathogen identification [12, 13] (Table 2).

Table 2. Microarrays used for the detection and diagnosis of category A bioterrorism agents and the main findings and conclusions from the studies

Microarray used	Category A agent(s) studied	Main findings and conclusion	Reference
DNA			
Oligonucleotide	*C. botulinum*	The array provides a rapid approach for the simultaneously detection of botulinum neurotoxin genes and determining the relatedness of type A *C. botulinum* strains isolated from different sources associated with a botulism outbreak.	10
	Y. pestis, B. anthracis	The oligonucleotide microarray can specifically detect and identify *Y. pestis* and *B. anthracis* from similar species in milk samples.	5
	Variola major	DNA from 52 samples of various ORV species were tested and accurately identified. The new microarray method is a valuable tool for the rapid and accurate detection and differentiation of various OPV species, including VARV species.	13
	Variola major, Ebola virus, Marburg virus	The presence of the viruses was confirmed after microarray analysis of nasopharyngeal aspirates, blood, urine, and tissue samples from persons infected.	17

Table 2. (Continued)

Microarray used	Category A agent(s) studied	Main findings and conclusion	Reference
Resequencing	*B. anthracis*	The arrays represent a "one reaction" genotyping technology that possesses the ability to discriminate highly similar isolates and other strains from *B. cereus* s.l.Clade 1.	12
	B. anthracis, Y. pestis, F. tularensis	The array platform can identify *B. anthracis*, *F. tularensis*, *Y. pestis* and distinguish them from similar species in a single assay.	14
	Ebola Zaire, Lassa Virus	The assay detects and identifies closely related strains of filoviruses of the Ebola Zaire group, or the Machupo and Lassa arenaviruses , but is also able to differentiate near neighbors of the same agent types.	15
Whole genome	Lassa virus	There are strong transcriptional changes in the immune response to LASV exposure, but this was paired with a lack of pro-inflammatory cytokine response. The results provide a foundation for biomarker identification for clinical diagnosis.	16
Multi-genome	*B. anthracis*	The sources of genome diversity and aspects of genome evolution of members of the *B. cereus* group, but especially *B. anthracis*, were elucidated, improving diagnostics and enabling the identification of biomarkers in the future.	11
Carbohydrate			
Multiplex (polysaccharide)	*B. anthracis, F. tularensis*	Antibodies resulting from infection of *B. anthracis* and *F. tularensis* were about to be detected and differentiated using this single platform.	19
Protein			
Multiplex (antibody-functionalized barcoded resins (BCRs))	*B. anthracis, F. tularensis*	Anthrax PA, *F. tularensis* lipopolysaccharide and CD14 antigens were accurately identified and quantified in tetraplex assays, showing potential for the use of BCRs as multiples antigen detection and identification on a microarray format.	21
ELISA based antibody	*C. botulinum*	Simultaneous detection of BoNT serotypes A, B, C, D, E, and F was achieved with high sensitivity providing a rapid detection tool for *C. botulinum* toxins in various samples.	22

DNA Arrays

DNA microarrays have been shown to be an effective high-throughput detection and diagnostic tool for the identification of pathogenic microorganisms in clinical, environmental, food, and water samples and have received considerable attention due to the ability to simultaneously analyze a very large number of nucleic acid sequence targets and detect multiple genetic targets or genomes from multiple pathogens on a single slide [14]. The assays are characterized based on the type of DNA fragments that are used on the array, such as complementary DNA (cDNA) or oligonucleotides [2]. DNA microarray technology allows for the entire genome of one or multiple organisms to be studied at one time with relatively simple techniques.

