

BIOSENSORS

1. Introduction

The history of biosensors started in the year 1962 with the development of enzyme electrodes by the scientist Leland C. Clark. Since then, research communities from various fields such as VLSI, Physics, Chemistry, and Material Science have come together to develop more sophisticated, reliable and mature biosensing devices for applications in the fields of medicine, agriculture, biotechnology, as well as the military and bioterrorism detection and prevention [1]. Biosensor is a device that consists of two main parts: A bioreceptor and a transducer. Bioreceptor is a biological component (tissue, microorganisms, organelles, cell receptors, enzymes, antibodies, nucleic acids, etc) that recognizes the target analyte. Other part is transducer, a physicochemical detector component that converts the recognition event into a measurable signal [2 and 3]. The function of a biosensor depends on the biochemical specificity of the biologically active material. The choice of the biological material will depend on a number of factors via the specificity, storage, operational and environmental stability [2 and 4]. Biosensors can have a variety of biomedical, industry, and military applications. The major application so far is in blood glucose sensing because of its abundant market potential [1 and 5]. Biomolecules such as enzymes, antibodies, receptors, organelles and microorganisms as well as animal and plant cells or tissues have been used as biological sensing elements [2]. Microorganisms have been integrated with a variety of transducers such as amperometric, potentiometric, calorimetric, conductimetric, colorimetric, luminescence and fluorescence to construct biosensor devices [3, 6 and 7]. In this paper, we review recent development in use of biosensors as a diagnostic tool, as well as some future applications of biosensor technology.

2. Types of Biosensors

2.1. Resonant Biosensor

In this type of biosensor, an acoustic wave transducer is coupled with an antibody (bio-element). When the analyte molecule (or antigen) gets attached to the membrane, the mass of the membrane changes. The resulting change in the mass subsequently changes the resonant frequency of the transducer. This frequency change is then measured [8].

2.2. Optical biosensors

The output transduced signal that is measured is light for this type of biosensor. The biosensor can be made based on optical diffraction or electrochemiluminescence. Optical transducers are particularly attractive for application to direct (label-free) detection of bacteria. These sensors are able to detect minute changes in the refractive index or thickness which occur when cells bind to receptors immobilized on the transducer surface. They correlate changes in concentration, mass or number of molecules to direct changes in characteristics of light. Several optical techniques have been reported for detection of bacterial pathogens including: monomode dielectric waveguides, surface plasmon resonance (SPR), ellipsometry, the resonant mirror and the interferometer etc [9-11].

2.2.1. Surface plasmon resonance (SPR) biosensor

This is an evanescent field based optical sensors using thin gold film for sensing applications. The interaction between analyte flowing over immobilized interactant on gold surface is probed through the detection of reflection minima on photo-detector array sensors. SPR has successfully been applied to the detection of pathogen bacteria by means of immunoreactions [11 and 12].

2.2.2. Piezoelectric biosensors

Piezoelectric (PZ) biosensor offers a real-time output, simplicity of use and cost effectiveness. The general idea is based on coating the surface of the PZ sensor with a selectively binding substance, for example, antibodies to bacteria, and then placing it in a solution containing bacteria. The bacteria will bind to the antibodies and the mass of the crystal will increase while the resonance frequency of oscillation will decrease proportionally [9 and 11].

2.3. Thermal Biosensors

This type of biosensor is exploiting one of the fundamental properties of biological reactions, namely absorption or production of heat, which in turn changes the temperature of the medium in which the reaction takes place. They are constructed by combining immobilized enzyme molecules with temperature sensors. When the analyte comes in contact with the enzyme, the heat reaction of the enzyme is measured and is calibrated against the analyte concentration. Common applications of this type of biosensor include the detection of pesticides and pathogenic bacteria [11].

2.4. Electrochemical Biosensors

Electrochemical biosensors are mainly used for the detection of hybridized DNA, DNA-binding drugs, glucose concentration, etc. Electrochemical biosensors can be classified based on the measuring electrical parameters as: (i) conductimetric, (ii) amperometric and (iii) potentiometric. Compared to optical methods, electrochemistry allows the analyst to work with turbid samples, and the capital cost of equipment is much lower. On the other hand, electrochemical methods present slightly more limited selectivity and sensitivity than their optical counterparts [9 and 13].

2.4.1. Conductimetric Biosensors

The measured parameter is the electrical conductance/resistance of the solution. When electrochemical reactions produce ions or electrons, the overall conductivity or resistivity of the solution changes. This change is measured and calibrated to a proper scale. Conductance measurements have relatively low sensitivity [9].

2.4.2. Amperometric Biosensors

This is perhaps the most common electrochemical detection method used in biosensors. This high sensitivity biosensor can detect electroactive species present in biological test samples. Amperometric biosensors produce a current proportional to the concentration of the

substance to be detected. The most common amperometric biosensors use the Clark Oxygen electrode [9 and 11].

2.4.3. Potentiometric Biosensors

These are the least common of all biosensors, but different strategies may be found nonetheless in this type of sensor the measured parameter is oxidation or reduction potential of an electrochemical reaction. The working principle relies on the fact that when a voltage is applied to an electrode in solution, a current flow occurs because of electrochemical reactions. The voltage at which these reactions occur indicates a particular reaction and particular species [2 and 11].

2.5. Bioluminescence sensors

Recent advances in bioanalytical sensors have led to the utilization of the ability of certain enzymes to emit photons as a byproduct of their reactions. This phenomenon is known as bioluminescence. The potential applications of bioluminescence for bacterial detection were initiated by the development of luciferase reporter phages. The bacterial luminescence *lux* gene has been widely applied as a reporter either in an inducible or constitutive manner. In the inducible manner, the reporter *lux* gene is fused to a promoter regulated by the concentration of a compound of interest. As a result, the concentration of the compound can be quantitatively analyzed by detecting the bioluminescence intensity. Bioluminescence systems have been used for detection of a wide range of microorganisms [2 and 11].

2.6. Nucleic Acid-based Biosensors

A nucleic acid biosensor is an analytical device that integrates an oligonucleotide with a signal transducer. The nucleic acid probe is immobilized on the transducer and acts as the bio-recognition molecule to detect DNA/RNA fragments [11].

2.7. Nanobiosensors

Nanosensors can be defined as sensors based on nanotechnology. Development of nanobiosensor is one of the most recent advancement in the field of Nanotechnology. The silver and certain other noble metal nanoparticles have many important applications in the field of biolabelling, drug delivery system, filters and also antimicrobial drugs, sensors [14].

3. Microbial Biosensors

Microbes have a number of advantages as biological sensing materials in the fabrication of biosensors. They are present ubiquitously and are able to metabolize a wide range of chemical compounds. Microorganisms have a great capacity to adapt to adverse conditions and to develop the ability to degrade new molecules with time. Microbes are also amenable for genetic modifications through mutation or through recombinant DNA technology and serve as an economical source of intracellular enzymes. Purified enzymes have been most commonly used in the construction of biosensors due to their high specific activities as well

as high analytical specificity. Over 90% of the enzymes known to date are intracellular. In this respect, the utilization of whole cells as a source of intracellular enzymes has been shown to be a better alternative to purified enzymes in various industrial processes. Whole cells have been used either in a viable or non-viable form. Viable cells are gaining considerable importance in the fabrication of biosensors. Viable microbes metabolize various organic compounds either anaerobically or aerobically resulting in various end products like ammonia, carbon dioxide, acids etc that can be monitored using a variety of transducers. Viable cells are mainly used when the overall substrate assimilation capacity of microorganisms is taken as an index of respiratory metabolic activity, as in the case of estimation of biological oxygen demand (BOD) or utilization of other growth or metabolically related nutrients like vitamins, sugars, organic acids and nitrogenous compounds. Another mechanism used for the viable microbial biosensor involves the inhibition of microbial respiration by the analyte of interest, like environmental pollutants. The major application of microbial biosensors is in the environmental field [4 and 15]. Environmental applications of biosensors include the detection of harmful bacteria or pesticides in air, water, or food. A microbial biosensor consisting of an oxygen microelectrode with microbial cells immobilized in polyvinyl alcohol has been fabricated for the measurement of bioavailable organic carbon in toxic sediments. Microbial biosensors have been developed for assaying BOD, a value related to total content of organic materials in wastewater. BOD sensors take advantage of the high reaction rates of microorganisms interfaced to electrodes to measure the oxygen depletion rates [2, 16 and 17].

4. Biosensors and cancer

Cancer diagnosis and treatment are of great interest due to the widespread occurrence of the diseases, high death rate, and recurrence after treatment. According to the National Vital Statistics Reports, from 2002 to 2006 the rate of incidence (per 100,000 persons) of cancer in White people was 470.6, in Black people 493.6, in Asians 311.1, indicating that cancer is wide-spread among all races. Cancer can take over 200 distinct forms, including lung, prostate, breast, ovarian, hematologic, skin, and colon cancer, and leukemia, and both environmental factors, and genetic factors are associated with an increased risk of developing cancer. Bacterial and viral infections are also strongly associated with some types of cancer [18 and 19]. In medicine, biosensors can be used to monitor blood glucose levels in diabetics, detect pathogens, and diagnose and monitor cancer [20]. The use of emerging biosensor technology could be instrumental in early cancer detection and more effective treatments, particularly for those cancers that are typically diagnosed at late stages and respond poorly to treatment, resulting in improvements in patient quality of life and overall chance of survival [19]. By measuring levels of certain proteins expressed and/or secreted by tumor cells, biosensors can detect whether a tumor is present, whether it is benign or cancerous, and whether treatment has been effective in reducing or eliminating cancerous cells [19 and 20].

5. Biosensors and Pathogen detection

Bacteria, viruses and other microorganisms are found widely in nature and environment. Microbial diseases constitute the major cause of deaths in developing countries [11]. Pathogen detection is of the utmost importance primarily for health and safety reasons. Polymerase chain reaction (PCR), culture and colony counting methods as well as immunology-based methods are the most common tools used for pathogen detection. They involve DNA analysis, counting of bacteria and antigen-antibody interactions, respectively. In spite of disadvantages such as the time required for the analysis or the complexity of their use, they still represent a field where progress is possible. Biosensors have recently been defined as analytical devices incorporating a biological material intimately associated with or integrated within a physicochemical transducer or transducing microsystem, which may be optical, electrochemical, thermometric, piezoelectric, magnetic or micromechanical [9 and 21]. There are three main classes of biological recognition elements which are used in biosensor applications. These are enzymes, antibodies and, nucleic acids. In the detection of pathogenic bacteria, however, enzymes tend to function as labels rather than actual bacterial recognition elements. Enzymes can be used to label either antibodies or DNA probes much in the same fashion as in an ELISA assay. In the case of amperometric biosensors enzymatic labels are critical. More advanced techniques may operate without labelling the recognition element, such as the case of surface plasmon resonance (SPR), piezoelectric or impedimetric biosensors. The use of antibodies in biosensors is currently more spread than that of DNA probes, the following sections deal mainly with antibody-based biosensors. Fig.1 shows the three most frequent antibody immobilisation routes, which are:

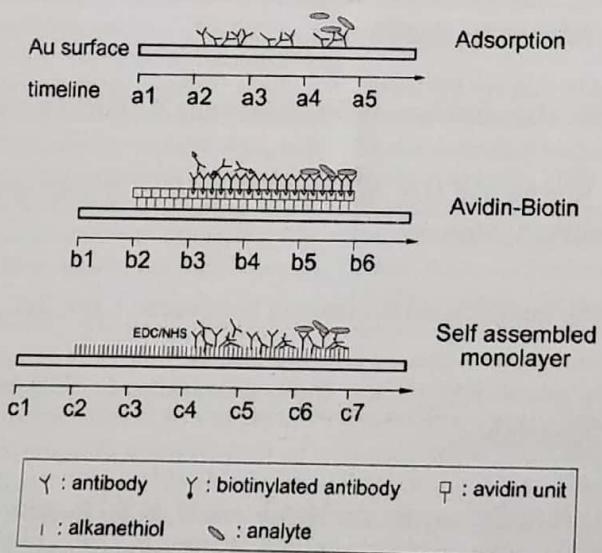


Figure 1. Main immobilization strategies

Different types of biosensor are being employed for detection of pathogenic microbes. Piezoelectric immunosensors were developed for *Listeria monocytogenes* and members of the Enterobacteriaceae family etc [22]. In the immunogravimetric microbial assay, a PZ crystal

coated with anti-*C. albicans* antibody was used for the detection of *C. albicans* concentrations in the range of 106–108 cells/ml [22]. In study pyle *et al.*, Indirect detection of *Escherichia coli* O157:H7 by fluorescent labeled antibody method [23]. Amperometric biosensors have been developed for indirect detection of *E. coli* by Nakamura *et al.* Brooks *et al.*, developed amperometric biosensor for Salmonella detection [24 and 25]. Light addressable potentiometric sensor array have been developed for *Neisseria meningitidis*, *Brucella melitensis* by Lee *et al.* [26]. Nucleic acid hybridization based biosensor schemes are being developed for pathogens such as *E. coli* and *Mycobacterium tuberculosis*. Bioluminescence systems have been used for detection of a wide range of microorganisms [11].

6. Conclusion

Biosensors have been miniaturised extensively in the recent years. Keeping in line with such developments, microbial cells with high enzyme activities may be required. This is essential especially when microbial cells are used as substitutes to enzyme based sensors. Microorganisms, due to their low cost, long lifetime and wide range of suitable pH and temperature, have been widely employed as the biosensing element in the construction of biosensors.

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Improving health care and laboratory medicine: the past, present, and future of molecular diagnostics

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In vitro diagnostics has many different disciplines. Histology tests are classic for diagnosis, followed by chemistry and serology tests. The newest in vitro diagnostic tools are molecular. These clinical tests are used to determine therapy type, duration, and dose; they are also used to determine if therapy has been successful. The choice of test depends upon the question being asked.

As molecular diagnostics has evolved, it has demonstrated clear advantages over some traditional methods, although it does not completely replace other methods. Molecular methods are extremely sensitive, which is critical for the direct detection of viral nucleic acids. When a pathologist states that patients have undetectable virus, that does not mean that they have no virus in their systems. It means that the level of virus is below the limits of detection for the assay used. A robust and sensitive assay can provide more useful information. Other advantages are that molecular assays often require minimal sample volumes and do not require culture. Molecular methods can be very accurate, as they measure DNA or RNA sequences specific for the virus or mutation of interest. Finally, molecular diagnostics usually offers tremendous time savings in the laboratory, allowing clinicians earlier access to data and thus earlier treatment for patients.

Nucleic acid amplification is the key to molecular diagnostics. A molecular test for a virus must detect a specific RNA or DNA sequence in the midst of a tremendous amount of other nucleic acids: human genomic DNA, mRNA, bacterial DNA, and RNA. This process is like pulling the proverbial needle out of the haystack. The power of a nucleic acid amplification technique is that it can amplify a specific sequence and do so in a predictable manner so that the number of molecules in the initial sample can be quantified.

Polymerase chain reaction (PCR) is the gold standard for amplification processes in diagnostics. Since the technique was first published in 1985 (1), it has become the most widely used nucleic acid amplification technology. With PCR, a target sequence of nucleic acid is amplified through a repetitive series of reactions catalyzed by a single enzyme, a thermal stable nucleic acid polymerase. PCR can amplify both DNA and RNA.

The process of PCR typically involves taking the sample through three different temperatures, each of which causes a different event. The sample is placed into a tube with the proper primers, thermal stable polymerase, nucleotides, and buffers. In the first step, denaturation by heating to 95°C causes the double-stranded DNA to form single strands. In the second step, the temperature is dropped to 55°C to 60°C, which allows the target-

specific primer to bind to only the intended nucleic acid sequence. The specificity of an assay depends on the quality of primer choice. Finally, in a third step, the temperature of the reaction is raised to about 72°C and the polymerase synthesizes an identical copy of the target sequence, called an amplicon. The cycle is repeated many times through the three different temperatures. Everything occurs rapidly in a closed tube in a thermocycling instrument.

