EMERGENT TECHNOLOGIES IN THE DETECTION OF EMERGING AND RE-EMERGING VIRUSES WITH REFERENCE TO INFECTIONS IN HUMANS.

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ABSTRACT:

Diagnostic virology, once limited to cell culture methods, microscopy and serology has now been revolutionized by newer technologies. The need to improve sensitivity, cut down instrumentation costs and augment portability for field studies have led to the emergence of nanotechnology based diagnostic tools. Nanotechnology for viral diagnostics is based on nanoscale reactions, the analyte is detected by a biosensor. These could be electrochemical, optical or piezoelectric biosensors. An amperometric biosensor was developed for rapid measurement of the concentrations of the virus measured by cyclic voltammetry. Potentiometric biosensors with nanoporous gold electrodes carrying conjugates of the secondary antibody-gold particles labeled with horseradish peroxidase (HRP), elecrochemical impedence spectroscopy (EIS) with antibody immobilized on the surface of a platinum electrode fabricated with colloidal gold and carbon nanotubes have all been used to detect viruses like influenza virus type A, HBV, HCV etc.

Nanowires are ultra-thin silicon wires that can be used to detect viruses with very high sensitivity and specificity in real time. Whispering-gallery mode (WGM) biosensors offer ultrasensitive, label-free detection in miniaturized formats making them highly suitable for use in real time detection of viruses in field situations. Surface Plasmon resonance (SPR) a form of reflectance spectroscopy is applicable as biosensors for virus detection. Quantum dots (QD) which are fluorescent semiconducting nanocrystals are now developed for the simultaneous detection of several respiratory viruses. This review is focused on promising new technologies that have emerged in the decade for the diagnosis of emerging and re-emerging viral infections. These technologies are especially useful in early diagnosis of infections with epidemic potential.

Key words: *Nanotechnology, viral infections.*

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INTRODUCTION:

Infectious diseases have always been a threat to mankind. Emerging and re-emerging infectious diseases include previously unknown diseases as well as known diseases with increasing incidence. Several factors including changing immigration patterns, ecological changes, climate changes, changes in social structure etc. contribute to emergence and re-emergence of infectious diseases. Most of these infections are caused by RNA viruses. These virsues contain / code for RNA polymerases required for their genome replication. These enzymes lack proof reading ability which increases the frequency of mutations that could be an important factor in the evolution RNA viruses. Appropriate disease surveillance and diagnosis is the key to eliminate or minimize the spread of the infectious agent during an outbreak.

Viruses associated with emerging and re-emerging human infections include dengue viruses, chikungunya virus, West Nile virus, Japanese B encehepahlitis virus, severe acute respiratory syndrome associated coronoviruses (SARS-CoV), human immunodeficiency virus (HIV), influenza A virus, hantaviruses, hepatitis C virus (HCV), hepatitis E virus (HEV) etc. Emerging and re-emerging viral infections pose significant challenges in surveillance and diagnosis. Direct methods of diagnosis of viral infections include antigen detection, cultivation of viruses in cell culture and the detection of viral nucleic acids from clinical samples. Detection of antibody against viruses represents an indirect method of diagnosis. Indirect methods of diagnosis have an inherent disadvantage of a longer window period and also have little or no prognostic value. With the advent of molecular techniques, nucleic acid detection methods are being increasingly used in diagnostic Virology. Necessities to reduce the window period, enhance sensitivity, circumvent expensive instrumentation, improve cost-effectiveness, reduce turnaround time and augment compatibility with field studies have led to the rapid development of newer technologies in diagnostic Virology. This review will focus on promising new technologies that have emerged in the last five to ten years for the diagnosis of emerging and re-emerging viral infections. Many of these technologies are available only in sophisticated public health laboratories in the west and only in a few advanced laboratories in India. India unfortunately lacks a network of good state-of-the-art public health laboratories.