Foodborne Detection

The public food supply is considered a vulnerable target for bioterrorism attacks, and events related to the deliberate contamination of food using conventional pathogens, such as Escherichia coli O157:H7 and Salmonella, but also nonconventional foodborne pathogens, such as weaponized category A agents [11]. Early detection of disease inducing pathogens in the food supply is critical in the event of an attack or a potential natural outbreak in order to spare the susceptible public populations from a potentially life threatening disease and to spare the food industry from economic havoc due to loss of productivity and business, legal issues, cost of medical treatments for the infected, and recall and destruction of contaminated products [15]. Conventional pathogen detection methods such as culture methods, polymerase chain reaction (PCR) and immunology-based methods may take hours to days to produce a definitive result [16]. Microarray technology offers a high throughput technique to detect multiple foodborne pathogens in food samples and poses as a useful laboratory tool to rapidly detect agents and eliminate contaminated products, preventing public consumption of harmful food items. Oligonucleotide DNA microarrays have been utilized in the detection of a variety of category A bioterrorism agents that have the potential to contaminate the public food supply, including *B. anthracis*, *C. botulinum*, and *Y. pestis*. A focused oligonucleotide microarray format was used by investigators to provide a rapid approach for neurotoxin gene detection and preliminary determination of the relatedness of strains isolated from different food sources associated with a *C. botulinum* outbreak [17]. The focused microarray provides useful information beyond the toxin type of the strain involved with a particular outbreak, namely epidemiological associations in an outbreak investigation [17]. Similarly to the previous study, a new generation low density oligonucleotide microarray was created to allow the simultaneous detection and identification of *Y. pestis* and *B. anthracis* to be used in a foodborne application [11]. The microarray was designated as the "Y-PESTIS/B-ANTHRACIS 4x2K Array" and contains 533 and 1,707 probes for *Y. pestis* and *B. anthracis*, respectively, that target the pathogen's virulence genes [11]. The specific targeting of these genes provides information on the virulence potential of strains implicated, rendering the microarray especially useful in the detection and profiling of potentially life threatening biothreat agents.

Pathogen Differentiation and Clinical Diagnosis

The Bacillus cereus sensu lato group includes multiple species including *B. cereus*, *B. thuringiensis, B. mycoides*, *B. pseudomycoides*, *B. weihenstephanensis* and *B. anthracis*, the causative agent of anthrax. These species share 99% sequence identity and belong to one phylogenetic group [18]. A multi-genome DNA microarray was utilized to investigate *B. cereus* group genomic diversity and to elucidate events associated with the emergence of *B. anthracis* [18]. The study found indications that the evolution of *B. anthracis* was associated with the acquisition and/or maintenance of a limited but specific set of chromosomally-encoded virulence-associated genes along with the genes encoded on the virulence plasmids. The data obtained from the study allows for the better understanding of events associated with the evolution of *B. anthracis* and will help to improve diagnostics for the pathogen. Similarly, a customized resequencing DNA microarray was used to distinguish infectious *B. anthracis*

from closely related B. cereus sensu lato strains utilizing isolated genomic DNA from each strain and results were compared with multi-locus sequencing typing (MLST) data, the traditional method of DNA genotyping [19]. The resulting data suggested that *B. anthracis* resequencing microarray technology is the most effective and valuable tool for genotyping Clade 1 *B. cereus* strains along with strains within the *B. anthracis* lineage, offering advantages over MLST. Other methods of species identification, including amplified fragment length polymorphism analysis and multi-locus sequence typing, are numerous, but have a lower genetic resolution sensitivity level [19].

An oligonucleotide DNA microarray method was developed for simultaneous detection and identification of six species of Orthopoxvirus (OPV) including Variola (VARV), Monkeypox (MPXV), Cowpox, Camelpox, Vaccinia (VACV), and Ectromelia (ECTV) viruses (20). The method allows for the discrimination of OPV species from other pathogens that cause infections with clinical manifestations similar to OPV infections. Being able to detect and differentiate OPV species is especially important in biodefense due to the fact that the majority of the world population does not have protective immunity to the viruses after the discontinuation of mass vaccination and is susceptible to deadly infection caused by various OPV, mainly VARV, the causative agent of smallpox. Other methods to discriminate OPV include differentiation of viral protein profiles using Western blotting and other serological assays, which require production, isolation, and unambiguous typing of live virus, which can prove difficult. PCR-based methods are also utilized, allowing for rapid and sensitive detection, but do not have output capacity comparable to microarray technology [20].