PCR causes exponential amplification of a target nucleic acid region. Starting with 1 copy of a molecule leads to 16 amplicons after 4 cycles. Starting with 2 copies leads to 32—4 copies after the first cycle, 8 after the second cycle, 16 after the third cycle, and finally 32 after the fourth. Clinicians do not know how many copies are present in a sample but use the result to extrapolate back. If they found 96 amplicons after 4 cycles, they could determine that the sample had 6 copies initially. Instrumentation now allows this to be done automatically.

Roche has developed a portfolio of PCR business units based on disease areas. In six areas, an expertise is already present: virology, women's health, genomics, microbiology, blood screening, and oncology. This article discusses applications of molecular diagnostics in these fields.

VIROLOGY: HIV AND HEPATITIS

The effectiveness of HIV drugs has been determined by molecular methods, which measure the viral loads. The PCR-based Roche Amplicor (1992) and COBAS Amplicor (1995) HIV-1 Monitor tests were instrumental in the Food and Drug Administration (FDA) approval of most HIV drugs on the market. In March 2004, the National Institutes of Health released revised Guidelines for the Use of Antiretroviral Agents in HIV-1 Infected Adults and Adolescents (2). The document stated: "The goal of therapy is suppression of viral replication to below the level of detection." Whereas the 2002 guidelines said that physicians should use the most sensitive assay available, the 2004 guidelines stated: "Results of therapy are evaluated through plasma HIV RNA levels." These statements highlight the clinical utility and importance of diagnostics in directing and monitoring therapy.

From Roche Molecular Systems, Pleasanton, California.

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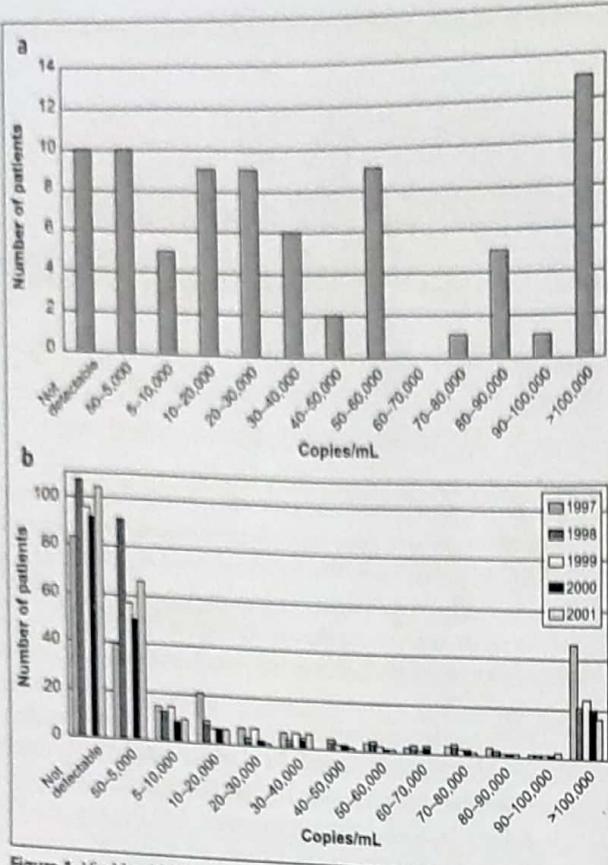


Figure 1. Viral load (a) in 1996, before molecular viral load testing, and (b) from 1997 to 2001, when molecular viral load testing was routinely used. With the molecular data, clinicians were able to better control viral loads of their patients with HIV by adjusting therapy. Data courtesy of Dr. Essmyer, St. Luke's Medical Center, Kansas City.

Because of its increased sensitivity, the Amplicor HIV-1 Monitor assay can reveal earlier when the antiretroviral therapy is failing, because viral loads that would have been below the limits of detectability with another assay are detectable with PCR. Such knowledge helps the clinician intervene earlier and keep the patient healthy.

Dr. Essmyer from St. Luke's Medical Center in Kansas City shared her data with me. In 1996, before HIV viral load testing was used to direct therapy, the patient pool had widely variable viral loads, and therapy was not controlling viral replication. Between 1997 and 2001, physicians began routine use of viral load testing to monitor treatment and adjust antiretroviral regimens. With such informed treatment, almost all patients had nondetectable viral loads (Figure 1). Those patients who continued to have a high viral load (>100,000) appear to have drug-resistant virus.

Hepatitis C virus (HCV) is another growing global health problem. Estimates suggest that 170 million to 200 million people worldwide are carriers. HCV is four times more prevalent than HIV. As many as two thirds of individuals infected with HCV have no symptoms and are therefore unaware of their infection. Unfortunately, symptoms usually appear decades after the virus was acquired. Over 280,000 deaths annually are associated with the effects of HCV infection, such as cirrhosis, fibrosis of the liver, and hepatocellular cancer (3-6).

There are three major types of molecular testing: qualitative, quantitative, and genotyping. Each helps answer a different clinical question. A highly sensitive HCV qualitative assay can

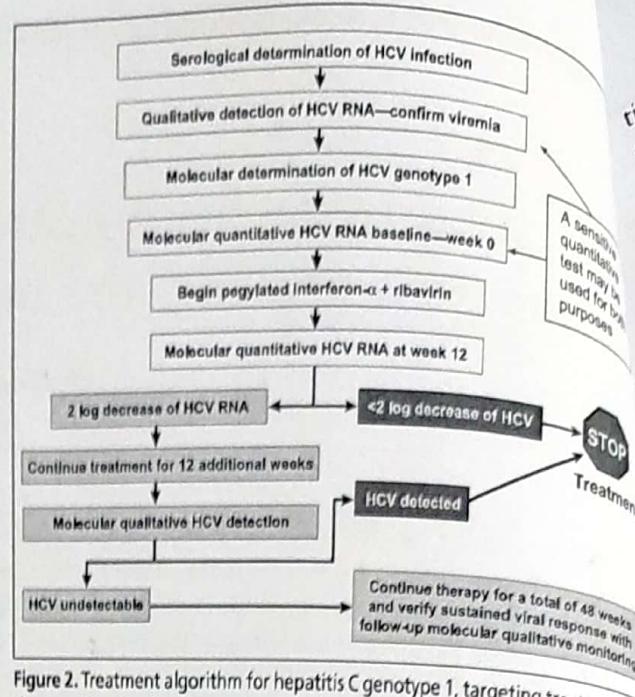


Figure 2. Treatment algorithm for hepatitis C genotype 1, targeting treatment with the use of molecular diagnostics. Based on reference 8.

determine if HCV is present and active; a quantitative viral load test can determine how much HCV is present; and a genotype assay can identify the HCV type. Diagnostics is quickly changing with the introduction of real-time PCR and the development of new clinical tests.

A treatment algorithm is used by many physicians for patients with genotype 1 HCV (Figure 2). Molecular diagnostic tests are critical in determining treatment course and duration. These tests help physicians identify patients who are unlikely to respond to therapy in the long term and justify the discontinuation of therapy. The process begins with the serological determination of HCV exposure and a molecular test to detect the presence of HCV RNA, which indicates an active infection. Since newer real-time PCR quantitative molecular tests have excellent sensitivity, some laboratories now use quantitative tests to confirm viremia. Then, if genotyping reveals HCV genotype 1, the original quantitative test result can be used as the baseline viral load before treatment. If a qualitative test is used to confirm viremia, a quantitative test should be performed before treatment to determine dosing of ribavirin. After 12 weeks of treatment with pegylated interferon and ribavirin, another quantitative assay is performed. If the patient's HCV RNA levels do not drop by two logs, therapy may be stopped, since the chance of achieving a sustained viral response with further treatment is very low. If a two-log drop is achieved, another 12 weeks of therapy should bring the HCV RNA levels to below detectable limits. A complete round of therapy—48 weeks—will often achieve a sustained viral response in patients who attain the 12- and 24-week milestones. In monoinfected genotype-1 HCV patients, 40% to 50% of patients have been reported to have a sustained viral response (7).

Use of the algorithm can result in cost savings and decreased morbidity. The retail cost of 48 weeks of treatment of pegylated interferon and ribavirin is \$29,000. Discontinuing therapy after 12 weeks in the group that has been shown not to benefit translates to a savings of \$21,750 per patient. When therapy is discontinued at 24 weeks, the savings is \$14,500. Further, the therapy has significant side effects; because of the morbidity, pa-

patient compliance is often low. It is easier to motivate patients to try the treatment for the shorter time periods when they know that its effectiveness will be evaluated. Similarly, those in whom the treatment is effective become motivated by the test result since continued treatment may lead to a sustained viral response.

A good example of the failure of serological tests alone is shown by a case study provided by Dr. Wohlfleifer from Florida. A 51-year-old man had been HIV positive for more than 15 years. He lived with an HIV+, HCV+ partner for 18 years. The patient was taking several antiretroviral drugs and had a CD4 count of 329 cells/mm³ (22.5%). His alanine aminotransferase was 304 IU/L, and his aspartate aminotransferase was 158 IU/L. Hepatitis panels showed the presence of hepatitis B and hepatitis A, but not HCV. The physician ordered a serum test of HCV antibody and a quantitative HCV RNA test. When the former test result came back as negative, the laboratory decided not to run the HCV RNA test. Dr. Wohlfleifer persisted in his request, and the result of the HCV RNA assay was amazingly high: a viral load of >850,000 IU/mL. Apparently, the HIV infection had affected the patient's immune response to HCV, explaining why antibodies were not detected. Without the molecular test, this patient would have been undiagnosed with active HCV infection.

WOMEN'S HEALTH: CHLAMYDIA

Roche has a PCR test that detects *Chlamydia trachomatis* and *Neisseria gonorrhoeae* at the same time from the same sample. A number of regulatory bodies and associations have recommended widespread screening for these diseases (8). For example, the Health Plan Employer Data and Information Set and the Centers for Disease Control and Prevention recommend screening all sexually active women up to age 25 for chlamydia and screening older women if risk factors are present (9).

The screening guidelines reflect the occurrence of the disease. For biologic reasons, women in their teens and twenties are particularly susceptible to this infection (Figure 3). Nationwide screening of women at a family planning clinic showed that in 5 states and territories, the prevalence rate was <4.0%; in 11, it was 4.0% to 4.9%; and in 37, it was >5.0%. Texas' rate in this study was 8.2% (10).

Rates of chlamydial infections rose significantly between 1987 and 2001. Several factors played a role in this rise, including increased awareness of the need for screening in public and private health care settings, improvement in the sensitivity of diagnostic tests, and improved surveillance and reporting systems. Screening does make a difference. A large-scale screening program initiated in family planning clinics of region 10 of the Department of Health and Human Services (i.e., Alaska, Idaho, Oregon, and Washington) has been followed by a 60% reduction in chlamydial infection rates. Other screening programs have been initiated based on such demonstration projects (11).

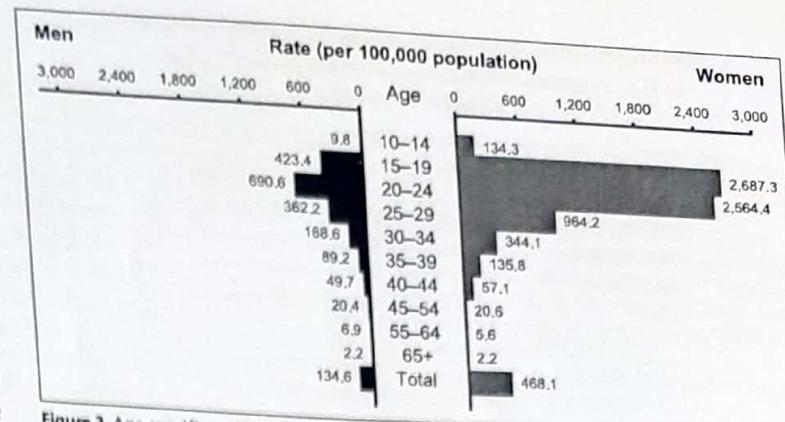


Figure 3. Age-specific and sex-specific rates of chlamydial infection in the USA, 2003. Source: Division of STD Prevention, Centers for Disease Control and Prevention.

Table 1. Comparison of diagnostic tests for chlamydia and gonorrhea

Test	Advantages	Disadvantages
Culture	<ul style="list-style-type: none"> Specificity nears 100%, thereby reducing the potential for false-positive results 	<ul style="list-style-type: none"> Requires a skilled laboratorian; is labor intensive and expensive Sensitivity is about 80% Cervical specimens only
DNA probe	<ul style="list-style-type: none"> More stable transport of specimens Less expensive than culture 	<ul style="list-style-type: none"> Cervical specimens only Sensitivity is about 65%
Enzyme-linked immunosorbent assay	<ul style="list-style-type: none"> Less technically demanding than culture Less expensive 	<ul style="list-style-type: none"> Cervical specimens only Sensitivity is about 60%
Nucleic acid amplification	<ul style="list-style-type: none"> >90% sensitivity and specificity Can use either urine or cervical swabs as specimens 	<ul style="list-style-type: none"> More expensive than DNA probe or enzyme-linked immunosorbent assay

Source: Health Plan Employer Data and Information Set.

While molecular diagnostic tests are available for *Chlamydia trachomatis* and *Neisseria gonorrhoeae*, they have to be compared with other diagnostic methods. Table 1 lists advantages and disadvantages of each method.

PERSONALIZED MEDICINE: PHARMACOGENETICS

The goal of personalized medicine has been touted in the popular press: both Wall Street Journal and US News & World Report have reported on it. Currently, drug therapy often relies on trial and error. Even "state-of-the-art" pharmaceutical therapy is applied in a one-size-fits-all manner. The response rate is far from perfect. With personalized medicine, therapy will be selected based on individual patient characteristics that become known through bioinformatics. The results will be response rates that approach 100%, as well as increased survival rates, improved quality of life, cost savings, and reduced morbidity and mortality.

In October 2000, Fortune published a story of a boy who died because personalized medicine was not available:

The death of nine-year-old Michael Adams-Conroy didn't seem at first like a signal event in medicine. . . . While recuperating from what seemed to be flu, Michael went into a prolonged grand mal

Table 2. Common CYP450 2D6 and 2C19 drugs, which patients metabolize differently

CYP2D6	CYP2C19
Antiarrhythmics	Amitriptyline (in part)
Antidepressants	Certain barbiturates
Beta-blockers	Proguanil
Neuroleptics	Citalopram
Others	Cyclophosphamide
—Atomoxetine	Diazepam
—Codeine	Imipramine
—Ondansetron	Mephenytoin
—Tamoxifen	Omeprazole

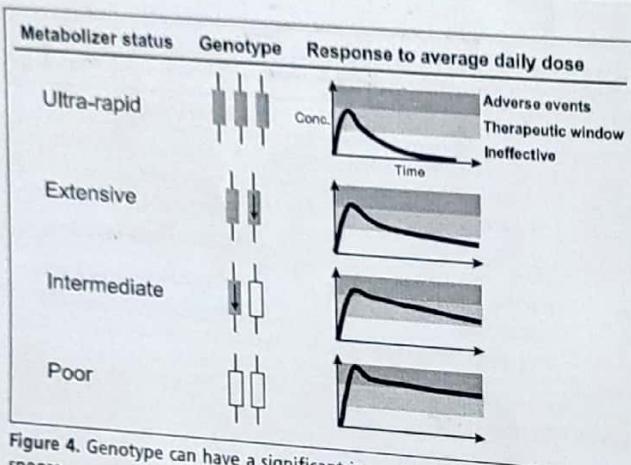


Figure 4. Genotype can have a significant impact on drug metabolism and response.

seizure and died. His grieving parents, Jayne and Neil, soon got another shock: An autopsy showed a massive overdose of Prozac in Michael's blood and tissues, raising the specter of a murder charge against them.... Thus began the Adams-Conroys' painful pilgrimage to a medical frontier known as pharmacogenetics, the study of how genetic idiosyncrasies influence responses to drugs (12).