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EMERGENT TECHNOLOGIES

Polymerase Chain Reaction (PCR)

Since the advent of PCR in the mid 1980s a wide range of PCR assays have evolved. Briefly, the target DNA is amplified using oligonucleotide primers (18-30bp in length) that bind specifically to the target DNA. The amplification involves denaturation of the target DNA followed by annealing of the oligonucleotide primers to the target DNA. The annealed primers are then extended by the enzyme *Taq polymerase* to create new copies of the target DNA. This cycle is repeated several times resulting in exponential amplification of the target DNA. The amplified DNA can be visualized using agarose gel electrophoresis. Amplification of viral nucleic acids using PCR allows for the detection of one or a few copies of the viral genome.

PCR has revolutionized molecular biology and its applications in the diagnosis of viral infections. The advantages of PCR over conventional diagnostic methods include high sensitivity, early diagnosis and rapid turnaround time and the ability to detect non-cultivable agents. Multiplex PCRs were developed to overcome the lack of cost effectiveness. The use of multiple pairs of primers, each pair specific for different targets allows the detection of multiple viruses in one reaction tube. Multiplexing significantly reduces the use of consumables and also manpower. In addition, multiplex PCRs are suitable for simultaneous screening of multiple viruses that are organ system specific or symptom specific. For example, a nasal swab could be used for screening several respiratory viruses that are etiological agents of respiratory disease. However, multiplex PCRs are more exacting in terms of primer design and optimization.

Real time PCR

The increasing need for quantitation of viral nucleic acids lead to the development of real time PCR. The accumulation of PCR products is monitored in real time using various platforms. SYBR green I is a cyanine dye that binds to nucleic acids and is hence acts as a fluorescent reporter to monitor accumulation of amplicons in real time. SYBR green is widely used for real time PCR owing to high sensitivity and cost effectiveness. Apart from SYBR green various chemistries such as Taqman probes, molecular beacons, Scorpion primers, Florescence resonance energy transfer (FRET) probes, primer probe energy transfer etc. are used for real time PCRs. Real time PCR offers several advantages over conventional PCRs: a) It provides quantitative data b) reduces the risk of cross contamination by minimizing the need for post-PCR handling c) hands-on time is reduced d) turnaround time is reduced e) offers high sensitivity and specificity.

Real time PCR has created phenomenal improvements in molecular diagnosis of viruses and should be considered as one of the major advancements in the field. Real time PCR is now widely use for the diagnosis and monitoring of HIV, hepatitis C virus (HCV), chikungunya, dengue, West Nile, Ebola, Marburg, Yellow fever viruses etc [1-3].

Mass tag PCR

Multiplex PCRs have a maximum limit of 3-4 targets per reaction, limiting the use of multiplex PCRs in the screening of viruses that are etiological agent of a syndrome (eg. respiratory disease / diarrhea). More recently, mass tag PCRs were developed to overcome this issue. Briefly, the target is amplified with 'mass tag' primers with a unique molecular weight tag. Subsequent to PCR amplification, the amplicons are purified using column based methods to remove unincorporated primers. The 'mass tag' is then photocleaved from the amplicons using ultra violet rays and analyzed using spectrophotometry. The detection and identification of viruses in clinical samples is based on their cognate tags. A single mass tag PCR reaction was able to rapidly detect up to 22 different respiratory pathogens from clinical specimens with high sensitivity[4].

Isothermal amplification

Isothermal amplification technologies such as loop-mediated isothermal amplification (LAMP) are becoming increasingly used. LAMP uses a set of six specially designed primers: 3 forward and 3 backward primers. In the non-cyclic step the strand displacement activity of *Bst* DNA polymerase helps in the synthesis of a complementary strand primed by the forward primers. The outermost forward primer displaces the complementary strand, which forms a stem loop structure at the 5′ end. This serves as a template for the backward primer initiated DNA synthesis. Subsequently, the outermost backward primer primed strand displacement DNA synthesis results in the formation of a dumbbell-shaped structure with stem loops at both ends. This dumbbell-shaped structure is the template for cyclic amplification using internal primers. The cyclic step is associated with exponential amplification of the template. The amplified template can be visualized using agarose gel electrophoresis. Owing to higher amplification efficiency, LAMP reactions yield large quantities of pyrophosphate ions, which can combine with magnesium ions to form an insoluble white precipitate of magnesium pyrophosphate in the reaction tube. The turbidity associated with the formation of magnesium pyrophosphate allows for real time monitoring of LAMP amplification. Turbidimetric analysis can be performed by an OD reading at 400nm. Turbidimetry is relatively inexpensive compared to fluorometry used in real time PCR[5]. The unique advantages of LAMP are a) isothermal amplification using a heating block or a water bath and b) compatibility with cost-efficient turbidimetric analysis for real time monitoring. Applications of LAMP include the detection of HIV-1[6] and SARS-CoV[7].