The respiratory pathogen microarray version 1 (RPM v.1) chip, a resequencing DNA microarray, was utilized under blinded conditions to detect and discriminate different strains of B. anthracis, Y. pestis, and F. tularensis in clinical samples [21]. Resequencing arrays were developed to combine the multiplexing power of standard microarrays with the discrimination capabilities of sequence analysis, while they offer the capability of having a single test that detects and discriminates between a target pathogen and its genetically similar pathogens. The RPM v.1 chip was primarily designed to identify common respiratory pathogens and has eleven targets devoted to the detection of six CDC category A bioterrorism agents that may present with nonspecific, flu-like symptoms at early stages of infection. The study demonstrated the potential of resequencing arrays to detect and identify specific sequences from a variety of inactivated biothreat organisms [21]. Similarly, a resequencing microarray (a broad-range resequencing pathogen microarray [RPM] for detection of tropical and emerging infectious agents [TEI] including biothreat agents: RPM-TEI v 1.0) was used to detect and differentiate multiple hemorrhagic fever-inducing filoviruses and arenaviruses, including Ebola Zaire and Lassa virus (LASV), respectively, along with multiple other infectious agents with high specificity and sensitivity [22]. LASV was further studied by utilizing a whole genome microarray to elucidate temporal host response in the peripheral blood mononuclear cells (PBMCs) of non-human primates following aerosol exposure to the virus [23]. The data obtained allowed the investigators to further elucidate the host immune responses during LASV infection and provided a foundation for LASV specific biomarker identification, a potential method of clinical diagnosis. A microarray platform, the GreeneChip microarray system, was designed for pathogen surveillance and discovery of multiple viral and bacterial infectious pathogens, including OPVs, Ebola virus (EBOV), and Marburg virus (MARV) [24]. The GreeneChip technology introduces sample preparation and

labeling methods that enhance sensitivity. The new array has been used in an outbreak investigation when other methods failed to detect a microorganism in a fatal hemorrhagic fever case and has been validated with cultured viral isolates and tested with blood, respiratory, urine, and tissue samples containing the viral pathogens [24].

Carbohydrate Arrays

The use of carbohydrate microarray technology for the detection of bacterial and viral infections is growing in interest. The technology holds great promise as a high-throughput means of detecting the interactions of proteins with diverse oligosaccharide sequences of glycoproteins, glycolipids and polysaccharides [25]. With increasing awareness of post-genome glycosylation as a potentially important form of post-translational modification of proteins, carbohydrate microarray technology is especially needed for the detection and characterization of carbohydrate ligands, which are largely diverse and unable to be cloned in the case of oligosaccharide ligands. Because of these limitations, conventional methods are not ideal and high-throughput microarray technology is being utilized to analyze carbohydrate-protein interactions to elucidate mechanisms of infection, inflammation, and immunity [25]. A microarray platform was developed in 2008 using bacterial 'signature' carbohydrates associated with *B. anthracis* and *F. tularensis* infection [26]. The trisaccharide derived from *F. tularensis* was converted to glycosylamines by reductive amination and was then immobilized on epoxide-functionalized glass slides along with anthrose, a *B. anthracis* specific carbohydrate, and the corresponding tetrasaccharides attached to a linker containing –NH2 group. The microarray was used to specifically detect antibodies in the serum of melioidosis patients, or animals immunized with the causative bacteria of tularemia or anthrax [26].

Protein Arrays

Protein microarrays, or protein chips, are miniaturized, parallel assay systems that contain small amounts of purified proteins in a high-density format [27]. Protein microarray technology offers a post-genomic strategy to investigate host-pathogen interactions by identifying multiple protein-molecule interactions as well as immunogenic characteristics of the host at any stage of disease. There are two types of protein microarrays: analytical and functional. Analytical protein microarrays are composed of well-characterized biomolecules with specific binding activities, such as antibodies, to analyze the components of complex biological samples or to determine whether a sample contains a specific protein of interest and have been used for protein expression profiling, biomarker identification, clinical diagnosis, and environmental/food safety analysis. Functional protein arrays are used for drug target identification/validation and studies of protein interaction, biochemical activity, and immune responses [27]. A multiplex assay based on antibody-functionalized barcoded resins (BCRs) was used to detect antibodies against human pathogens *F. tularensis* lipopolysaccharide (LPS) and *B. anthracis* protective antigen (PA) toxin, demonstrating an alternative platform for multiplex immunoassays capable of detecting antigens [28]. Enzyme-linked immunosorbent assay (ELISA)-based protein microarrays allow for the simultaneous