Michael's Prozac levels were accumulating after every dose; little drug was being cleared from his system. He was a poor metabolizer of drugs through his CYP450 2D6 genotype. Drugs metabolized through the CYP450 2C19 gene make up a quarter of all prescription drugs (Table 2). In contrast to Michael's poor metabolism, some people are ultra-rapid, extensive, or intermediate metabolizers of these drugs (Figure 4). Knowledge of the category of drug metabolism phenotype helps physicians tailor dosing.

Information on drug responses can be gained through a molecular method called microarray genotyping analysis using the AmpliChip P450 test, which is currently available as an FDA-approved product from Roche.

MICROBIOLOGY, BLOOD SCREENING, AND ONCOLOGY

As the USA was being afflicted with the scare of anthrax as a bioterrorism agent, a molecular assay was developed and put into use in 4 weeks. That speed is nothing short of revolutionary. Patterns of anthrax infection differ globally as well as geographi-

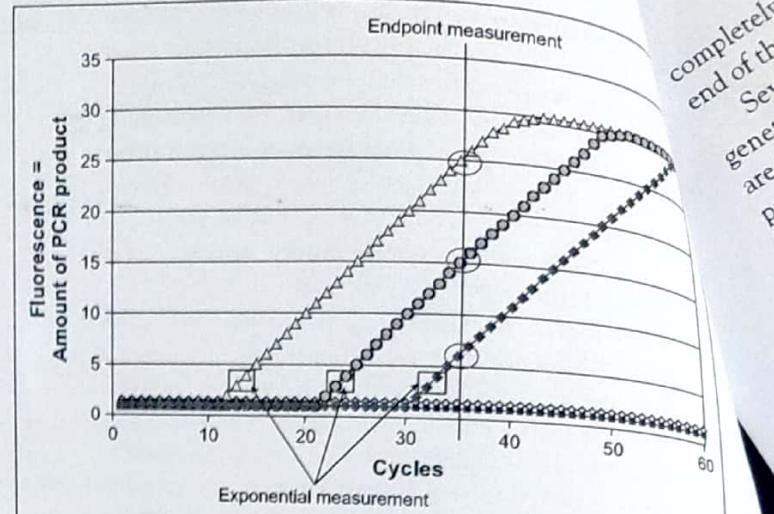


Figure 5. Real-time PCR measures at the base of the exponential phase (rectangles) rather than at the endpoint (ovals). The exponential phase is the start of predictable amplification; the earlier the exponential phase begins, the more copies of the product present in the initial sample. After a certain point, a plateau effect occurs. Each line in the table represents a different sample. A negative sample shows up as a horizontal line at the threshold level.

cally within the USA; an assay can be customized to a local or regional pattern.

When the USA faced the West Nile virus epidemic, with a corresponding threat for the blood supply, the FDA and the Centers for Disease Control and Prevention turned to molecular diagnostic companies to rapidly design and manufacture an assay. An assay with an automated platform was made available in only 8 months. Such an accomplishment would have been impossible without molecular methodologies.

One example of the impact of molecular diagnostics in oncology was featured in the *New York Times* (13). Two types of childhood medulloblastoma have been identified based on clinical course; however, the two types look similar under a microscope. When samples were taken from both groups of patients and put into a DNA microarray system, two different patterns emerged that now allow physicians to provide the right level and type of chemotherapy for these young patients. It spares some children unnecessary morbidity and offers a better chance of a cure for children who can benefit.

FUTURE OF CLINICAL MOLECULAR TECHNOLOGY

Only 51 years ago, Watson and Crick proposed the structure of DNA and alluded to the significance of their findings: "It has not escaped our notice that the specific pairing we have postulated immediately suggests a possible copying mechanism for the genetic material" (14). In 2001, the human genome sequence was published and created an opportunity for revolutionizing health care with individualized medicine, the curing of diseases, and new horizons for preventive medicine.

Real-time PCR is the next evolution of PCR; it is currently in clinical use. As PCR generates specific amplicon molecules, real-time PCR uses a fluorescent reporter signal to measure the amount of amplicon as it is generated (Figure 5). This kinetic PCR allows for data collection after each cycle of PCR instead of only at the end of the 20 to 40 cycles. Real-time PCR has an increased dynamic range compared with running the PCR

and of the reaction. Several tasks lie ahead in molecular technology. First, the genetic correlations with disease need to be validated. They are based on retrospective studies but will need to be based on prospective studies. Second, clear guidance is needed from the FDA on genetic tests. Third, education is needed—for physicians, health care workers, and the general public. It will also be important to ensure the protection of human subjects and patients in this process and to examine the economic value of molecular diagnostics.

We are in the midst of a change in diagnostic paradigm. Now tests are usually used for diagnosis, therapy, and monitoring of therapy. The focus in the near future will be on wellness: predisposition testing, targeted monitoring, and prevention of diseases through nutrition, lifestyle, and medications. The goal is to give people a greater opportunity to attack diseases before they become patients.

Clearly, health care will be more complicated in the future. The complexity will require cooperation among disciplines. A new, shared language will develop based on the molecular foundations of disease. With such knowledge, health care will also become more individualized. As Sir William Osler said, "If it were not for the great variability among individuals, Medicine might be a Science, not an Art" (15).

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Diseases and Molecular Diagnostics: A Step Closer to Precision Medicine

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Abstract The current advent of molecular technologies together with a multidisciplinary interplay of several fields led to the development of genomics, which concentrates on the detection of pathogenic events at the genome level. The structural and functional genomics approaches have now pinpointed the technical challenge in the exploration of disease-related genes and the recognition of their structural alterations or elucidation of gene function. Various promising technologies and diagnostic applications of structural genomics are currently preparing a large database of disease-genes, genetic alterations etc., by mutation scanning and DNA chip technology. Further the functional genomics also exploring the expression genetics (hybridization-, PCR- and sequence-based technologies), two-hybrid technology, next generation sequencing with Bioinformatics and computational biology. Advances in microarray "chip" technology as microarrays have allowed the parallel analysis of gene expression patterns of thousands of genes simultaneously. Sequence information collected

from the genomes of many individuals is leading to the rapid discovery of single nucleotide polymorphisms or SNPs. Further advances of genetic engineering have also revolutionized immunoassay biotechnology via engineering of antibody-encoding genes and the phage display technology. The Biotechnology plays an important role in the development of diagnostic assays in response to an outbreak or critical disease response need. However, there is also need to pinpoint various obstacles and issues related to the commercialization and widespread dispersal of genetic knowledge derived from the exploitation of the biotechnology industry and the development and marketing of diagnostic services. Implementation of genetic criteria for patient selection and individual assessment of the risks and benefits of treatment emerges as a major challenge to the pharmaceutical industry. Thus this field is revolutionizing current era and further it may open new vistas in the field of disease management.

Keywords Molecular signature · Molecular techniques · Molecular diagnostics · Genetic diseases and disorders · Precision medicine

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Introduction

Our healthcare system is critically and crucially dependent upon diagnostics. Today's medical decision making is strongly based upon the diagnostics results. Right from the genetic tests that can help notify personalized cancer treatment to the microbial culture for recognition of right antibiotic combating an infection, diagnostics provide critical insights at every stage of medical care prevention, detection, diagnosis, treatment and successful management of health conditions. The main categories of diagnostics are

clinical chemistry, immunology, hematology, microbiology and molecular diagnostics. The molecular diagnostics has attracted particular attention in recent years owing to deep insights it brings to diagnosis and treatment.

Molecular diagnostics has transformed diagnostics dynamically, leading to insights in research and treatment in many disease states that are revolutionizing health care. The current review will provide an overview of the current scenario of molecular diagnostics, with the glimpse of the key technology that are driving the molecular revolution with pin-pointing some specific diseases and disorders. We conclude by noting emergence of two novel therapeutic approaches of pharmaco-genomics and nutri-genomics that have the power to influence the diseases management plan.

Table 1 Historical significance-development and progression of molecular biology/techniques

Year/ decades	Discovery/event	Discoverer/company	Remarks
1869	Deoxyribonucleic acid, or DNA	Johann Friedrich Miescher	—
1944	Transforming material is DNA	Oswald Avery, McCarty and Colin MacLeod	DNA seems to be genetic material
1928	Transformation	Franklin Griffith	Genetic material is a heat-stable chemical
1949	DNA composition was species specific	Erwin Chargaff	A = T; G = C
1949	Characterization of sickle cell anaemia as a molecular disease	Linus Pauling	Discovery that a single amino acid change at the β -globin chain leads to sickle cell anemia
1953	Double helical model of DNA	Watson-Crick	Led the foundation of molecular biology
1958	Isolation of DNA Polymerases	Arthur Kornberg	Important milestone for DNA replication
1960	First Hybridization techniques	Roy Britten	—
1969	In situ-hybridization	Gall and Pardue	—
1970,	Isolated the first restriction enzyme	Hamilton Smith	An enzyme that cuts DNA at a very specific nucleotide sequence
1972	Assembled the first DNA molecule	Paul Berg	Crucial steps in the subsequent development of recombinant genetic engineering
1961	First "triplet"—a sequence of three bases of DNA	Marshall Nirenberg	Triplet-codes for one of the twenty amino acids
1961	Theory of genetic regulatory mechanisms	François Jacob and Jacques Monod	Showed on a molecular level, how certain genes are activated and suppressed
1973	Efforts to create the construction of functional organisms	Stanley Cohen and Herbert Boyer	Experiments try to demonstrate the potential impact of DNA recombinant engineering
1977	Developed new techniques for rapid DNA sequencing	Walter Gilbert (with graduate student Allan M. Maxam) and Frederick Sanger	Made it possible to read the nucleotide sequence for entire genes
1970s,	Nucleic acid hybridization methods and DNA probes	—	Highly specific for detecting targets
1983	Polymerase chain reaction (PCR)	Kary Mullis	For rapidly multiplying fragments of DNA
1985	New method to detect patient's beta-globin gene for diagnosis of sickle cell anaemia	Saiki and his colleagues	—
1987	Identified human immunodeficiency virus (HIV) by using PCR method	Kwok and colleagues	The first report the application of PCR in clinical diagnosis infectious disease
1992	Conception of real time PCR	Higuchi et al.	Amplification in real time
1996	First application of DNA microarrays	Derisi et al.	DNA arrays to be made on glass substrates
2001	First draft versions of the human genome sequence	International Human Genome Sequencing Consortium	—

History of Molecular Diagnostics: Advancements and Discovery

The field of molecular biology grew in the late twentieth century (Table 1). In 1980 [1] advised a prenatal genetic test for Thalassemia that was not based on DNA sequencing, but on restriction enzymes that cut DNA at specific short sequences, creating various bands of DNA strand depending upon the presence of allele (genetic variation). In the 1980s, the term "Molecular Diagnostics" was utilized in the names of companies such as Molecular Diagnostics Incorporated and Bethesda Research Laboratories Molecular Diagnostics [2]. The 1990s saw an era of identification of newly discovered genes and new techniques for DNA sequencing.

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known mutation within a single day, rather than months. With the advent of PCR, molecular diagnostics to cross the threshold of the clinical laboratory for the provision of genetic services, such as carrier or population screening for known mutations, prenatal diagnosis of inherited diseases, or in recent years, identification of unknown mutations. Therefore, being moved to their proper environment, the clinical laboratory, and molecular diagnostics could provide the services for which they have been initially considered. Each PCR cycle theoretically doubles the amount of specific DNA sequence present resulting in an exponential accumulation of the DNA fragment being amplified. PCR is an enzyme-driven, primer-mediated (forward and reverse primer), temperature-dependent process for replicating a specific DNA sequence in vitro. The principle of PCR is based on the repetitive cycling of three simple reactions of amplification that include: (1) Denaturing: At 95 °C template DNA double strand separates into two single strands. (2) Annealing: Next the temperature is reduced to 55 °C and two specific oligonucleotide primers attach to the DNA template complementarily. (3) Extension: The temperature is then raised again but this time to 72 °C, facilitating the DNA polymerase to extend the primers at the 3' terminus of each primer and synthesize the complementary strands along 5' to 3' terminus of each template DNA using deoxynucleotides contained in media.

With commercialization of these techniques, debut the patents/exclusive right for their achievements. In 1998, the European Union's Directive 98/44/EC clarified that patents on DNA sequences were allowable. An example of the patents use in molecular diagnostics was seen in 2010 in the US, when AMP sued Myriad Genetics to defy the latter's patents regarding two genes, BRCA1, BRCA2, which are associated with breast cancer. However, in 2013, the US Supreme Court ordered, a ruling that a naturally occurring gene sequence could not be patented.

The term "Molecular disease" was introduced by Pauling and his colleagues in 1949, based on their finding that change in a single amino acid at the β -globin chain causes sickle cell anaemia. In principle, their explorations have set the foundations of molecular diagnostics, even though the big transformations occurred many years later.

Molecular Diagnostic Techniques in Identification and Characterization

Correct and accurate identification of causative agents like microbes in microbial diseases, particular genetic sequences in genetic diseases and protein levels are very essential for the management of these patients, making specificity and sensitivity important tools in diagnosis. Classical molecular techniques like normal PCR and blotting although played satisfactory role in diagnosis. However, currently molecular techniques like gene and peptide sequencer, real-time PCR and microarrays may detect more precisely and specifically without consuming much time.

Polymerase Chain Reactions (PCR)

The discovery of PCR [4] and its quick optimization, by a thermo stable Taq DNA polymerase from *Thermus aquaticus* [5] has greatly facilitated and revolutionized molecular diagnostics. It is a robust technique as a large amount of copies of the target sequence generated by its exponential amplification, permitting the identification of a

known mutation within a single day, rather than months. With the advent of PCR, molecular diagnostics to cross the threshold of the clinical laboratory for the provision of genetic services, such as carrier or population screening for known mutations, prenatal diagnosis of inherited diseases, or in recent years, identification of unknown mutations. Therefore, being moved to their proper environment, the clinical laboratory, and molecular diagnostics could provide the services for which they have been initially considered. Each PCR cycle theoretically doubles the amount of specific DNA sequence present resulting in an exponential accumulation of the DNA fragment being amplified. PCR is an enzyme-driven, primer-mediated (forward and reverse primer), temperature-dependent process for replicating a specific DNA sequence in vitro. The principle of PCR is based on the repetitive cycling of three simple reactions of amplification that include: (1) Denaturing: At 95 °C template DNA double strand separates into two single strands. (2) Annealing: Next the temperature is reduced to 55 °C and two specific oligonucleotide primers attach to the DNA template complementarily. (3) Extension: The temperature is then raised again but this time to 72 °C, facilitating the DNA polymerase to extend the primers at the 3' terminus of each primer and synthesize the complementary strands along 5' to 3' terminus of each template DNA using deoxynucleotides contained in media.

Extension leads to formation of new double strand DNA copies comprising of two single template DNA strands and two synthesized complementary DNA strands. After extension, the reaction will repeat above steps. Each copy of DNA then serves as another template for further amplification. PCR products will be doubled in each cycle. After n cycles (approx. 30), the final PCR products will have double no. copies of template DNA in theory. The whole process just needs 2–5 h depending on the no. and types of nucleotide.

Multiplex PCR

Multiplex PCR is like performing multiple PCR reactions simultaneously in single PCR tube. Here two or more primer pairs are included in one reaction tube and two or more DNA templates are targeted simultaneously. It is a relatively effortless molecular way to detect few different bacteria in one PCR reaction. In multiplex PCR, the primer pairs should be specific to the target gene and the PCR products (amplicons) should be in different sizes that are specific to different DNA sequences. This technique saves time and reagents by targeting multiple genes at a time, therefore extra information may be gained from a single test-run that otherwise would require several times the reagents and more time to perform. Annealing

temperatures for each of the primer sets must be optimized to work correctly within a single reaction, and amplicon sizes. That is, their base pair length should be different enough to form distinct bands when visualized by gel electrophoresis.