Other nucleic acid amplification techniques, including signal amplification techniques such as branched DNA, hybrid capture, etc have limited applications.

Nucleic acid testing (NAT) for blood borne viruses

The surveillance methods of blood borne viruses in blood banks have undergone significant improvements in the last few years. Blood and blood products are screened by NAT for HCV, HIV and hepatitis B virus (HBV) in many developed countries[8,9]. Multiple samples (8-96 samples) are pooled and NATs are performed; if a pool tests positive for any of the blood borne viruses, each sample in the pool is retested individually. NATs combine the advantages of high throughput screening with a sensitivity several orders of magnitude higher than conventional serological techniques. The use of NATs has reduced the risk of transmission of blood borne viruses through transfusion of blood and blood products to negligible levels[9].

Robotics in diagnostic virology

With the extensive use of nucleic acid detection techniques, there is an increasing need for robust and high throughput nucleic acid extraction techniques. Several robotic options are now available for automated nucleic acid extraction[10]. These automated methods offer improved efficiency and robustness and reduce the hands-on time and

labour input compared to manual methods of extraction. In addition, robotics minimizes the risk of cross contamination and allows for high throughput applications. It is a matter of time before extraction, amplification and detection of nucleic acids are all done on a single automated machine.

Portable thermal cyclers

Battery operated, stand-alone portable thermal cyclers as well as portable real time PCR machines are now available. These portable machines are especially useful for rapid on-site diagnosis during disease outbreaks.

Nanotechnology

Nanotechnology for viral diagnostics is based on nanoscale reactions where the interaction of the bioreceptor with the analyte is detected by a transducer. The bioreceptor plays a key role in providing specificity by selective interaction with the analyte. The transducer converts the biorecognition event into interpretable signals. Expertise from inherently different fields of biochemistry and physics has led to the development of biosensors. The biosensors used in nanotechnology may be classified into electrochemical biosensors, optical biosensors and piezoelectric biosensors.

Electrochemical biosensors

Majority of the biosensors used for detection of viruses are electrochemical in nature. The advantages of electeochemical biosensors include high sensitivity, suitability for microfabrication, and simple instrumentation.

Amperometric biosensors

Antigen-antibody binding and DNA hybridization do not generate a significant signal of their own, necessitating the use of an active electrochemical marker. An amperometric biosensor was developed for rapid evaluation of the concentrations of the Japanese B encehapahlitis vaccine. An antibody to the Japanese B encehepahlitis virus was immobilized on a gold nanoparticle / o-phenylenediamine polymer film bilayer was used with a platinum electrode[11] and Prussian blue was used as an electrical mediator. Ferrous and ferric ions were used as probes on the surface of the platinum electrode. In a temperature and pH controlled microenvironment the concentration of the Japanese B encehepahlitis vaccine was measured by cyclic voltammetry. The assay was highly sensitive, reproducible and had a correlation coefficient of 0.995.

Potentiometric biosensors

Potentiometric biosensors measure the change in pH, redox potential or changes in ionic concentration. Hepatitis B virus in serum was detected using nanoporous gold electrodes with conjugates of the secondary antibody-gold particles labelled with horseradish peroxidase (HRP). The potential shift generated by the electroactive product of HRP catalyzed *o*-phenylenediamine oxidization with hydrogen peroxide was directly proportional to the concentration of hepatitis B surface antigen (HBsAg). This assay was highly sensitive, reproducible and rapid[12]. Similar methodologies have been described to detect Japanese B encephalitis virus[13]. Potentiometric sensors in combination with a flow-through immunofiltration-enzyme assay system has been developed for the rapid and specific identification of upto 8 different biological warfare (BW) agents within a span of 15 minutes[14]. The lower limit of detection was as low as 3×10^3 cfu/mL. Thus making this assay superior to many of the existing technologies.