Reverse Transcription-Polymerase Chain Reaction (RT-PCR)

RT-PCR is the technique of synthesis of cDNA from RNA by reverse transcription (RT) firstly, which is then followed with amplification of a specific cDNA by PCR. This is the most valuable and sensitive technique for mRNA detection and quantitation that is currently available. RT-PCR is mostly used to detect viruses and the viability of microbial cells through the assessment of microbial mRNA. The discovery of PCR also has provided the foundations for the design and development of many mutation detection schemes, based on amplified DNA. In general, PCR either is used for the generation of the DNA fragments to be examined, or is part of the recognition method. The first attempt was the use of restriction enzymes [6] or oligonucleotide probes, immobilized onto membranes or in solution [7] in order to spot the existing genetic variation, in particular the sickle cell disease-causing mutation. In the following years, an even larger number of mutation detection approaches have been built up and implemented. RT-PCR may be single step or two step procedure. As the name suggests single step RT-PCR shall combine first-strand cDNA synthesis (RT) and subsequent PCR in a single reaction tube. This technique requires gene specific primer and is useful in high throughput applications. However it is less sensitive and efficient since the reaction is a compromise between reverse transcription and amplification conditions. Two step PCR requires two steps—first one for the synthesis of cDNA in a PCR tube followed by second step in a separate PCR tube for amplification of gene of interest. This technique is more sensitive, flexible and allows analysis of multiple genes.

Real-Time PCR

Real time PCR is a simple, quantitative assay for any amplifiable DNA sequence. It was illustrated for the first time by, Higuchi et al. [8]. It is based on using fluorescent labelled probes to detect, confirm, and quantify the PCR products as they are being generated in real time. The real time PCR, which has three novel features as temperature cycling occurs considerably faster than in standard PCR assays, hybridization of specific DNA probes occurs continuously during the amplification reaction and a fluorescent dye is coupled to the probe and fluoresces only when

hybridization takes place. Lack of post PCR processing of amplified products makes this technique convenient. The production of amplified products is observed automatically by real time monitoring of fluorescence. Depending on the amount of target gene, a small signal can be produced within 30–45 min. Since the tubes do not have to be opened at the time of reaction, the risk of carry over contamination gets considerably reduced. In recent years, some commercial automated real-time PCR systems have been available (Light Cycler & TaqMan). In these systems, such as the Light Cycler TM and the Smart Cycler®, these systems perform the real-time fluorescence monitoring by using fluorescent dyes such as SYBR-Green I, which binds non-specifically to double-stranded DNA generated during the PCR amplification. Others, such as the TaqMan, use fluorescent probes that bind specifically to amplification target sequences.

These techniques can be divided roughly into three categories, depending on the basis for discriminating the allelic variants:

Enzymatic-Based Methods

The first restriction enzyme based approach used extensively was RFLP analysis. It exploits the alterations in restriction enzyme sites, leading to the gain or loss of restriction events [6]. Since then a number of enzymatic approaches for mutation detection have been conceived, based on the dependence of a secondary structure on the primary DNA sequence. These methods utilize the activity of resolvase enzymes T4 endonuclease VII, and more recently, T7 endonuclease I to digest heteroduplex DNA formed by annealing wild type and mutant DNA [9]. Mutations are indicated by the size of the various fragments. A variation of the theme involves the use of chemical agents for the same purpose [10]. Another enzymatic approach for mutation detection is the oligonucleotide ligation assay [11]. This technique involves the hybridization of two oligonucleotides to complementary DNA stretches at sites of possible mutations. The oligonucleotide primers are designed such that the 3" end of the first primer is immediately adjacent to the 5" end of the second primer. Therefore, if the first primer matches completely with the target DNA, then the primers can be ligated by DNA ligase. On the other hand, if a mismatch occurs at the 3" end of the first primer, then no ligation products will be obtained.

Electrophoretic-Based Techniques

This category is distinguished by a number of different approaches intended for screening of known or unknown mutations, based on the different electrophoretic mobility of the mutant alleles, under denaturing or nondenaturing

evolutions. Using this technique, Single strand conformation polymorphism (SSCP) and heteroduplex (HDA) analyses [12], were among the first methods designed to spot molecular defects in genomic loci. In combination with capillary electrophoresis, SSCP and HDA analysis now provide an excellent, simple, and rapid mutation detection platform with low operation costs and, most fascinatingly, the potential of easily being automated, thus allowing for high-throughput analysis of patient's DNA. Another equally well employed technique for mutation detection is, Denaturing and Temperature Gradient Gel Electrophoresis (DGGE and TGGE, respectively). In this case, electrophoretic mobility differences between a wild type and mutant allele can be "visualized" in a gradient of denaturing agents, such as urea and formamide, or of increasing temperature. Lastly, an increasingly used mutation detection technique is the two-dimensional gene scanning, based on two-dimensional electrophoretic separation of amplified DNA fragments, according to their size and base pair sequence. The latter involves DGGE, following the size separation step.

Solid Phase-Based Techniques/Hybridization or Blotting Techniques

This set of techniques consists of the foundation for most of the present-day mutation detection technologies owing to their extra advantage of being effortlessly automated and hence are highly recommended for high throughput mutation detection or screening. In 1970s there was out break on Nucleic acid hybridization techniques that is based on the pairing of two complimentary nucleotide strands mainly due to involvement of hydrogen thus duplex or hybrid results. The hybrids may be resultant of DNA–DNA, RNA–RNA, or DNA–RNA, thus single stranded molecule may be DNA or RNA in which one nucleic acid strand (the probe) originates from an organism of known identity and the other strand (the target) originates from an unknown organism to be detected or identified.

Saiki et al. [13] developed a fast, accurate, and convenient method for the detection of known mutations reverse dot-blot, and implemented it for the detection of b-thalassemia mutations. The essence of this method is the utilization of oligonucleotides, bound to a membrane, as hybridization targets for amplified DNA. Some of this technique's advantages are that one membrane strip can be utilized to detect many different known mutations in a single individual (a one strip-one patient type of assay), the potential of automation, and the ease of interpretation of the results, using a classical avidin–biotin system. However, the disadvantage is that this technique cannot be used for the detection of unknown mutations. Continuous development has given rise to allele-specific hybridization

of amplified DNA (PCR-ASO), on filters and newly extended on DNA oligonucleotide microarrays for high throughput mutation analysis [14]. Recently, Petralia et al. (2016) have reported a point of care real time PCR platform for the detection of pathogen species HBV [15]. The core of the platform is a hybrid microchip composed by a silicon part and a polycarbonate portion mounted on the plastic ring. The experiments were conducted at different annealing temperature and probe amounts and the results indicated an improvement of sensitivity of about 1 Ct.

Microarrays

A microarray is a battery of enhanced features of microscopic technique. This technique usually involves hybridization of DNA with target molecule, for quantitative (gene expression) or qualitative (diagnostic) analysis of large numbers of genes simultaneously or to genotype multiple regions of a genome. Each DNA spot contains approx. picomoles (10^{-12} mol) of a specific DNA sequence, known as probes (or reporters). There has been a tremendous improvement in the microarray technology in terms of efficiency, discriminatory power, reproducibility, sensitivity and specificity due to advances in fabrication, robotics, and bioinformatics, these improvements have allowed the transition of microarrays from strictly research bench site to bed site in clinical diagnostic applications. Microarrays can be differentiated on the basis of characteristics such as the nature of the probe, the solid-surface support used, and the specific method used for probe addressing and/or target detection.

Further, advances appear in the form of In-situ-synthesized arrays, which are extremely-high-density microarrays that use oligonucleotide probes, for example Gene Chips (Affymetrix, Santa Clara, CA) are the most widely known. In-situ synthesized arrays are synthesized directly on the surface of the microarray made up of 1.2 cm^2 quartz wafer unlike the printed oligonucleotide arrays described above because in situ-synthesized probes are typically short (20–25 bp), multiple probes per target are included to improve sensitivity, specificity, and statistical accuracy. Yet another type are the bead array, similar to the above mentioned printed and in situ-hybridized microarrays, Bead Arrays (Illumina, San Diego, CA) provide a patterned substrate for the high-density detection of target nucleic acids. However, instead of glass slides or silicon wafers as direct substrates, Bead Arrays rely on 3- μm silica beads that randomly self-assemble onto one of two available substrates: the Sentrix Array Matrix (SAM) or the Sentrix Bead Chip [15]. Unlike the other array the exclusive feature of Bead Arrays rely on passive transport for the hybridization of nucleic acids. One more type of array,

electronic microarrays utilize active hybridization via electric fields to control nucleic acid transport. Micro-electronic cartridges (NanoChip 400; Nanogen, San Diego, CA) use complementary metal oxide semiconductor technology for the electronic addressing of nucleic acids [16]. Each Nano Chip cartridge has 12 connectors that control 400 individual test sites. In contrast to the above mentioned arrays, which are 2-D based, Suspension bead arrays are essentially three-dimensional arrays based on the use of microscopic polystyrene spheres (microspheres or beads) as the solid support and flow cytometry for bead and target detection. Furthermore, they are distinct from the high-density Illumina Bead Arrays, in which the beads are immobilized on fiber-optic strands or silicon slides. Most of the clinical diagnostic laboratories have not chosen expensive high technology infrastructure, as the number of tests expected to be performed, have not been large enough to justify the capital outlay. Therefore, simple screening tests such as SSCP and HDA were and still are the methods of choice for many clinical laboratories, as they allow for rapid and simultaneous detection of different sequence variations at a detection rate of close to 100%.

Sequencing

Although PCR has significantly facilitated the expansion of molecular diagnostics, it nonetheless has a number of limitations. First of all, it is problematic for Taq Polymerase to amplify CG repeat-rich regions which sometimes leads to the classic alternative of Southern blot analysis. Also, Taq Polymerase is error-prone at a range of 10^4 – 10^5 nucleotide, which is strongly influenced by the conditions of the amplification reaction, such as magnesium or deoxyribonucleotide concentration, pH, temperature, and so on. Due to Polymerase errors there can be unspecific background, depending on the detection method, resulting in limiting the detection level. To overcome these technical problems, positive results should be confirmed by alternative methods or by using high fidelity thermo stable polymerases. Finally, it needs to be stressed that despite the wealth of mutation detection methodologies, DNA sequencing technology has immensely contributed to detection of unknown mutations. Until the late 1970s, determining the sequence of a nucleic acid containing even five or ten nucleotide was an extremely difficult and laborious task. After introduction of new technique of DNA sequencing, which includes several methods and technologies that are used for determining the order of the nucleotide bases—adenine, guanine, cytosine, and thymine—in a molecule of DNA, the knowledge of DNA sequences has become indispensable for basic biological research and in numerous applied fields such as diagnostic, biotechnology, microbiology etc. There has been significant

acceleration in the field of biological research and discovery with the advent of DNA sequencing. Several microbial genomes have been sequenced using this technique. In 1977, two different methods for sequencing DNA were developed, that is, the chain termination method and the chemical degradation method. In 1976–1977, A. Maxam and W. Gilbert developed a DNA sequencing method based on chemical modification of DNA and subsequent cleavage at specific bases. This rapidly became more accepted, since purified DNA could be used directly, while the initial Sanger method required that each read start be cloned for production of single-stranded DNA. However, with the improvement of the Sanger method, Maxam–Gilbert sequencing has less popularity due to its technical complexity, prohibiting its use in standard molecular biology kits, extensive use of hazardous chemicals, and difficulties with scale-up. Each of four reactions (G, A + G, C, and C + T). Thus a series of labeled fragments are generated, from the radio labeled end to the first “cut” site in each molecule. Size based separation of the fragments in the four reactions is done by parallel electrophoresis on a denaturing acrylamide gel. Visualization is done by exposure of gel to X-ray film for autoradiography, yielding a series of dark bands each corresponding to a radiolabeled DNA fragment, from which the sequence may be inferred. This method is sometimes called “chemical sequencing” method. The chain-terminator method or Frederick Sanger method became a popular method of DNA sequencing due to its greater efficiency and use of fewer toxic chemicals and lower amounts of radioactivity than the method of Maxam and Gilbert. The key principle of the Sanger method was the use of dideoxynucleotide triphosphates (ddNTPs) as DNA chain terminators.

Molecular Diagnostics in the Post-genomic Era

A new era of molecular biology dawned in February 2001, when the first draft sequence of the human genome was declared (International Human Genome Sequencing Consortium 2001) and was followed up with the genomic sequences of other organisms, with the new era came new opportunities and challenges. Post human genome draft publication, the major challenge, was to improve the existing mutation detection technologies to achieve robust cost effective, rapid, and high-throughput analysis of genomic variation. In the last decade, technology has improved rapidly and new mutation-detection techniques have become available, whereas old methodologies have evolved to fit into the increasing demand for automated and high throughput screening. Denaturing high performance liquid chromatography (DHPLC) used for detection of polymorphic changes of disease-causing mutations one of the new technologies that emerged. It detects the presence

of a genetic variation by the differential retention of homo- and heteroduplex DNA on reversed-phase chromatography under partial denaturation. DHPLC has proved to be one of the most powerful tools of mutation detection and is capable of detecting single-base substitutions, deletions, and insertions can be detected successfully by UV or fluorescence monitoring within 2–3 min in unpurified PCR products as large as 1.5-kilo bases. Another useful technique of mutation analysis is Pyro sequencing, which is a non-gel-based genotyping technology, and provides a very reliable method and an attractive alternative to DHPLC. Pyrosequencing detects de novo incorporation of nucleotides based on the specific template, causing release of a pyrophosphate, which is converted to ATP and followed by luciferase stimulation. The light produced, detected by a charge couple device camera, is translated to a pyrogram, from which the nucleotide sequence can be deducted [17].

There has been a steady increase in the number of samples being sent for molecular analysis. This is due to sensitive and robust techniques like real time PCR and high throughput techniques like microarray. Among the various molecular techniques discussed above, the use of the PCR in molecular diagnostics is considered the gold standard for detecting nucleic acids and it has become an essential tool in the research laboratory. There is a wider acceptance for Real-time PCR [17] due to its improved rapidity, sensitivity, and reproducibility. There is a real time detection of the PCR product during the exponential phase of the reaction, thereby combining amplification and detection in one single step. The reduced number of cycles, use of fluorogenic labels and sensitive methods of their detection has largely removed post-PCR detection procedures, making the technique sensitive. Currently, newborns can be screened for phenylketonuria and other treatable genetic diseases. In the future, we might see children at high risk for coronary artery disease getting characterized and treated to prevent changes in their vascular walls during early adulthood. In the near future, we could witness as a part of standard medical practice, the individual drug monitoring response profiles throughout life, using genetic testing for the identification of their individual DNA signature. Shortly, genetic testing will comprise a wide spectrum of different analyses with a host of consequences for individuals and their families, which is worth emphasizing when explaining molecular diagnostics to the public.

Current Scenario of Molecular Diagnosis in Various Diseases

Clinical laboratories have seen a boost in a number of molecular techniques finding utility in diagnosis and monitoring of disease conditions. Right from plasmid profiling, various methods for generating restriction

fragment length polymorphisms, to polymerase chain reaction (PCR) and micro-arrays have all made increasing inroads into clinical laboratories. Among the molecular techniques, the most popular one for characterization of etiologic agents of disease directly from clinical samples has been PCR based methods as it allows for rapid detection of unculturable or fastidious microorganisms directly from clinical samples. Over the past two decades, the relentless progress and application of molecular diagnostic techniques has opened new vistas in the diagnosis of not only microbial diseases but also in other diseases and disorders like neurodegenerative disorders, cancers and genetic diseases. The various classes of mRNA have like non coding (lnc and nc), micro RNAs and genetic variants of various diseases like cancer, Alzheimer, Parkinson etc. have been identified and characterized, which may be useful in management of these patients.

Application of Various Molecular Techniques in Microbial Diseases

Role of Hybridization Technique in Microbial Disease

At present it is used for DNA and RNA for microbial characterization and identification. With some modification in traditional probe hybridization, the fluorescent in situ hybridization (FISH) evolved, which is a highly valuable tool for the specific and rapid detection of pathogenic bacteria in clinical samples without cultivation [18]. As reported in patients with exacerbations due to *H. influenzae*, *S. aureus*, and *P. aeruginosa* and FISH is used for the rapid detection of microorganisms that cause acute pulmonary infections with 100% sensitivity. The phylogenetic identification of single cell microbes has been successfully done based upon their 16S rRNA sequences as some of these sequences are in all organisms. FISH probes complementary to specific sequence of 16s rRNA can detect malaria infection in blood samples. This assay is highly sensitive and can identify all five species of Plasmodium known to cause malaria in humans and its better than that of Giemsa stain [19].

FISH technique has allowed for the development of low cost molecular tests like the DNA probe methods that detect *C. trachomatis* or *N. gonorrhoea* are examples of low-cost molecular tests. For hybridization assays such as the INNO-LiPA® Rif.TB (Innogenetics) and GenoType® MTBDR (plus) (Hain Life Science GmbH) line-probe assays displays a pooled good sensitivity and a specificity of 0.99 for detecting rifampin resistance in isolates or directly from clinical specimens. Amplification of the detection signal after probe hybridization improves

sensitivity to as low as 500 gene copies per micro liter and provides quantitative capabilities. This approach has been widely utilized for quantitative assays of viral load (hepatitis B virus [HBV] and hepatitis C virus [HCV]). Further the development of dual colour FISH recently has allowed the detection of *M. tuberculosis* and *M. avium* from other pathogens in bacterial cultures with a high sensitivity and at a rapid pace of less than 2 h detection time [20].

FISH probes commercially available in market uses solution-phase hybridization and chemiluminescence for direct recognition of microbial agents in sample material like PACE2 products of Gen-Probe and the hybrid capture assay systems of Digene and Murex. These systems are flexible as they are adaptable to small or large numbers of specimen, are simple to handle and work, besides having a long shelf life. The PACE2 products now help in detection of both *Neisseria gonorrhoeae* and *Chlamydia trachomatis* in a single specimen (one specimen, two separate probes). The hybrid capture systems identify human papillomavirus (HPV) in cervical scrapings, herpes simplex virus (HSV) in vesicle material, and cytomegalovirus (CMV) in blood and other fluids. Although less sensitive than the target amplification based methods for detection of viruses, these methods do give the quantitative results which have proven fruitful for determining viral load and prognosis and for monitoring response to therapy [21]. Probe hybridization is useful for identifying slow growing organisms after isolation in culture using either liquid or solid media. Identification of mycobacteria and other slow-growing organisms such as the dimorphic fungi (*Histoplasma capsulatum*, *Coccidioides immitis*, and *Blastomyces dermatitidis*) has certainly been facilitated by commercially available probes. All commercial probes for recognizing organisms are produced by Gen-Probe and use acridinium ester-labeled probes directed at species-specific rRNA sequences. Gen-Probe products are available for the culture identification of *Mycobacterium tuberculosis*, *M. avium-intracellulare complex*, *M. gordonae*, *M. kansasii*, *Cryptococcus neoformans*, the dimorphic fungi, *N. gonorrhoeae*, *Staphylococcus aureus*, *Streptococcus pneumoniae*, *Escherichia coli*, *Haemophilus influenzae*, *Enterococcus* spp., *S. agalactiae*, and *Listeria monocytogenes*. The sensitivity and specificity of these probes are excellent, and they provide species identification within 4–6 h. The mycobacterial probes, on the other hand, are accepted as a common method for the identification of *M. tuberculosis* and related species [22]. Shah et al. 2017 have successfully utilized MN Genus-MTBC and MTBC-MAC FISH assays as effective diagnostic tools for detecting Mycobacteria from solid and liquid cultures and for their identification as MTBC, MAC or NTM other than MAC. The two FISH assays have a LOD of at least 5.1×10^4 cfu of bacilli per ml which can help minimize delays in diagnosis by being applicable relatively early after initiation of cultures [20].

Role of Microarray Technique in Microbial Disease

Nübel et al. [23] used the PCR amplification, in combination with an oligonucleotide microarray, to identify *Bacillus anthracis* based on the rRNA ITS region. Several studies reported the use of microarrays to identify pathogenic yeasts and molds by targeting the ITS regions in fungal rRNA genes [24]. Recently, a DNA microarray was established to detect and identify 14 commonly encountered fungal pathogens in clinical specimens collected from neutropenic patients [25]. A microarray technique for the detection and identification of enteropathogenic bacteria at the species and subspecies levels was developed, covering pathogenic *E. coli*, *Vibrio cholerae*, *Vibrio parahaemolyticus*, *Salmonella enterica*, *Campylobacter jejuni*, *Shigella* spp., *Yersinia enterocolitica*, and *Listeria monocytogenes* [26]. Recently a study has developed a new oligonucleotide microarray comprising 16 identical subarrays for simultaneous rapid detection of avian viruses: avian influenza virus (AIV), Newcastle disease virus (NDV), infection bronchitis virus (IBV), and infectious bursal disease virus (IBDV) in single- and mixed-virus infections. Diagnostic effectiveness of the developed DNA microarray has been 99.18% and therefore can prove highly effective in mass survey for specific detection of AIV, NDV, IBV and IBDV circulating in the region in the course of epidemiological surveillance [27].

Role of Polymerase Chain Reactions (Multiplex, Nested/Semi-nested, Broad Range, Reverse Transcription and Real Time) in Microbial Disease

PCR is a very robust technique for the detection of minute quantities of specific microbial DNA sequences owing to its ability to amplify minute amounts (less than 3 copies) of specific microbial DNA sequences in a background mixture of host DNA. Many micro-organisms, have been identified using this technique for example *Mycobacterium tuberculosis*, pneumococci, meningococci and *Burkholderia cenocepacia*. Over 100 viruses are known to cause acute viral encephalitis in humans. There is a battery of infective states of CNS which can be accounted for by viral infections like those involving the spinal cord (myelitis), the brain stem (e.g., rhombencephalitis), the cerebellum (cerebellitis), or the cerebrum (encephalitis) and almost all acute viral infections of the CNS produce some degree of meningeal as well as parenchymal inflammation. Rarely, such as West Nile virus (WNV) meningoencephalitis or cytomegalovirus (CMV) radiculomyelitis, polymorphonuclear cells rather than lymphocytes may be the predominant cell type, and thus provide some diagnostic guidelines. Even with such a diverse symptoms routine CSF studies only rarely lead to identification of a specific etiologic

agent. The entire picture of diagnosis of viral infections of CNS has changed for good now a days due to the discovery of new molecular diagnostic technologies, such as the PCR to amplify viral nucleic acid from CSF [28, 29]. The real-time multiplex PCR assay designed by researchers is extremely rapid and permits the confirmation of bacterial isolates as *Brucella* spp., *B. abortus*, or *B. melitensis* within 2–3 h. Further this technique has assisted in the identification of infrequently isolated *Brucella* species and the recognition of atypical *Brucella* strains by utilizing genus specific primers-probe sets. This is advantageous since the conventional methods for *Brucella* isolation and characterization may take days to weeks to perform and often requiring the preparation of heavy suspensions of these highly infectious pathogens [30]. Since viral disease severity and viral load are linked, use of real-time PCR quantitation has proven beneficial when studying the role of viral reactivation or persistence in the progression of disease [31]. Nonetheless, the general diagnosis of invasive diseases caused by *Aspergillus fumigatus* and *Aspergillus flavus* has been made easier by real-time PCR assays [31]. The diagnosis of efflux-mediated resistance in *Pseudomonas aeruginosa* has been performed by using molecular diagnostic approach. Real time PCR has helped in the detection of mexA and mexX and reverse transcription pPCR for mex C and mex E, thus helping in the analysis of multidrug resistance in *P. aeruginosa* [28].

Recently Jiang et al. developed an assay combining multiplex PCR and Luminex technology (MPLT) for the detection of nine important respiratory bacterial pathogens, which frequently cause LRTIs including *S. pneumoniae*, *Moraxella catarrhalis*, *S. aureus*, *Streptococcus pyogenes*, *Haemophilus influenzae*, *Mycoplasma pneumoniae*, *Legionella* spp., *P. aeruginosa*, and *Klebsiella pneumonia* assay demonstrated a high diagnostic accuracy for *S. pneumoniae* (sensitivity, 87.5% and specificity, 100%). Furthermore, sensitivity and specificity for the other eight pathogens all attained 100% diagnostic accuracy. Indeed, this assay may be a promising supplement to conventional methods used to diagnose LRTIs [32].

HIV Qualitative Nucleic Acid Assays

The qualitative detection of HIV nucleic acids finds application in three main areas: the identification of acute infection, assurance of blood safety, and in early infant diagnosis. Shortcomings of western blot analysis in confirmation of HIV infection have been overcome by an amended testing algorithm employing qualitative nucleic acid testing [33]. The APTIMA HIV-1 RNA Qualitative

Assay and the Procleix HIV- 1/HCV assay (Gen-Probe, San Diego, CA, USA) are both Food and Drug Administration (FDA)-approved for blood-donor screening to exclude blood from donors with acute HIV infection [34]. Transcription-mediated amplification technology is used in these two assays [35]. Infants born to mothers infected with HIV-1 have maternal antibodies directed against HIV up to 18 months of age and prevents the use of antibody-based assays for the early diagnosis of HIV infection. Such infected infants have a high morbidity and mortality in the first 2 years of life; thus, an early diagnosis is important to establish the infection status of the exposed infant in order to employ appropriate ART sufficiently early. Qualitative nucleic acid assays for the detection of HIV pro-viral DNA, viral RNA and total nucleic acid [36], have become the methods of choice for diagnosis in infants born to HIV-1-infected mothers. Cobas AmpliPrep/COBAS® TaqMan® HIV-1 Qualitative Test is one such test that uses 70 µl of whole blood or dry blood spot, and is directed against gag and LTR regions of HIV-1 genome.

H CD-4 T cells numbers and HIV RNA viral load are two routinely used laboratory markers in chronically infected patients and serve as guide ART initiation, monitor treatment effectiveness, determine clinical progression [37], and determine treatment regimens. An HIV RNA level below the detection limit is indicative of excellent compliance by patients and ART efficacy [37]. HIV-1 viral load determination is typically performed with HIV RNA amplification by reverse transcription-polymerase chain reaction (RT-PCR), nucleic acid sequence-based amplification (NASBA) or branched chain DNA tests. New microarray techniques including those from Affymetrix (Santa Clara, CA, USA), have currently allowed host transcriptome analyses in individuals infected with HIV-1 [38], and 34 studies involving HIV-1 and microarrays from 2000 to 2006 yielded important data on HIV-1-mediated effects on gene expression and provided new insights into the intricate interactions occurring during infection [39]. The host transcriptome profiles may prove to extremely important for the evaluation of disease progression and prognosis and RNA-Seq approach using deep-sequencing technologies for transcriptome profiling will further add to the precision of transcriptome analyses [38]. Gen-Probe's (APTIMA®) HIV-1 RNA qualitative assay, is the only molecular assay that is FDA approved for diagnosis of acute infections and as a confirmatory test for diagnosing HIV-1 in samples that test reactive for HIV-1 antibodies. Other commercially available molecular assays were developed as quantitative viral load assays for therapeutic monitoring, and are currently only available in expensive, high-throughput formats.

Role of Sequencing in Microbial Disease

Sequencing of broad range of PCR products has facilitated the identification of almost any bacterial species by comparing the resulting sequences with known sequences in GenBank or other databases, the identification of the unknown bacteria is possible. Sequencing of 16S rRNA has become an important tool, which has been used more and more in microbial detection and identification algorithms, especially for unusual, non-culturable, fastidious and slow growing pathogens, or after antibiotics that have been administered to the patient. Thus this technique is helping in combat infectious diseases, where a consistent detection of bacteria in specimens would prove critical in diagnosis and future therapy. Under these circumstances, PCR is the most reliable assay for detection of microbes in clinical specimens. The major challenge in the rapid detection, identification and characterization of microbial pathogens lies in the accurate recognition of a traits, species, sub species and genus, or combination of traits, that is unique to a specific microbial strain [40]. Increasingly, DNA based assays are replacing the conventional serotyping based methods. Rather DNA based assays detect known genomic signatures generally on 16S rRNA-based almost that is conserved by nature And offer rapid and reliable identification of microbial pathogens in most precise manner [41]. New DNA sequencing platforms are already enabling novel approaches to explore and characterize microbial genomes, while at the same time profoundly altering our understanding of the natural genetic diversity exist in microbial populations. Table 2 representing various established techniques currently used in screening of microbial infections.

Genetic Diseases, Disorders and Molecular Diagnosis

The worth of the interaction between basic science and clinical practice has very close association but it has become even more apparent in the past sixty years with the notable rate of development in the field of molecular genetics.

Fragile X Syndrome

Tri-nucleotide repeat DNA sequences positioned within the transcribed region of a gene can expand, by a process of vibrant mutation, likely due to a mechanism of 'strand slippage' during DNA replication and ultimately compromise the function of the gene dynamic mutations of CGG triplets give rise to folate-sensitive fragile sites of human

chromosomes. Primarily these fragile sites to be cloned and characterized which is accountable for the single most common form of inherited mental retardation, the Fragile X Syndrome. This syndrome, clinically well-defined by the presence of mental retardation of variable severity, modest facial dysmorphism and macroorchidism in adult males, has been documented for years as an X-linked Mendelian disorder offering abnormal and confusing features of inheritance, penetrance, cytogenetic expression and clinical variability. The mainstream of males who carry the Fragile X Mutation (FRAXA) are exaggerated by mental retardation and show a cytogenetically inducible fragile site, but 20% of obligate male carrier, the so called 'normal transmitting males', are both clinically and cytogenetically negative. Overall, characteristic can be defined by the nature of the FRAXA dynamic mutation and the inactivation of the FMR1 (Fragile X Mental Retardation 1) gene, coding for the RNA-binding protein (FMRP), typically expressed in human brain and testis. The 59 untranslated region of the FMR1 gene harbors a series of CGG triplet repeats, highly polymorphic in normal individuals; the total of these repeated units varies from 4 to about 54. A FRAXA mutation is demarcated as a 'premutation' when the repeats increase beyond the upper-normal limits to a size of about 200 triplets, characteristic of carrier females and normal transmitting males in whom, despite the structural modification, the gene is expressed and the protein is functional [42]. The altered allele transmitted by a premutated mother to her offspring scarcely ever shows small decreases of the repeats; more commonly, with a risk that is higher for larger premutations, it undertakes a process of expansion that can reach a size increase of more than 2000 repeats and is referred to as the 'full mutation' [43].

Molecular Diagnosis of FRAXA Mutations

Direct molecular diagnosis of Fragile X Syndrome is marked at the discovery and measurement of the abnormal expansion of the CGG repeats region and at the characterization of the state of methylation of the FMR1 promoter, a reflection of the residual function of the gene. There is a rational international consensus on the detail that DNA-based testing for the FRAXA mutation should be skilled through the combined use of two techniques: PCR amplification and Southern blot/Hybridization.

This is the tactic commonly selected to adopt for the molecular analysis of FRAXA mutations that is regularly done by diagnostic service as part of the different diagnosis of mental retardation of unknown cause. The other way is screened by Southern blotting of genomic DNA digested with a combination of restriction enzymes that includes

Table 2 Molecular techniques utilized for characterization of various microbes
Organism

	Techniques	Infections
CMV	Qualitative PCR Real-time PCR RT-PCR Real-time PCR Multiples PCR Real-time quantitative PCR (Viral load Detection)	CNs Infection Congenital Infection Flu Bronchiolitis Croup HIV/AIDS
HIV	Real-time PCR Hybridization RT-PCR Real-time PCR 16s rDNA PCR 16s rDNA PCR	Hepatitis (chronic) MERS-CoV pneumonia Vaginitis Meningitis Osteomyelitis
Hepatitis	Nested PCR Multiples PCR Real-time PCR (Gene Xpert)	Malaria Health care associated Infections Tuberculosis MDR-TB
Middle-East Respiratory Syndrome Coronavirus (MERS-CoV) Group-B Streptococcus (GBS) Neisseria Meningitidis Helicobacter species <i>Plasmodium falciparum</i> Methicillin-resistant <i>Staphylococcus aureus</i> (MRSA) Multi-drug resistant <i>M. tuberculosis</i>	Real-time PCR 23S rRNA based Specific oligonucleotide probes Real-time PCR	Brucellosis Cystic fibrosis
<i>Brucella</i> spp., <i>B. abortus</i> , or <i>melitensis</i> <i>Stenotrophomonas maltophilia</i>		

methylation-sensitive rare cutters, followed by hybridization to a suitable labeled probe. This is a more time-consuming and labor-intensive procedure, it may be not as accurate in discriminating small permutations from large normal alleles, but it can be used as the only test since it ensures a reliable and complete diagnosis [44].