Elecrochemical impedence spectroscopy (EIS)

Antibody to hepatitis B surface antigen (anti-HBs) was immobilized on the surface of a platinum electrode fabricated with colloidal gold and polyvinyl butyral. The binding of HBsAg to the immobilized anti-HBs results in an impedance change that is detected using EIS. The sensitivity of impedance measurements is scaled up via the encapsulated effect of polyvinyl butyral and the large specific surface area and high surface free energy of gold nanoparticles. HBsAg could be detected in concentrations as little as 7.8 ng/ mL and the linear range was 20-160 ng/mL[15].

Carbon nanotubes

Carbon nanotubes are created with allotropes of carbon with a cylindrical nanostructure. The carbon nanotubes with cylindrical carbon molecules display extraordinary strength and unique electrical properties. Multiwalled carbon nanotube-modified glassy carbon electrode (GCE) with covalently immobilized 4, 4′-diaminoazobenzene (4,4′-DAAB) were used to detect hybridization of HBV DNA sequences. The binding of HBV DNA to ss-HBV DNA probe on the modified GCE with immobilized 4,4′-DAAB induced a decrease in peak current of (associated with redox reaction) as measured by differential pulse voltammetry[16]. Hybridization of non-complementary DNA did not alter the peak current. The decrease in the peak current following the hybridization of specific DNA is attributed to DNA helices blocking the electron transfer between the 4,4′-DAAB and the electrode surface associated with the low conductivity of DNA molecules. Carbon nanotubes have also been used to detect influenza virus (type A) and HCV RNA[17, 18].

Nanowire arrays

Nanowires are ultra-thin silicon wires that can be used to detect viruses with very high sensitivity and specificity in real time. Silicon nanowires are synthesized with silane as reactant, 20-nm gold nanoclusters as catalysts and diborane as p-type dopant using chemical vapor deposition. The nanowires are covalently coupled with the virus specific antibody receptors. Fluidic channels formed by a flexible polymer channel or a 0.1-mm-thick glass coverslip sealed to the array device were used to deliver the samples. Electrical measurements were made by using lock-in detection with a prime number between 17 and 79 Hz chosen as the modulation frequency. Conductance vs. time data was recorded. The binding of the virus to the antibody induces a telltale change in conductance (Figure 1).

Concurrent detection of electrical and optical signals confirmed that the changes in conductance correspond to the binding and unbinding of the virus. Nanowire arrays

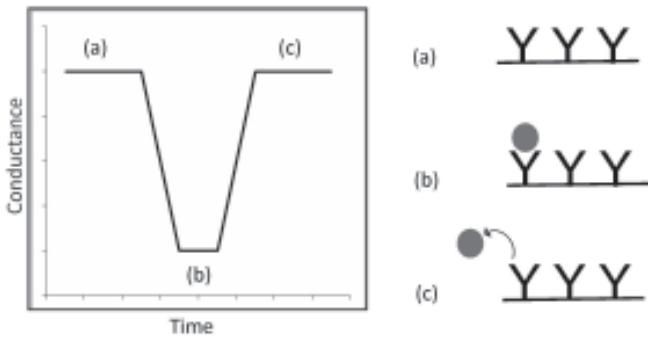

Figure 1: Detection of a single virus using nanowire devices.The panel on the left shows the changes in conductance associated with the binding and release of a single virus. The panel on the right explains how the changes in conductance are occurring (a) A nanowire device modified with a virus specific antibody. (b) a drop in conductance associated with the binding of a single virus to the nanowire device with the specific antibody. (c) Conductance returns to baseline levels as the virus unbinds.

have been used to detect Influenza viruses, Paramyxoviruses and adenoviruses[19]. The high sensitivity of nanowire arrays allows the detection of single viruses. Hence they could detect the presence of the viruses very early in the incubation period even before the onset of disease. Nanowire arrays could be adapted to detect several viruses from one clinical sample. In addition, these arrays could potentially be modified for use in the detection of viral variants and genetically engineered strains.