Familial Cancer Syndromes: The Von Hippel-Lindau Disease

Mutations of a gene mapped to the distal end of the short arm of chromosome 3 (3p25-26), have now been found to be accountable for the von Hippel-Lindau disease, a highly penetrant, dominantly inherited syndrome showed by susceptibility to a variety of benign and malignant tumors. Pathologic conclusions are retinal and central nervous system hemangioblastomas, pheochromocytomas, and renal and pancreatic cancer. In von Hippel-Lindau disease both sexes are equally involved, there is a penetrance of about 90% at the age of 65 with a mean age at diagnosis of 26 years. The VHL gene is 1810 bp in length, with an open reading frame (ORF) of 852 bp and two in-frame starting

codons; it is composed of three exons, with exon 2 alternatively spliced. The alternative transcripts are ubiquitously expressed in a tissue-specific and developmentally selective manner [45]. The study of the normal function of this tumor suppressor gene promises a wide gain in our knowledge of the mechanisms of gene transcription; VHL acts as a competitor of Elongin (SII), one of the transcription elongation factors, and can modify vascular endothelial growth factor and neoangiogenesis, upon which tumor growth is dependent. The practical approach is precise for the finding of medium small-size mutations, the type of alteration commonly found in the VHL pheochromocytoma phenotype [46]. Detection is done with the procedure of PCR amplification of partially overlapping genomic DNA fragments covering the entire coding sequence of the VHL gene and including exon-flanking intronic regions. The amplification products are subjected to polyacrilamide gel electrophoresis (PAGE), for detection of deletion/insertion mutations that may change the molecular weight of the product, and to Single Strand Conformation Polymorphism (SSCP) analysis. This method, designed to specifically identify single base substitutions, is the most widely used mutation scanning

technique; it is based on the tendency of single stranded DNA molecules to assume a three-dimensional conformation which is dependent on the primary sequence, is very specific and can be simply detected on a native polyacrylamide gel. DNA fragments that show size alterations on PAGE analysis or aberrant SSCP bands are subsequently subjected to direct automated sequencing to confirm and characterize the mutation. This strategy has allowed to identify different mutations, in unrelated VHL patients, and to detect case of somatic mosaicism in an asymptomatic subject [47].

Cystic Fibrosis (CF)

Cystic fibrosis (CF) is the most common autosomal recessive inherited disease in Caucasians and affects approximately 1 in 2500 individuals. It is a multifaceted disorder that influence Pulmonary, Pancreatic, Gastro-intestinal, and Reproductive organ system. The pathological processes influencing these organ systems happen due to mutations in the CFTR gene which encodes the cystic fibrosis transmembrane conductance regulator, a membrane chloride channel present in the apical membrane of secretory epithelia. The CFTR protein is a cyclic-AMP dependent channel, activation of protein kinase A occurs when there is increase in levels of c-AMP inside a secretory epithelial cell ultimately it binds to the phosphorylation site on the (regulatory) R-domain of the CFTR protein thus opening the channel. The CFTR chloride channel essentially works as an electrostatic attractant by directing intracellular and extracellular anions toward positively charged transmembrane domains inside the channel. The CFTR protein includes 12 transmembrane (TM) domains. Two of these (TM1 and TM6) attract and bind chloride (and/or bicarbonate) ions. After binding chloride ions to these sites in the pore, the mutual repulsion hastens expulsion of the ions from the cell [48].

When CFTR is activated in general, chloride ions are secreted out of the cell. But, additionally with chloride ion secretion, the epithelial sodium channel (ENaC) is also inhibited by CFTR (Konig et al. 2001), and less sodium is absorbed into the cell, maintaining a higher combined ionic gradient to allow water to depart the cell by osmosis providing fluid for epithelial tissue secretions. In cystic fibrosis these mucus secretions become hyperviscous that accounts for the main features of cystic fibrosis. There are more than 1950 individual CFTR mutations in recent times reported to cause CF (<http://www.genet.sickkids.on.ca/cftr/app> and <http://www.hgmd.cf.ac.uk/ac/index.php>). These are inactivating (loss-of-function) mutations and include deletions, insertions, splice site mutations, nonsense mutations as well as more than 650 missense mutations. The severity

and appearance of the disease may depend on the type of mutation [49].

Testing of Cystic Fibrosis

Preliminary investigation of a sample is commonly done by means of a commercially available kit as there is no gold standard for routine testing; commercially available kit allows analysis of approximately 30 sequences variants, which are accountable for more than 90% of CF disease causing mutations. The mutations tested should be capable of identifying at least 80% of mutations e.g., at least p.Phe508del (F508del), p.Gly551Asp, (G551D), p.Gly542X (G542X) and c.489 + 1G > T (621 + 1G > T). Current methods used in CFTR testing can be categorized into two groups: targeting at known established mutations, and scanning methods. These now include exploration of large unknown CFTR rearrangements, including large deletions, insertions and duplications, by semi-quantitative PCR experiments, i.e. Multiplex Ligation-dependant Probe Amplification CFTR mutations may be missed by scanning techniques, especially when homozygous, and even direct sequencing cannot identify 100% of mutations [50]. Undetected CFTR mutations may penetrate deep within introns or regulatory regions which are not generally analyzed. For example 3849 + 10kbC > T (c.3718-2477C > T) and 1811 + 1.6kbA > G (c.1679 + 1.6kbA > G), the detection of which require meticulous methodologies. Attention should also be pinpointed on heterogeneity, as it has been stated in patients with the classical form of CF, including a positive sweat test, but this possibly concerns less than 1% of cases. Additionally mutations in the SCNN1 genes, encoding sodium channel (ENaC) subunits have been revealed in non-classic CF cases where no CFTR mutations could be recognized by extensive mutation scanning [51].

Eye Diseases and Molecular Diagnostics

Present progress in molecular diagnosis has unknotted several monogenetic and multi-factorial relationships in eye diseases. In this segment we will focus on common genetic diseases and disorders that have known causative genes and available measures for prevention or treatment.

Glaucoma

Glaucoma is well documented for its heterogeneity and presently affecting more than 2% population globally over age 40 years. At least 15 genetic loci have been mapped for

385
on the type of
POAG and 2 loci for PCG, of which only GLC1A (myo-fibrillarin, MYOC), GLC1E (optineurin, OPTN) GLC1G (WD repeat domain 36, WDR36, and GLC3A (cytochrome P4501B1, CYP1B1) have been described. Mutations in MYOC account for about 2–4% of POAG cases in European and American studies [52]. In Chinese populations prevalence of MYOC mutations to be 1.1–1.8% and OPTN mutations in 16.7% POAG patients [53]. Mutations in CYP1B1 were shown in 48% of French PCG patients, but only 20% of Japanese patients. In addition, CYP1B1 mutations were also identified in early-onset POAG, whereas MYOC mutations were linked with PCG. These studies suggested that CYP1B1 may act as a modifier of MYOC expression and that these two genes may interact through a common pathway [54].

Age-Related Macular Degeneration (AMD)

AMD is revealed by progressive demolition of the macula, leading to central vision loss. AMD approximately affects 1.5% of the general population in Western Europe. Stargardt macular dystrophy (STGD) is the most common hereditary form of macular degeneration. STGD is an autosomal recessive or less often dominant disorder of the retina and is commonly related with early-onset macular degeneration. Three contributing genes have been linked with AMD, complement factor H (CFH), ATP-binding cassette transporter (ABCA4), and apolipoprotein E (APOE). CFH informed to be a main gene for AMD [55]. One mutation in CFH, Y402H, was found to be related for up to 50% of the attributable risk of AMD. The APOE allele ε2 showed an increased risk for AMD while ε4 conferred a protective role against AMD and ABCA4 mutation, T1428 M, was found in 8% of Japanese STGD patients [56].

Retinitis Pigmentosa (RP)

RP is a heterogeneous group of retinal degenerations often affecting the rod photoreceptors. RP causes night blindness, loss of peripheral vision, and eventually to a loss of central vision. The prevalence of RP is estimated to be about 1 in 3500 worldwide. (Rivolta et al. 2002) Currently 40 genetic loci have been associated with non-syndromic RP, from which 32 genes have been recognized. Rhodopsin (RHO) mutations identified for more than 25% of adRP cases. Mutations in retinitis pigmentosa 1 (RP1) account for 6–8% of adRP cases and Retinitis pigmentosa GTPase regulator (RPGR) is a most important gene for XLRP [57].

Retinoblastoma (RB)

Retinoblastoma (RB) is the most predominant intraocular malignancy in children. It can be familial or sporadic, with an incidence of 1 in 15,000–20,000 live births in nearly all populations out of which about 50% of RB cases are heritable. Generally familial, bilateral, or unilateral multifocal RB is observed as carriers of a RB1 germ-line mutation. RB is transmitted in an autosomal dominant manner with 80–90% penetrance. Secondary genetic and epigenetic changes in another gene(s) are necessary to precipitate tumor development [58].

The prevalence rate of germ-line mutation in sporadic RB in Chinese population is 19% (8/42), with 11% (3/28) among unilateral cases. The direct genetic testing can be achieved by sequencer, after exploring nucleotide sequence. Alternative approach for mutation analysis is by single strand conformational polymorphism (SSCP) or by confirmation-sensitive gel electrophoresis (CSGE), or by denaturing high-performance liquid chromatography (DHPLC). Other technical procedures can also be utilized to cover the whole spectrum of gene mutations, such as fluorescent in situ hybridization (FISH) or methylation-specific polymerase chain reaction (PCR). Presently Taq-man probe based real time PCR is also utilized in exploring nucleotide sequence at particular locus. Pre-natal diagnosis is commonly restricted to untreatable and severe congenital eye disorders such as RB. For individuals with familial or bilateral RB, the RB1 genetic testing should be first performed on both peripheral blood and tumor DNA. The 27 known RB1 coding exons, splice boundaries, and the promoter can be amplified by PCR followed by direct sequencing [59].

Molecular Diagnosis in Neurological Disorders and Diseases

Transmissible Spongiform Encephalopathies (TSEs)

TSEs or prion diseases are critical disorders of the central nervous system initiated by unconventional infectious agents, prions, which do not seemingly contain any nucleic acid nor induce any specific immunological reaction in the host. Lack of nucleic acid makes unsuitability to use PCR or real time technique in this type of disorder. Various common transmissible spongiform encephalopathy are, (1) CJD (Creutzfeldt–Jakob disease), (2) GSS (Gerstmann–Sträussler–Scheinker syndrome), (3) FFI (Fatal familial insomnia), (4) vCJD (variant Creutzfeldt–Jakob disease), (7) Scrapie, (6) CWD (Chronic wasting disease) and (7) BSE (Bovine spongiform encephalopathy) [60].

The main pathogenetic event in TSEs is the conformational change of a host protein, cellular PrP (PrP^c), encoded by the prion gene PRNP, into a pathological isoform. This conformer, called PrP^{Sc} (after its first identification in experimentally scrapie-infected rodents) aggregates into amyloid fibrils and accumulates into neural and, often, lymphoreticular cells [61]. Although blood taken from vCJD patients and inoculated into susceptible mice does not always cause disease, it is still possible that low level of infectivity and, as a consequence, PrP^{Sc} might be present in blood. Further efficiency and species barrier may upset its manifestations. Numerous Animal studies have shown that the pathogenesis of TSE as after experimental inoculation of rodents/Mice with TSE agents, PrP^{Sc} is characteristically noticeable in the CNS weeks before the expression of disease and its level augments until the animal dies. As the rise of PrP^{Sc} corresponds to that of infectivity, PrP^{Sc} is commonly used as a surrogate marker for assessing the amount of infectivity in biological samples. The ratio of infectivity and PrP^{Sc} is relatively constant in the hamster brain experimentally infected with the 263 K strain of scrapie at a mean value of ~105–106 protein molecules for one lethal dose 50 (LD50) [62]. But recently few techniques at pre-clinical levels have sufficient potency to be proved as good biomarkers as they can detect very low levels of these proteins like chemicophysical precipitation based protocols, affinity chromatography or affinity precipitation techniques. The amended isolation method for PrP^{Sc} with sodium phosphotungstate [63], and novel molecules plasminogen, and protocadherin-2 binding with high affinity to PrP^{Sc}, might enhance new expectations for preclinical diagnosis of TSEs. Among the immunological methods of PrP^{Sc} screening, western blotting is mainly characterized and widely validated method. It offers the advantage of recognizing different forms of PrP^{Sc} through the analysis of the molecular mass and the relative abundance of di-, mono- and non-glycosylated bands. These parameters characterize the so-called PrP glyotype, a kind of 'PrP signature', which varies among different forms of TSEs. PrP^{Sc} glyotyping has been projected for differentiating various forms of TSEs (e.g., scrapie from BSE, sporadic from variant CJD and for improving the classification of human TSEs. In sporadic CJD, for example, the combination of the two most frequent PrP^{Sc} glyotypes (I and II) with the three possible genotypes of PrP at the polymorphic codon 129 (methionine homozygous, valine homozygous, or heterozygous) enables the sub classification of this form into six distinct groups, each of which presents distinct clinical and pathological features [64]. But this technique is time consuming and only few samples can be assessed at a time so ELISA can overcome these problems. Further

development and up gradation in immune assay in the form of Dissociation-enhanced lanthanide fluorescence immunoassay/conformation-dependent immunoassay (DELFIA/CDI) is the latest-generation immunoassay with an ELISA format, where the detection system is sensitive time-resolved lanthanide fluorescence instead of chemiluminescence. It can measure picograms (10–12 g) of PrP^{Sc} per ml and thus represents one of the most sensitive technique for the detection of PrP^{Sc} [65]. Further one more technique MUFs (Multi-Spectral Ultraviolet Fluorescence Spectroscopy) characterizes proteins by their specific fluorescent pattern of emission when they are excited by ultraviolet radiation and bypasses the need for pre-treatment steps to eliminate PrP^c or for antibody binding. It has the potential to discriminate cellular from pathological prion protein, and various forms of PrP^{Sc} from different strains. Moreover most sensitive and specific technique which uses fluorescent antibody with confocal microscopy in FCS (Fluorescence Correlation Spectroscopy) which recognizes single fluorescent molecules in solution as they pass between the exciting laser beam and the objective of a confocal microscope, equipped with a single-photon counter. It is performed quickly and requires only small amounts of samples. The assay solution is mixed with anti-PrP antibodies tagged with fluorophores that bind strongly to PrP^{Sc} aggregates, which become highly fluorescent and easily visible against the background of monomeric PrP^c. This technique is ~20-fold more sensitive than western blot and was able to detect, for the first time, PrP^{Sc} in the CSF of ~20% of CJD patients.

Further 14-3-3 proteins were also detected in the CSF of patients with genetic CJD carrying the codon 200 or the codon 210 mutation of the PRNP gene, in ~50% of CSF samples taken from patients with vCJD. Further proteins released in the CSF after cerebral damage, such as tau, neuron-specific enolase (NSE), and S-100, are also increased in CJD patients, although their specificity, sensitivity and predictive values are always inferior, or at most equal, to those of 14-3-3 proteins. Genetic susceptibility to the disease is also seen in sporadic and iatrogenic CJD where ~70% of affected individuals are homozygous for methionine at the polymorphic 129 site of PrP (The EUROCD Group 2001) in vCJD, 100% of patients are methionine homozygous [66]. This susceptibility, however, cannot be used to predict who will develop the disease since ~40% of the Caucasian population is methionine homozygous. (The EUROCD Group 2001) The finding of a PrP-like gene (PRNDgene) located downstream from the PrP gene in mammalian species has provided hope that PRND and its encoded protein (Doppel, Dpl) contributes to the pathogenesis of TSEs, and be of help in the recognition of at-risk individuals.