Optical biosensors

When light comes in contact with a sample, it pass through the sample or be reflected through the sample depending on the angle of incidence, wavelength of light, refractive index of the medium, the composition and concentration of the sample. Based on these principles, optical biosensors provide information on the presence and concentration of the analyte.

Whispering-gallery mode (WGM) biosensors

Light can be confined within a transparent material if it impacts the material at a specific angle (determined by the refractive index of the material), by a phenomenon known as total internal reflection. The reflection of this light induces an evanescent wave that extends approximately 200nm outward in the surrounding medium. The binding of a polarizable molecule to the surface of the light guide results in increased path length and the number of wavelengths of light through the optical path length, this 'reactive' effect is used for interferometry based detection. When a total internal reflection guided photon is recirculated several times within a silica sphere of with an approximate radius of 100nm it increases the sensitivity of the interferometry based detection several fold. A photodetector measures the discrete changes in resonance frequency/wavelength of a WGM which are dependent on the size and the mass of the analyte[20].

Microspheres are made from a tapered optical fiber tips that are melted in focused CO_2 laser. Virus sized polystyrene (PS) particles with a radius of 250nm were designed. These PS particles were diluted in PBS. The microspheres are exposed to PBS containing the PS particles within a sample cell that limits air flow and stabilizes ambient humidity. The photodetector measures the changes in the transmission spectrum every 20ms. After successful detection of the binding of the PS particles similar experiments confirmed that individual influenza A virions could be detected from aqueous buffers using this technique[21]. Biosensing using this technique is especially attractive for small volume samples. Apart from offering high sensitivity, label-free detection is an unique advantage of WGM biosensors. Labels could structurally and functionally interfere with the assay. Furthermore, the interference associated with labels may vary from sample to sample, making it difficult to troubleshoot. WGM biosensors offer ultrasensitive, label-free detection in miniaturized formats making them highly suitable for use in on-site, real time detection of viruses.

Surface Plasmon resonance

Surface plasmons are coherent electron oscillations that exist at the interface between any two materials. The excitation of surface plasmons by light is referred to as surface plasmon resonance (SPR). SPR is a form of reflectance spectroscopy that has been extensively used for developing biosensors.

Viruses are detected by SPR using a thin metallic layer interfaced with virus. Light is transformed into a surface wave of the free electrons at the metal surface, which propagates until it is completely absorbed and transformed into heat. This surface wave is very sensitive to molecules that are in proximity to the surface. The binding of the virus to its respective antibody will alter the refractive index as well as the resonance angle. With the increase in binding of the analyte the drift in the resonance angle is more. A sharp drop of reflected light indicates a drift in resonance angle and confirms the presence of the analyte. Linear and branched synthetic peptides of hepatitis A virus (HAV) have been used for the detection of HAV from serum using SPR biosensors[22]. Assays for cost effective and rapid detection of influenza viruses using SPR based biosensors are now under development. Though SPR biosensors offer label-free detection, the dynamic range of measurement is a limiting factor.

The Raman scattering by molecules adsorbed on rough metal surfaces can be enhanced 10^{14}-10^{15} fold and is referred to as surface enhanced Raman spectroscopy (SERS). This enhancement results in exponential increase in sensitivity allowing the detection of single viruses. The principle of SERS has been used to detect herpes simplex viruses in tear films from cases with conjunctivitis[23].

Quantum dots

Quantum dots (QD) are fluorescent semiconducting nanocrystals which have broad excitation spectra and narrow emission spectra. The conducting properties of QDs are

closely related to the size and shape of the individual crystal. Furthermore, QDs exhibit good stability on exposure to light. Respiratory syncytial virus (RSV) detection with high sensitivity using QDs has been reported[24]. QD mixes are now being developed for the simultaneous detection of several respiratory viruses.

Piezoelectric biosensors

Ability of certain types of material (eg. quartz crystals) to generate an electric potential corresponding to changes in mass as a result of a biorecognition event such as antigen-antibody interaction and DNA hybridization is the basis of piezoelectric sensors. The change in frequency of a quartz crystal resonator that reflects the mass per unit area is measured by a Quartz crystal microbalance (QCM). QCM represents one the most widely used piezoelectric biosensors. Piezoelectric sensors have been developed for the detection and genotyping of human papilloma virus (HPV). The presence of HPV was detected using degenerate probes. Subsequently, specific probes were used for HPV genotyping[25]. A peptide nucleic acid based piezoelectric biosensor was used to detect HBV DNA. The lower limit of detection of HBV DNA was 8.6 pg/L. Thus making this a highly sensitive method for the rapid and label free detection of HBV DNA[26]. Monoclonal antibodies to dengue E and NS-1 were immobilized QCM using protein A to create a piezoelectric immunochip to detect dengue virus from clinical samples[27]. A horse polyclonal antibody against SARS-CoV on piezoelectric crystal surface could detect SARS-CoV from sputum samples[28].

Oligonucleotide arrays

Palacios *et al.*,[29] devised an oligonucleotide array (GreenechipVr) with probes from 3 genomic targets for every family or genus of vertebrate virus. The probes were 60-mers and targeted both structural and non-structural proteins. The Greenechip Vr was able to accurately able to identify all the 49 viruses from cultured cells as well as clinical samples. The viruses studied include adenovirus, West Nile virus, Saint Louis encephalitis virus and influenza virus. The sensitivity differed for different viruses and ranged from 1.9×10^3 to 1.1×10^4 copies / assay. The authors claim that Greenechip Vr may be particularly useful in the diagnosis of clinical syndromes such as viral haemorrhagic fevers. Furthermore, reduced signals from Greenechip associated with the binding of viral nucleic acids that are not an exact match to the probe. These viral nucleic acids may represent viral variants or related viruses from the same family or novel agents. The viral nucleic acids that yield weak signals may be eluted for amplification and sequence analysis. Therefore, oligonucleotide arrays may also help identify novel agents or variants without prior information of nucleotide sequence.

Aptamers or aptasensors

Aptamers are single stranded DNA or RNA oligonucleotides that can bind to a variety of non-nucleotide targets including proteins with high affinity and high specificity. Aptamers represent a superior alternative to antibodies as recognition agents. An *in vitro* process called SELEX (systematic evaluation of ligands by exponential enrichment) is used to identify aptamer sequences. A random oligonucleotide sequence library is incubated with the target (eg. proteins or enzymes). Subsequently, the binding oligonucleotides are separated, eluted from the target and amplified resulting in an enriched pool of aptamers that bind the target. After several iterative cycles, the best aptamers are identified. The nucleotide sequence of the identified aptamers is obtained by cloning and sequencing. Several targets including proteins, enzymes, toxins and antibiotics may be identified using SELEX. Yamamoto *et al.*,[30] used a molecular beacon aptamer with a flurophore and a quencher to detect detect the Tat protein of HIV. Similarly, aptamer-based biosensors have been developed for the detection of HCV core antigen[31]. More recently, DNA aptamers have also been used as probes for recognizing virus infected cells[32].

Implantable virus detection systems

Studies are now underway to combine biosensors with radiofrequency identification devices for use as implantable virus detection systems. Presently this system is being developed for live stock. These devices will provide 3 levels of information: a) identification of the presence of a virus b) classify the virus and identify pandemics and c) precise identification of the infecting virus. However, these studies are still in their infancy and it may take several years for the product development, validation and assessment of safety and efficacy.

Despite the publication of several thousands of articles on biosensors in the last few years, biosensors are yet to revolutionize molecular diagnostic Virology. However, it is evident that several novel and robust technologies for the diagnosis of viral infections will emerge in the coming years.

Table 1: Key Challenges in the implementation of nanotechnology based devices in a diagnostic Virology laboratory

Key Challenges
(1) Validation of assays Sensitivity / Specificity Robustness Reproducibility
(2) Suitability of clinical specimens Integrity and stability of the virus Presence of inhibitors Compatibility with appropriate sample processing methods
(3) Quality control Globally reproducible and comparable results Development of internationally accepted standards Precision and accuracy
(4) Others Production of reliable and low-cost devices Device storage and transportation Staff competence

Several key challenges that remain include suitability of clinical specimens, global reproducibility, cost-effectiveness and storage issues (Table 1). Critical evaluation of these technologies for their sensitivities, specificities and applicability to clinical samples are required prior to routine use in clinical Virology laboratories.

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