Alzheimer is an aging-related neurodegenerative disorder demonstrated by irreversible loss of higher cognitive functions. The pathological hallmarks of AD include deposition of extracellular amyloid plaques, cerebrovascular amyloidosis and intracellular (Neurofibrillary tangles) NFTs. NFTs are made by hyper-phosphorylation of the microtubule-associated protein tau (MAPT), while proteolytic processing of the Amyloid beta Precursor Protein (APP) generates the neurotoxic Ab peptide, which has been involved in the formation of neuritic amyloid plaques. Recently Salvadores et al. defined a very sensitive method for biochemical diagnosis of AD based on specific detection of misfolded A β oligomers. They were able to distinguish AD patients from control individuals influenced by a variety of other neurodegenerative disorders or no degenerative neurological diseases with overall sensitivity of 90% and specificity of 92%. The protein misfolding cyclic amplification assay (Ab-PMCA), utilizes the functional property of Ab oligomers to seed the polymerization of monomeric Ab. Ab-PMCA allowed detection of as little as 3 fmol of Ab oligomers. These findings provide the proof-of-principle basis for developing a highly sensitive and specific biochemical test for AD diagnosis [67].

Recently a study has shown reduced expression of novel gene P9TLDR, potentially a microtubule-associated protein involved in neuronal migration, from an intra-cerebral brain-site-specific (AD temporal lobe vs. AD occipital lobe) polymerase chain reaction (PCR)-select cDNA suppression subtractive hybridization (PCR-cDNA-SSH) expression analysis. Additionally, this study also validated by *in vitro* AD-related cell model, amyloid- β peptide (Ab)-treated neurons which reduced P9TLDR expression correlated with increased tau protein phosphorylation [68].

Late-Onset Alzheimer's Disease (LOAD)

Recently Zhang et al. have discovered molecular systems linked with late-onset Alzheimer's disease (LOAD), by constructing gene-regulatory networks in 1647 post-mortem brain tissues from LOAD patients and nondemented subjects, they showed that LOAD reconfigures specific portions of the molecular interaction structure. Thus emphasised an immune- and microglia-specific module that is dominated by genes involved in phagocytosis, contains TYROBP as a key regulator, and is up regulated in LOAD. Mouse microglia cells over expressing intact or truncated TYROBP revealed expression changes that significantly overlapped the human brain TYROBP network [68].

Molecular Diagnosis of Genetically Transmitted Cardiovascular Diseases

Advances in molecular biology have improved our knowledge of the primary defects and basic mechanisms accountable for the pathogenesis of numerous diseases including cardio-vascular disease conditions and their phenotypic expression, and in the process, new approaches on cardiac diagnosis have been formulated.

Hypertrophic Cardiomyopathy (HCM)

HCM is a main and normally familial cardiac disease characterized by complex pathophysiology and unlimited heterogeneity in its morphological, functional, and clinical course. This wide diversity is pin-pointed by the fact that HCM may be seen in all phases of life, from the new-born to the elderly. The clinical course is extremely variable, with few patients remaining asymptomatic throughout life and others developing severe symptoms of heart failure, few die prematurely, either suddenly (often in the absence of prior symptoms) or owing to progressive heart failure. It has been marked, even from the preliminary descriptions of the disease, that HCM is commonly inherited as a Mendelian autosomal dominant trait [69]. HCM can be caused by a mutation in any 1 of 5 genes that encode proteins of the cardiac sarcomere: b-myosin heavy chain (on chromosome 14), cardiac troponin T (chromosome 1troponin I (chromosome 19), a-tropomyosin (chromosome 15), and cardiac myosin-binding protein C (chromosome 11). Moreover, mutations in 2 genes encoding crucial and regulatory myosin light chains have been reported in what may be an extremely rare form of HCM. This genetic diversity is further compounded by intragenic heterogeneity, with a total of more than 100 individual disease-causing mutations identified for these genes. Available data support that mutations in the b-myosin heavy chain gene (myosin is the primary contractile protein in thick filaments of myofibrils) may responsible for as much as 35% of familial HCM. All the known genetic myosin defects have proved to be missense mutations [70]. Cardiac troponin T mutations [71], responsible for an estimated 10–20% of familial HCM. Troponin T binds the troponin complex to tropomyosin and plays a major role in calcium regulation of cardiac contraction and relaxation.

Long-QT Syndrome (LQTS)

The long-QT syndrome (LQTS; Romano-Ward) is a rare familial disease transmitted as an autosomal dominant trait, causing a predisposition to syncope and sudden cardiac

death (often related to emotional or physical stress, vigorous activity, or arousal stimuli). Unexpected collapse is mediated through ventricular tachyarrhythmias such as polymorphic ventricular tachycardia (*torsade de pointes*) and ventricular fibrillation. Presently, there are three key LQTS genes (KCNQ1, KCNH2, and SCN5A) that provide explanation for approximately 75% of the disorder. For the major LQTS genotypes, genotype-phenotype correlations of gene-specific arrhythmogenic triggers, electrocardiogram (ECG) patterns, response to therapies, and intragenic and increasingly mutation-specific risk stratification. The 10 minor LQTS-susceptibility genes collectively account for less than 5% of LQTS cases [72]. The key diagnostic and phenotypic hallmark of LQTS is abnormal prolongation of ventricular depolarization, measured as lengthening of the QT interval on the 12-lead ECG.

Marfan Syndrome (MFS)

Marfan syndrome (MFS) is a systemic connective tissue disorder with autosomal dominant inheritance, first showed in 1896 by Antoine Marfan. Life expectancy may be reduced, usually due to involvement of the cardiovascular system with progressive aortic root dilatation, dissection and rupture, or valvular regurgitation. The primary defect responsible for MFS, first described in 1991, resides in a gene (FBN1) localized to the long arm of chromosome 15 encoding the connective tissue protein fibrillin-1 [73]. Fibrillin is a structural glycoprotein component of microfibrils, which are extracellular components that participate in the formation of mature elastic fibers and which provide structural functions independent of elastin. Linkage analysis has shown no locus heterogeneity for MFS; the cause-and-effect relation with the clinical Marfan phenotype has been confined to fibrillin mutations [74].

Molecular Diagnosis and Various Cancer

The enigma of carcinogenesis has been now streamlined into few significant hallmarks such as sustaining proliferative signalling, evading growth suppressors, resisting cell death, enabling replicative immortality, inducing angiogenesis and activating invasion and metastasis. Underlying these hallmarks is genome instability, which generates the genetic diversity that expedites their acquisition. Thus persistent progress in molecular research has quite simplified the puzzle although its pathogenesis is still in infancy in various cancers. Cancer is a change in the cellular processes that cause a tumour to grow out of control. Cancerous cells sometimes have mutations in oncogenes, such as KRAS and CTNNB1 (β -catenin) [75]. Analyzing

the molecular signature of cancerous cells, the DNA and its levels of expression via messenger RNA enables physicians to characterize the cancer and to choose the best therapy for their patients. As of 2010, assays that incorporate an array of antibodies against specific protein marker molecules are an emerging technology; there are hopes for these multiplex assays that could measure many markers at once [76].

Prostate Carcinoma

Globally, prostate cancer (PCa) is one of the commonest cancers in men influencing 33% of global burden. The prostate biopsy remains invasive method for detecting PCa like others and currently, serum prostate-specific antigen (PSA) is considered as one of the best available tumour marker for detecting PCa at early stage which also has prognostic value. However, there are certain limitations of PSA in which the most significant one is that it is prostate-specific and not cancer specific.

Genomics/Epigenetic and SNPs

Over the past thirty years epigenetic has broadened its field and played an important role in the study of cancer genetics. Epigenetic gene regulation denotes to non-coded heritable changes in gene expression which includes DNA methylation, histone modifications and noncoding RNA-induced transcriptional changes. These are desirable for the transcriptional regulation and genomic stability. Two histone modifiers HAT p300 and HDM EZH2 are promising PCa biomarkers which have shown to be over expressed in PCa and its expression levels precisely linked with different disease stages. This characteristics may make it a standard dual biomarker. Hypermethylation and gene silencing have been documented for cell cycle regulation such as anaphase promoting complex (APC) and Ras association domain-containing protein 1 (RASSF1a), detoxification enzymes e.g., glutathione S-transferase Pi 1 (GSTP1). Furthermore, combined assays for GSTP1 and APC hyper methylation have unlimited potential for detecting PCa in clinical samples up to 100% sensitivity. The risk of PCa can also be evaluated from single nucleotide polymorphisms (SNPs) of alleles in different region of chromosome (EHBPI, THADA, ITGA6, EEFSEC, PDLIM5, FU20032, SLC22A3, JAF1, LMTK2, NKX3, CMYC, MSMB, CTBP2, HNF1B, KLK2-3, TNRC6B, BIK, IL-10, IL-18 [77], NUDT10-11) which influence the behavior of the disease and its progression by changing expressions of mRNA and protein [78]. This has been explored and documented in more than 9000 patients (9893–61, 388 patients) [79].

Transcriptomics

The noncoding RNA (ncRNA) is a relatively novel field in PCa research. The term ncRNA encompasses the well-studied functional RNAs like rRNA and tRNA, as well as microRNA (miRNA; previously known as small ncRNA) including long ncRNA (lncRNA) and small interfering RNA (siRNA). Three known lncRNAs which have validated their significance in detecting, screening and monitoring PCa [80], because of their high specificity and sensitivity are PCa non coding RNA-1 (PRNCR1), prostate-specific gene 1 (PSGEM1), and PCa antigen 3 (PCA3); also referred to as differential display 3 (DD3). Recently it has also been proposed that PCGEM1 gene, which encodes a lncRNA is highly prostate-specific. Moreover, the screening of TMPRSS2-ERG fusion (TEF) techniques as examined by Immunohistochemistry, FISH and RT PCR found to have significance in the diagnosis PCa. However the TE fusion in combination with PCA3 mRNA may prove more beneficial in diagnosis [81]. Circulating microRNAs (miRNA) have newly been supposed to be biomarkers for non-invasive diagnosis in various tumors [82]. Several gene expression studies also reported altered interleukins expression in prostate cancer patients [83]. These differentially regulated miRNAs lead to changes in the expression and activity of their targets in PCa. The miRNA expression changes with the development and progression of PCa as some of the cancer-related genes are regulated by them and thus its dysregulation has significance in PCa. Using a mouse xenograft model, Mitchell et al. [84] have demonstrated that miRNAs originated from the human PCa xenografts enter the circulation and thus reported that miR-141 is up regulated in sera of metastatic PCa patients which can distinguish PCa patients from healthy controls with high sensitivity and more accuracy.

Proteomics

Proteomics also play a dynamic role in the field of biomarker specially in non-invasively collected bio fluids as for prognosis [CGRP, VEGF, endoglin (CD105), chromogranin-A, neuron-specific enolase, interleukin-6 transforming growth factor- β , other methylated genes including RASSF1a, APC, RARB2 and CDH1, prostate-specific cell antigen, testosterone, estrogen, sex hormone binding globulin, caveolin-1, E-cadherin, b-catenin, MMP-9, tissue inhibitor of MMPs (TIMP 1, 2) progastrin-releasing peptide (ProGRP 31–98)] and PSP94, ZAG, prostasome (auto-antibodies), huntingtin interacting protein 1 (auto-antibodies), TSP-1, leptin, ILGF-1, -2, human kallikrein 2, a-methylacyl-CoA racemase (auto-antibodies), early prostate cell antigen-1, -2, GSTP1 hypermethylation, cytokine

macrophage MIF, hK11, apolipoprotein A-II for diagnosis. Few as urokinase-type plasminogen activator system, prostate membrane-specific antigen, hepatocyte growth factor, MIC-1, EGFR family (c-erbB-1 (EGFR), c-erbB-2 (HER2/neu), c-erbB-3 (HER3) and c-erbB-4 (HER4)) [85], have shown their unique potency in diagnosis as well as prognosis [83]. More recently Dwivedi et al. [86–91] have proposed circulating serum interleukin-18 as a diagnostic biomarker and interleukin-10 for prognosis. Metastatic castration resistant PCa (MCRPCa) and metastasis associated protein-1 (MTA-1) have been widely explored for their role in PCa mainly in vascularization of the progressing tumor. The significance of WNT5A, EZH2, MAPK pathway members, AR, various androgen metabolism genes are also over expressed in metastatic PCa and c-FOS jun-B down-regulated thus also have significance as biomarker. Other promising molecular markers for this cancer which are reportedly over expressed are human kallikrein-related peptidase 2 (hK2), early PCa antigen (EPCA), a-methylacyl-coA racemase (AMACR), insulin-like growth factors and binding proteins (IGFBP-2 and IGFBP-3), TGF- β 1, elevated circulating levels of the interleukin-6 (IL-6), and its receptors, urokinase plasminogen activator (uPA) and receptor (uPAR), enhancer of zeste homolog 2 (EZH2), and prostate-specific membrane antigen (PSMA) [92].

Breast Cancer

Breast cancer is a foremost public health issue globally. The number of new cases of female breast cancer was 124.9 per 100,000 women per year. The number of deaths was 21.2 per 100,000 women per year. These rates are age-adjusted and based on 2010–2014 cases and deaths. (National Cancer Institute stats report <https://seer.cancer.gov/statfacts/html/breast.html>). The more typical approach to breast cancer diagnostics via hormone receptor analysis is IHC. IHC involves the use of antibodies and enzymes, such as horseradish peroxidase, to stain tissue sections for the tumor antigens of interest. This analysis method can be performed on either frozen or formalin-fixed paraffin-embedded (FFPE) tissue, as well as on small amounts of tissue acquired in procedures such as core biopsies. IHC also has the advantage of not only determining the percentage of positive nuclei but also the intensity of staining in individual nuclei. Unfortunately, in addition to a lack of inter-laboratory standardization of the IHC technique, the process for characterizing the positivity of either ER or PR staining is performed subjectively by a pathologist, thereby introducing variability in interpretation. Regardless of this subjectivity in staining intensity, IHC is by far the most common approach to evaluating hormone status in breast

cancer today. Another major prognostic marker that is currently recommended for the evaluation of primary invasive breast cancer is the human epidermal growth factor receptor 2, also known as HER2. HER2 is an oncogene belonging to the EGF receptor (EGFR) family. Gene amplification of HER2 has been shown to occur in 10–40% of primary tumors and HER2 protein overexpression is found in almost 25% of breast cancers [93].

HERmark™ Assay

In an effort to expand the available methods of HER2/neu analysis, Monogram Biosciences has recently released the HERmark™ breast cancer assay. This assay measures total HER2 protein (H2T) and functional HER2 homodimer (H2D) levels on the cell surface of FFPE breast cancer tissue. It practices a dual antibody system in which a fluorescent tag on one antibody is cleaved by a second antibody containing a photo-activated molecule. The fluorescent tags are then quantified using capillary electrophoresis (CE). HERmark reports whether a patient is HER2-negative, -positive or -equivocal based on quantified HER2 protein levels expressed as numeric values (HERmark, Monogram Biosciences, Inc. www.hermarkassay.com).

Transcriptomics Based Biomarkers: Theros H/ISM and MGISM

Theros H/ISM is a molecular diagnostic test that assesses the ratio of HOXB13:IL17BR gene expression as a predictor of clinical outcome for breast cancer patients treated with tamoxifen. A high level of expression of the two-gene ratio has been associated with tumor aggressiveness and failure to respond to tamoxifen [94]. Theros MGISM is an additional test that uses a five-gene expression index to stratify ER + breast cancer patients into high or low risk of recurrence by reclassifying grade 2 (intermediate proliferative) tumors into grade 1-like or grade 3-like outcomes [95].

Mamma Print™

The Mamma Print test is a molecular diagnostic tool that evaluates a breast cancer patient's chance for tumor recurrence. The Mamma Print uses a 70-gene signature that has been reported to have independent prognostic value over clinicopathologic risk assessment in patients with node-negative breast cancer. The test needs a fresh sample (at least 3 mm in diameter) obtained during a surgical

biopsy to be sent to the Agendia laboratory in Amsterdam in an RNA-stabilizing solution for analysis. RNA is isolated from the sample, amplified and hybridized with a standard reference to the Mamma Print microarray to obtain the 70-gene expression profile [96]. This method has been shown to have an extremely high correlation of prognostic prediction to tumor recurrence ($p < 0.0001$). In 2007, the US FDA approved the Mamma Print test for use on freshly frozen tissue. Although the Mamma Print gene expression profile has the potential to be a useful diagnostic tool, there are many limitations that need to be taken into consideration.

Oncotype DX®

Oncotype DX is a 21-gene expression assay that uses qRT-PCR and microarray technologies to characterize patients who may be positively treated with chemotherapy and estimate the likelihood that invasive breast cancer will recur after treatment. The Oncotype DX assay uses FFPE tissue blocks that can be shipped from anywhere in the USA and internationally. Currently, Oncotype DX is the standard breast cancer screening test for women with early-stage (Stage I or II), node-negative, ER + invasive breast cancer. The assay reports a recurrence score that ranges from 0 to 100, indicating the probability of cancer recurring within 10 years of the original diagnosis. The recurrence score is then categorized into one of three groups: low, intermediate or high risk. There is a particular urgency for such information in women with early-stage breast cancer, where the great variety of treatment options can be narrowed down and tailored to each patient. Both ASCO and the National Comprehensive Cancer Network (NCCN) have incorporated the Oncotype DX assay into their guidelines [97].

MicroRNA

MicroRNA deregulation in breast cancer was primarily described by Iorio and colleagues in 2005. Since this first study, there has been a surge of data added on the expression of various microRNAs and their roles in breast cancer. miR-21 has surfaced in multiple studies as having consistent and significant increased expression in breast cancer cell lines and human tissue when compared with normal cells and tissues. Additionally miR-10b was one of the three microRNAs in the Iorio et al. study that demonstrated significant down regulation in breast cancer cells (HMECs). However, in a successive study, miR-10b appeared to be highly expressed in metastatic cancer cells.

³⁹¹ Functional studies have described that miR-10b over expression promotes cell migration and invasion in vitro, and initiates tumor invasion and metastasis in vivo. Multiple studies have also demonstrated a significant association between expression of miR-206 and the expression of estrogen receptors in breast cancer. Iorio et al. [98] were the first to show that miR-206 expression was raised in those tumors that were ER. miR-125a and miR-125b were first reported in a microRNA profile study to be significantly down regulated in HER2-positive breast cancers. Computation analysis then confirmed target sites at the 3'UTR regions of HER2 and HER3 for these microRNAs. (Matti et al. 2006) A tissue culture analysis showed that overexpression of miR-125a or miR-125b in an ErbB2-dependent cancer cell line (SKBR3) suppressed HER2 and HER3 transcript and protein levels, which decreased cell motility and invasiveness [99]. Recent advancements and investigations in the field of liquid biopsy-based biomarkers, especially DTCs and CTCs bearing molecular signature have the capability to behave as potential biomarkers and can discriminate breast cancer between localized to metastasizing one. Further our current project SERB-NPDF 2015/000322 DST, New Delhi also showing unique trends of various miRNA with cancer stem cells in breast cancer patients.

Lung Cancer

Lung cancer is the major cause of cancer-related death in the world. Non-small cell lung carcinoma (NSCLC) explains for 80–85% of all lung cancers, with lung adenocarcinoma being the most typical histologic type in the United States. Enhancements in our knowledge of molecular alterations at multiple levels (genetic, epigenetic, protein expression) and their functional importance have the potential to impact lung cancer diagnosis, prognostication and treatment. In lung cancer as in other malignancies, tumourigenesis narrates to activation of growth promoting proteins [e.g., v-Kras2 Kirsten rat sarcoma viral oncogene homolog (KRAS), epidermal growth factor receptor (EGFR), BRAF, MEK-1, HER2, MET, ALK and rearranged during transfection (RET)] as well as inactivation of tumour suppressor genes [e.g., P53, phosphatase with tensin homology (PTEN), LKB-1] [100]. Lung cancers have extremely complex genomes with a recent large scale exome sequencing study of 31 non-small cell lung cancer (NSCLC) identifying 727 mutated genes not previously described in the literature or in the COSMIC database. Genomic studies have established previously well-known alterations in lung cancer such as KRAS, EGFR and BRAF and also identified low frequency but recurrent mutations that are novel in lung cancer including potentially

targetable alterations in JAK2, ERBB4, RET, fibroblast growth factor receptor 1 (FGFR1), and discoidin domain receptor 2 (DDR2) [101]. Lung cancer mutations have been recognized in v-Ki-ras2 Kirsten rat sarcoma viral oncogene homolog (KRAS), epidermal growth factor receptor (EGFR), BRAF and the parallel phosphatidylinositol 3-kinase (PI3 K) pathway oncogenes and more recently in MEK and HER2 while structural rearrangements in ALK, ROS1 and possibly rearranged during transfection (RET) provide new therapeutic targets. Amplification is another mechanism of activation of oncogenes such as MET in adenocarcinoma, fibroblast growth factor receptor 1 (FGFR1) and discoidin domain receptor 2 (DDR2) in SCC.

The role of tumour suppressor genes is increasingly recognized with aberrations reported in TP53, PTEN, RB1, LKB11 and p16/CDKN2A. The occurrence of these molecular targets as labelled above now defines the characteristics of NSCLC, with EGFR mutation and ALK rearrangements being the most clinically relevant at present. The prevalence of these mutations varies in lung cancer arising from patient in different regions. Activating EGFR mutations were found in up to 20% of Caucasians while in the Asian populations these EGFR mutations can be present in up to 40% of patients with NSCLC. These ethnic difference in NSCLC properties seems to be not limited to the presence of activating EGFR mutations but is also evident in other driver oncogenic mutation profiles (including ALK, KRAS, MET etc.), histology and hence tumour response to targeted therapy treatment. The presence of these driver mutations is normally found to be mutually exclusive to others in the same tumour [102]. In lung ADC among Asians, ALK rearrangement is seen in up to 7% of patients with lung ADC. Based on current reports of therapeutic molecular targets of EGFR mutation and ALK gene rearrangement in NSCLC and the availability of corresponding targeted agents, an algorithm of testing for molecular targets in NSCLC is proposed, which signifies a stepwise approach to testing for individual targets, beginning with EGFR then, if negative, ALK fusion gene or other potential targets if appropriate. Among NSCLC, adenocarcinoma accounts for up to 80% of histological subtypes. There are previous reports of correlations between histological subtypes of ADC demonstrating micropapillary features with presence of activating EGFR mutations, leading to the suggestions that the presence of specific mutations in NSCLC in fact represent heterogeneity in cancer biology and also response to therapy [103]. Given the heterogeneity of lung cancer histology, however, histological subtypes are hard to be used as the sole reliable marker for guidance to molecular phenotyping and selection of targeted therapy. Targeting therapeutic oncogenic mutations like EGFR and ALK can give dramatic initial treatment response or at least an initial stable clinical disease.

Oral Cancer

Oral cancer is among the 10 most common cancers worldwide, and is particularly seen in disadvantaged elderly males. Early detection and quick treatment provide the best chance for cure. The most predictive of the molecular markers thus far available and assessed in OSCC development include the TSG p53 protein expression, chromosomal polysomy (DNA ploidy), and changes (termed loss of heterozygosity; LOH) in chromosomes 3p or 9p (probably due to changes in the TSG p16). The practise of such biomarkers as adjuncts to routine histopathological assessment may help prognostication and effective management of PMLs but their routine use is still hindered by the cost and complexity of the tests, the lack of facilities in some laboratories, and limited outcome studies to date. More readily available markers, such as those of cell proliferation (Ki-67 antigen) and apoptosis (Bax, Bcl-2), may also play a diagnostic role: apoptotic Bcl-2 expression decreases significantly in dysplastic and early invasive lesions and then increases almost to normal tissue level in consequent stages while Ki-67 expression increases sharply in initial stages of OSCC, but significantly decreases in later stages [104].

The brush biopsy utilizes a small nylon brush to gather cytology samples then sent for computer scanning and analysis (Oral CDx) to identify and display individual cells. If suspect cells are identified, a pathologist then examines them to determine the final diagnosis and, in samples judged to be cancerous, a printout of the abnormal cells from the computer display and a written pathologist's report are returned to the clinician with the recommendation that a positive result be followed with a conventional incisional biopsy. The technique has proved rather controversial, with concern largely related to the question of false negative results. In the first published study, 59 of 945 patients in USA, the brush biopsy reportedly detected correctly all cases of OSCC, even when dentists did not suspect the presence of cancer from the lesion, but this trial showed a multi-center convenience sample, not all lesions were biopsied and inadequate specimens were excluded from the analysis. In a further US study, 61 brush biopsy results when compared with scalpel biopsy and histology to determine the positive predictive value of an abnormal brush biopsy finding showed that, of 243 patients with abnormal brush biopsies, 93 proved positive either for dysplasia [105] or carcinoma [106], and 150 were negative for either dysplasia or carcinoma, giving the positive predictive value of an abnormal brush biopsy of 38% (93/243).

Promoter hyper-methylation patterns of TSG p16, O6-methylguanine-DNA-methyltransferase, and death-associated protein-kinase have been characterized in the saliva of head and neck cancer patients [107]. Forensic science has

since shown that saliva can contain a number of messenger ribo-nucleic acid (mRNA) fragments including salivary specific statherin, histatin 3, and the proline-rich proteins PRB1, PRB2 and PRB3, as well as the ubiquitously expressed spermidine N1-acetyltransferase (SAT), β -actin, and glyceraldehyde-3-phosphate dehydrogenase (GAPDH). The mRNAs in saliva such as β -actin, SAT and interleukin-8 are relatively stable despite the presence of salivary ribonucleases. mRNAs in saliva have been tested in over 300 saliva samples from OSCC patients and healthy people, and the signature was always present in higher levels in the saliva of OSCC patients than in saliva from healthy people, with an overall accuracy rate of about 85%. Four salivary mRNAs (OLF/EBF associated zinc finger protein [OAZ], SAT, IL8, and IL1b) collectively have a discriminatory power of 91% sensitivity and specificity for OSCC detection [108]. Seven mRNA molecules: transcripts of: 1. IL8 (interleukin 8) playing a role in angiogenesis; replication; calcium-mediated signaling pathway; cell adhesion; chemotaxis; cell cycle arrest; immune response, 2. IL1B (interleukin 1B) which takes part in signal transduction; proliferation, inflammation and apoptosis 3. DUSP1 (dual specificity phosphatase 1) with a role in protein modification; signal transduction and oxidative stress, 4. H3F3A (H3 histone, family 3A) having a DNA binding activity, 5. OAZ1 (ornithine decarboxylase antizyme 1) taking part in polyamine biosynthesis 6. S100P (S100 calcium binding protein P) with a role in protein binding and calcium ion binding, and 7. SAT (spermidine/spermine N1-acetyltransferase) which takes part in enzyme and transferase activity were found significantly elevated in OSCC patients rather than in healthy controls [109].

Now, genetics has become the driving force in medical research and is now ready for integration into medical practice. Human genome draft (bio-informatics) with advancement in current techniques now opens new vistas in the fields of novel therapeutics such as Pharmacogenomics, Nutrigenomics that may transform the management of untreated disease and disorders [110–112].

Personalized Medicine: An Integration of Diagnostics with Therapeutics

In a large patient population size, a medicine and drug that is advantageous in many patients often flops to work in some other patients. Moreover, when it does work, it may cause adverse side effects, even death, in a small number of patients. Although large individual variability in drug efficacy and safety has been known to exist since the establishment of human medicine but the cause was beyond knowledge. On the other hand, the request to

overcome such variation has received more consideration now after the draft of human genome than ever before. The human genome sequence offers a special record of human evolution that varies among populations and individuals. Sequence variations in drug target proteins, drug-metabolizing enzymes, and drug transporters can alter drug efficacy, drug side effects, or both to cause variable drug responses in individual patients. From this prospect, the availability of the complete human genome sequence has made it possible to analyse the influence of variations of the human genome sequence on the pathogenesis of significant diseases and the response to drug therapy at an accelerating rate in recent years. The fast build-up of knowledge on genome-disease and genome-drug interactions has also encouraged the transformation of pharmacogenetics into a new entity of human genetics, pharmacogenomics and, at the same time, provided a rationale for the hope that individualized medicine can be achieved in the near future [113, 114].

The utilization of high throughput genotyping tools for the characterization and screening of single nucleotide polymorphisms (SNPs) ultimately can lead to the determination of the unique molecular signature of an individual in a relatively short period of time and it can predict the susceptibility or risk of any diseases. As The double mutation, A to T transversion at 1762 and G to A transition at 1764, is often present in patients with chronic hepatitis, hepatocellular carcinoma, and hepatitis and less often in asymptomatic carriers, in immunosuppressed patients, and in carriers without HBV markers. Further, individual drug responses can be forecasted from known genetic variances correlated with a drug effect, like drug response of Maraviroc efficacy depends on CCR5 alleles in HIV-1 infections and Interferon-alpha drug efficacy also decided by the alleles of IL28B in Hepatitis C infections [115]. Thus, this will allow the physician to decide the patient with a selective drug treatment.

Few pharmaceutical companies and research development agencies are developing a precise haplotyping scheme to identify individuals/patients who will be benefiting from which type of drug in a particular disease and disorders. Further, Nutrigenomics is also evolving and promising a better management of patients. Several bioactive food components, including both essential and nonessential nutrients, can regulate gene expression patterns. Thus, nutrigenomics is providing the effects of ingested nutrients and other food components on gene expression and gene regulation, i.e. diet–gene interaction in order to spot the dietary components having beneficial or detrimental health effects. Nutritional genomics (nutrigenomics), the junction between health, diet, and genomics, is influenced via epigenetic, transcriptomics, and proteomics processes of biology. Thus, it will help in determining the individual nutritional requirements based on the

genetic makeup of the person (personalized diet) as well as the association between diet and chronic diseases like cancer, opening new vistas to understanding the complexity of various diseases [116].

Conclusions

In the upcoming years, molecular diagnostics will continue to be of critical importance to public health worldwide. Molecular diagnostic offers physicians with critical information based on the early exploration of pathogens and subtle changes in patients' genes and chromosomes, allowing for earlier diagnosis, selection of appropriate therapies and monitoring of disease progression. A wide range of molecular based tests is available to evaluate DNA difference and changes in gene expression of patient DNA and RNA through Real-Time PCR, FISH and Sequencing Technologies [117]. Further, the complex relationship between diseases like Fertility and Obesity or Metabolic syndrome with Cancer have also gained momentum due to exploration of common pathways, that only become possible due to such advancement in molecular techniques [118]. However, there are many obstacles to overcome before the execution of these tests in clinical laboratories, such as which test to employ, the choice of technology and equipment, and issues such as cost-effectiveness, accuracy, reproducibility i.e. robustness, personnel training etc. Currently PCR-based testing outweigh; however, alternative technologies aimed to explore genome complexity without PCR are anticipated to gain momentum in the coming years as sequencing devices are more costly at present. Furthermore, development of integrated silicon chips mounted with biomolecules is now going to change the concept of traditional wet lab to "lab-on-a-chip". Thus it would be possible to analyse thousands of genes/proteins in hours from low amount/single cell sample. Thus coming era will be revolutionary, it will not only going to change our diagnostic systems but also plan of treatment and therapy [119, 120].

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